ATR-FTIR Spectroscopy of Membrane-Bound Ras Protein

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ERKLÄRUNG

Hiermit erkläre ich, dass die Arbeit selbständig verfasst und bei keiner anderen Fakultät eingereicht und dass ich keine anderen als die angegebenen Hilfsmittel verwendet habe. Es handelt sich bei der heute von mir eingereichten Dissertation um fünf in Wort und Bild völlig übereinstimmende Exemplare.

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CONTENTS

ABBREVIATIONS	i
Abstract	iv
Summary	vi
1 Introduction	1
2 State of the Art	4
2.1 Former Studies of Interest	4
2.2 Semisynthetic Ras Lipoprotein	5
2.3. Techniques	7
2.3.1. Fourier Transform Infrared (FTIR) Spectroscopy	7
2.3.1.1 Infrared Spectroscopy	7
2.3.1.2 Instrumentation of the FTIR Spectroscopy	10
2.3.2. Difference Spectroscopy	11
2.3.3. ATR-technology	12
2.3.4 Protein Structure Analysis	14
2.3.4.1 Hierarchal Organization of the Protein Structure	14
2.3.4.2 Domains, Motifs, and Folds	16
2.3.4.3 Protein structure determination	17
2.3.4.4 Protein structure determination <i>via</i> FTIR	18
2.3.4.4.1 Curve-Fitting	19
2.3.4.4.2 Side Chain Subtraction	21
2.3.4.4.3 Performing Curve-Fit	21
2.3.5 Lipid-to-Protein Ratio	23
2.3.5.1 Lipids and Liposomes	23

2.3.5.2 Lipid-to-Protein Ratio Calculation	24
3 Results and Discussion for Membrane-Bound Ras Protein Structure	25
3.1 Set-up	25
3.2 Measurements	27
3.3 Curve-Fit Analysis	30
3.4 Structural and Functional Interpretations	34
3.4.1 Curve-Fit Results	34
3.4.2 Second Derivative Analysis	40
3.4.2.1 Second Derivative Results	40
3.4.2.1.1 Tyrosine Side Chain	40
3.4.2.1.2 Membrane-Bound Ras Structure in Detail	41
3.4.2.1.3 Measurements with Polarized Light	45
3.4.2.1.4 Curve-Fit Results	48
3.4.2.2 Discussion	49
3.5 Summary and Outlook for Membrane-Bound Ras Structure Investigation	55
4 Ras Protein Nitric Oxide Interaction	57
4.1 Nitric Oxide Overview	57
4.1.1 Physical, Chemical Properties and Reactions of NO	59
4.1.2 NO Detection and Interaction Studies	62
4.2 NO Studies of Ras GTPases	64
4.3 ATR-FTIR Studies with Membrane-Bound Ras Protein and NO	67
5 Experimental Results and Data Analysis	68
5.1 Experimental Results	69
5.1.1 Stabilization Period	71

5.1.2 GTP Nucleotide Circulation	74
5.1.3 NO Solution Injection	76
5.2 Data Analysis	79
5.2.1 Difference of Amide I Normalized Spectra with respect to the GTP spectrum	82
5.2.1.1 GDP Nucleotide Circulation	82
5.2.1.2 NO Solution Injection after GTP Addition	83
5.2.1.2.1 GTP Hydrolysis	85
5.2.1.2.2 Membrane Modulation	89
5.2.2 Difference with Amide II Normalized Spectra	94
5.3 Control for the NO effect on POPC model membrane	98
5.4 Indirect Test of the Ras Protein Membrane Affinity	101
5.5 Discussion for the ATR-FTIR Studies with Membrane-Bound Ras Protein and NO	103
5.6 Summary of the ATR-FTIR studies with membrane-bound Ras protein and NO	110
6 Methods	111
6.1 General Methods and Procedure for the ATR-FTIR Measurement	111
6.2 Procedure for the ATR-FTIR studies with the membrane-bound Ras and NO	113
7 Chemicals and Materials	114
REFERENCES	116
Curriculum Vitae	144

ABBREVIATIONS

AC, analogue circuit

AM, amplitude mode

Å, angstrom

ATP, adenosine triphosphate

ATR, attenuated total reflection

CAAX, cysteine-aliphatic amino acid-aliphatic amino acid-any amino acid

cGMP, cyclic guanosine monophosphate

DC, digital circuit

DEAE, diethyl amino-ethyl

DMPC, 1,2-dimyristoglycerol-3-phosphocholine

DNA, deoxyribonucleic acid

DPPC, dipalmitoylphosphatidylcholine

DTE, dithioerythriol

e, endothelial

EDRF, endothelium derived relaxation factor

EPR, electron paramagnetic resonance

ERK, extracellular signal-regulated kinase

ESR, electron spin resonance

FAD, flavin adenine dinucleotide

FM, frequency mode

FMN, flavin mononucleotide

FSD, Fourier Self Deconvolution

FTIR, Fourier Transform Infrared

FWHH, full-width at half-height

GAP, GTPase activating protein

GC, guanylate cyclase

GDP, guanosine diphosphate

GEF, guanosine exchange factor

GTP, guanosine triphosphate

GTPase, guanosine triphosphatase

H, Harvey

h, horizontal

HD, hexadecyl

i, inducible

IRE, internal reflection element

K, Kirsten

L, loop

MALDI-TOF, Matrix Assisted Laser Desorption Ionisation – Time of Flight

MAP, mitogen activated protein

ME, modulated excitation

MIC, maleimidocaproyl

MLV, multilamellar vesicle

N, neuroblastoma

n, neuronal

NF1, neurofibromatosis type-1

NO, nitric oxide

NOS, nitric oxide synthase

NMR, nuclear magnetic resonance

PMA, phorbol 12-acetate 13-myristate

POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine

RBD, Ras binding domain

RMSD, root mean square deviation

RNS, reactive nitrogen species

ROS, reactive oxygen species

SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SOD, superoxide dismutase

StDev, standard deviation

ULV, unilamellar vesicle

v, vertical

α , alpha

β , beta

γ , gamma

ν , vibrational stretching

ν , frequency

λ , wavelength

Abstract

An ATR-FTIR set-up for the membrane-bound Ras protein studies was established. The first high quality Amide I bands of membrane-bound Ras were obtained. Protein secondary structure analyses were performed by an improved curve-fitting technique. We looked at the structural properties of lipidated N-Ras (1-188), truncated H-Ras (1-166) and membrane bound N-Ras (1-188) proteins. Truncated H-Ras and membrane-bound N-Ras protein results were in accordance with the previous structural studies of the Ras protein. When there was no membrane available to the protein for binding, some structural deviations of the lipidated N-Ras from the membrane-bound form were observed. This was suggested to be related to the second anchoring moiety of the lipidated protein. Also, Tyr side chain band splitting was observed for the membrane-bound Ras. This was indicating membrane interaction. Polarized measurements were performed and the outcomes were pointing at a modification of orientation which was observed mainly in the β -sheet contents of the membrane-bound Ras protein. Inspection of the second derivative spectra revealed indicative bands of β -hairpin motif. This could be relevant to the protein clustering on the membrane.

For the first time, direct interaction of the membrane-bound Ras protein with the nitric oxide as the GEF was investigated in a model system. Protein activity was achieved and the lipid absorption intensity was modulated. This effect on the membrane could have been triggered by the concerted GTP exchange of the Ras protein and succeeding GTPase activity. This observation was offered to have biological relevance to the membrane ruffling. In conjunction, dynamic random to sheet transitions of the membrane-bound Ras protein were observed, and stabilizing interactions of the lipid C=O region were accompanying. This could be associated with the conformational changes in the Ras protein.

Zusammenfassung

In dieser Arbeit wurde ein Aufbau zur Untersuchung von membrangebundenem Ras entwickelt. Erstmals wurden Amid I Banden mit gutem Signal-Rausch Verhältnis gemessen. Protein Sekundärstrukturanalysen wurden mit einer verbesserten Bandenzerlegungstechnik durchgeführt. Es wurden die strukturellen Eigenschaften von lipidiertem N-Ras, trunziertem H-Ras (1-166) und membrangebundenem N-Ras (1-188) untersucht. Die Ergebnisse für das trunzierte H-Ras und das membrangebundene N-Ras waren in Übereinstimmung mit bisherigen strukturellen Untersuchungen des Ras Proteins. Das nicht an eine Membran gebundene lipidierte N-Ras wies einige Strukturunterschiede zum membrangebundenen Protein auf, die dem Einfluss des zweiten Membranankers zugeschrieben wurden. Zusätzlich wurde ein Bandensplitting der Tyrosin Bande beobachtet, was ein Anzeichen für Membraninteraktion ist. Polarisierte Messungen wurden durchgeführt und die Ergebnisse deuteten auf eine Änderung der Proteinorientierung hin, die hauptsächlich im β -Faltblatt Anteil des membrangebundenen Ras Proteins beobachtet wurde. Die Untersuchung der zweiten Ableitung der Spektren zeigte Banden, die typisch für β -Harnadelschleifen Motive sind. Dies könnte für die Clusterbildung des Proteins an der Membran wichtig sein.

Zum ersten mal wurde die direkte Interaktion von membrangebundenem Ras mit Stickstoffmonoxid als Austauschfaktor in einem Modell-System untersucht. Die Proteinaktivität wurde erhalten und die Absorptionsintensität des Lipids wurde moduliert. Dieser Effekt auf die Membran könnte durch den konzertierten GTP-Austausch des Ras Proteins und die nachfolgende GTPase Aktivität ausgelöst worden sein. Es wurde vermutet, dass diese Beobachtung biologische Bedeutung für "membrane ruffling" haben könnte. In Verbindung damit wurden dynamische Zufallsknäuel zu β -Faltblatt Übergänge des membrangebundenen Ras Proteins beobachtet, die von stabilisierenden Interaktionen der C=O Region begleitet wurden. Dies könnte mit einem Konformationswechsel der Ras Proteins verbunden sein.

Summary

Ras protein participates in the signal transduction of cellular events such as cell division, proliferation, differentiation, morphology changes and apoptosis. During signal transduction, it is bound to the inner leaflet of the cellular membrane and shows activity through a guanosine triphosphate (GTP)-bound to guanosine diphosphate (GDP)-bound switch mechanism. Its malfunctioning is estimated to occur in 15-to-30 percent of the malignant tumour cells. Former studies were mainly carried out on the truncated protein which excludes the hypervariable C-terminus that targets the protein to the membrane. In the present study, doubly lipidated neuroblastoma (N)-Ras protein possessing the farnesyl and the hexadecyl lipid anchors was investigated. This study demonstrates the application of solid supported model membranes as a tool to membrane protein research with the use of a Fourier Transform Infrared – Attenuated Total Reflectance (FTIR-ATR) set-up. We looked at the structural properties of the membrane-bound lipidated N-Ras protein in comparison to the truncated H-Ras protein and to the lipidated N-Ras protein structures, both in a lipid-free environment. Truncated H-Ras and lipidated N-Ras proteins revealed similar secondary structures when the latter was bound to the membrane, whereas some structural deviations were observed in case of the lipidated protein when there was no membrane available to the protein for binding. The reason might have been that the membrane binding was preventing any probable deviations in the structure of the lipidated protein. This would possibly be related to the role of the second anchoring moiety. Further, polarized measurements were performed and the outcomes were pointing at a modification of orientation which was observed mainly in the β -sheet contents of the membrane-bound Ras protein. Inspection of the second derivative spectra revealed indicative bands of β -hairpin motif. This could be relevant to the protein clustering on the membrane. Also, membrane interaction was detected through the Tyr side chain band splitting. Eventually, our established set-up can be utilised for elaborating the details and for studying the membrane-bound Ras protein interactions with the guanosine exchange factors (GEFs), and the GTPase activating proteins (GAPs). These concepts can also be studied with the complex lipid mixtures to elucidate this intricate nature.

The initial application of the membrane-bound Ras protein-GEFs interaction was performed with the nitric oxide (NO) as the GEF. The lipids were responding typically as a modulated excitation (ME), *in vivo* conditions. Namely, we observed a modulation of the lipid concentration through the changes in its absorbance. It was a cyclic stimulation and the initial spectrum was restored at the end of measurement. Interpretation of the data was further leading to the conclusion that; NO-aided loosening of the membrane networking was occurring along with the stabilizing interactions of the lipid C=O region. In relation, a dynamic random to sheet transition in the membrane-bound Ras protein was taking place, which could be pertinent to the conformational changes in the protein. Such characteristic features were observed only when the protein was bound to the membrane and active on the surface. Hence, the membrane was modulated by the Ras protein activity. Interpretation of the data aided us for proposing a simple theory on the action mechanism of the Ras protein in distinct signal transduction pathways. It is summarized below.

Our measurements revealed that the NO-mediated GTP exchange of Ras protein and the subsequent GTPase activity were leading to a modulated excitation of the membrane. This excitation was suggested to be due to a pressure, or a force applied somehow to the membrane by the protein going under a conformational change, during the nucleotide exchange and the subsequent GTPase activity. The biological relevance of such an observation could be the membrane ruffling, which was postulated to be accompanied to the Ras protein activity in the live cell. Ruffling is common to distinct cellular actions and is stimulated in response to varying factors inherent to a cell, and in response to the extra-cellular agents. It was suggested that the multi-functionality of the Ras protein may be achieved through varying the switching frequency of itself according to the input stimuli; and thus, creating frequencies of particular output circuits in the membrane, to be the input of its downstream effectors and regulators. The significance of this proposal is underlying the co-ordination of multiple pathways with regard to the possible interference patterns of distinct signal inputs, including those arousing from the Ras signal transduction pathways. In the future, the validity and the details of this theory need to be investigated along with the possible practical applications.

1 Introduction

After the initial discovery of a 21 kDa (p21) protein [1] coded by Harvey [2] or by Kirsten rat sarcoma viruses [3], it was soon reported that the Guanine nucleotide binding is specific for this protein [4]. Its function as a molecular switch in signal transduction was soon realized and a great deal of effort was put to investigate its structural [5, 6], functional [7, 8], biochemical [9] and the biophysical properties [10, 11] including the details of its regulation [12-14], its interaction partners [15], downstream effectors [16], and upstream regulators [17].

Ras superfamily GTPases are among the small GTPases, and are present in all eukaryotes [18]. Ras superfamily possess 30-55 % sequence homology with a common molecular topology among its more than 200 members which are classified into at least five sub-families; the Ras, Rab, Rho, Sar1/Arf, and Ran families [19-23]. The residues important for nucleotide interaction are conserved and composed of four consensus sequences for the nucleotide binding [24]. Shortly, those are the P-loop GxxxxGK(S/T) and DxxG motifs which are involved in magnesium phosphate interactions and which contain residues important for GTPase activity, in addition to the NKXD and SAX motifs which consist of residues important for binding the ribose and the base moieties of guanosine nucleotide.

Ras proteins operate on distinct cellular activities as mediators in the cell signaling cascade from the receptor tyrosine kinases to the nucleus through activating the downstream effectors (Figure 1) to stimulate growth and differentiation [25, 26]. During its activity, Ras is bound to the inner leaflet of the cellular membrane with its C-terminus [27]. Functional importance of the C-terminus is known, since the 1980s, in triggering the membrane recognition, binding and localization [27]. C-terminus is hyper-variable. This results in different Ras isoforms (H-, N-, and K-Ras), those recruited to different membrane platforms. All isoforms are otherwise very similar in structure and function. They end with a CAAX motif initially, which undergoes sequentially: farnesylation, AAX proteolysis and methylesterification. Then H-Ras is palmitoylated at the residues Cys 181 and 184; whereas N-Ras is palmitoylated only at the Cys 181, besides the residing farnesyl at the residue Cys 186. K-Ras has a polybasic domain

instead, which is spanning the residues 175 to 180 [28]. The farnesyl group has the modest selectivity for the membrane binding [29]. Affinity of binding is regulated by the attachment or removal of the lipid anchoring moieties. Fully processed (farnesylated, carboxymethylated and palmitoylated) N-Ras peptide has the highest binding half-life which is correlated to the signaling duration; it is almost irreversibly bound to the membrane [29]. Inhibiting post-translational modifications with the forthcoming defects in localizations were offered as an alternative cancer therapy [30].

Figure 1. Extracellular signal is transmitted to the downstream effectors through the GTPase activity of the Ras protein. Intrinsically slow GTPase activity of the Ras protein is up-regulated by the guanosine exchange factors (GEF) and down-regulated by the GTPase activating proteins (GAP).

Truncated Ras with the initial 166 or 172 residues has been investigated so far. As already mentioned, the biochemical and structural properties of the isoforms, also in their full-length versions, are similar though not completely identical in sequence [31]. However the exact function and structure of the protein, and its localization in transforming *versus* non-transforming cellular environments are yet to be revealed by further investigations utilizing the lipidated Ras isoforms. Existing studies are pointing at the functional and regulatory importance of the Ras isoforms' differential segregation through the lateral organizations of

sphingolipid and cholesterol rich domains [32], revealing the complex crosstalk among those isoforms and different membrane microdomains [33-40]. Membrane microdomains, namely the lipid rafts, are highly organized platforms trafficking the signalling molecules and bringing them in close vicinity. Ras-signalling up regulation and down regulation is thus controlled by the availability of the effectors and regulator molecules. Referring to the possible mechanism of lipid raft targeting and clustering within [41-43], model systems enable stepwise tracking of the determinants dictating the segregation of Ras isoforms associated with the plasma membrane.

2 State of the Art

2.1 Former Studies of Interest

Figure 2. Illustration of membrane bound lipidated Ras. The 3D-model was built according to the X-ray [8] and NMR structures [36, 43]. (Illustration by Till Rudack)

In addition to those already mentioned above, further studies on the membrane-bound Ras peptide and protein (Figure 2) were performed by other groups using the techniques NMR,

neutron diffraction, fluorescence, ATR-FTIR, modeling and simulations. Insertion of the Ras proteins into the lipid monolayer and their orientation was investigated *via* IRRAS [40]. Doubly-lipidated N-Ras protein was inserted into the Langmuir monolayer of POPC/Sphingomyelin/Cholesterol (2:1:1) mixture. Shortly; the C-terminal backbone of the lipid modified N-Ras peptide was found to be located at the lipid/water interface, while the hydrophobic side chains and the lipid anchors were inserted into the hydrophobic core of the membrane, intriguingly showing extensive dynamics [40]. The results were pointing at the free energy contributions of different anchoring moieties; and orientation change in the protein was observed during the insertion process [40]. Recently, grazing incidence X-ray diffraction and specular reflectivity measurements were performed [41] for the binding of lipidated N-Ras protein to DPPC Langmuir monolayer at the air/liquid interface. It was concluded that the electron density profile of the monolayer was altered rather than the tilt angles and the structures of the lipid molecules. Also, a structural model of H-Ras protein in complex with DMPC bilayer was obtained from the modeling and all-atom explicit solvent molecular dynamics simulations [42]. It was suggested that the H-Ras membrane binding was involving direct interaction of residues in the catalytic domain with the phosphates of the lipids, in addition to the anchoring interactions of the C-Term. Finally, NMR characterization of the full-length, farnesylated *versus* non-farnesylated H-Ras was done [43]. The details will be given in comparison to the secondary structure results of our measurements, in the coming sections.

2.2 Semisynthetic Ras Lipoprotein

Doubly-lipid anchored N-Ras protein possessing one farnesyl and one hexadecyl lipid moiety [44-49] was obtained by attaching the Ras lipopeptide to the C-terminus, through a Maleimidocaproyl (MIC) group (Figure 3). The natural palmitoyl moiety was replaced with a hexadecyl moiety to prevent self-cleavage during measurements. Former studies with this

protein were carried out by other groups as well. Those were detailed above. Our laboratory carried out the ATR-FTIR spectroscopic investigations on the N-Ras protein structural perturbations upon binding to the solid supported 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) model membranes. Additionally, direct interaction studies of the nitric oxide (NO) with the membrane-bound Ras protein were initiated. Supported bilayer membranes possessing the structural and dynamic properties of conventional planar lipid bilayers are the working models for protein adsorption studies and for studying the lipid/water interface of the membrane [50, 51].

Figure 3. Natural N-Ras protein possesses the lipid anchors at the 181th and 186th residues (a). Semisynthetic peptide attached to the cysteine 181 of N-Ras *via* MIC group and the anchors end up to be attached to the 183th and 188th residues (b), ending in an insertion of 2 residues (encircled).

2.3. Techniques

2.3.1. Fourier Transform Infrared Spectroscopy (FTIR) spectroscopy

Shortly, infrared spectroscopy is based on the interaction of electro magnetic radiation (0.78-1000 μm) with the molecules' electric dipoles, and the resulting absorption at particular energies. [52] Molecular vibrations in the sample are excited as a result of IR absorption at characteristic frequencies which are determined by the properties of the atomic molecular bonds in a sample, with their unique spatial orientations. Hence, the sample has unique absorption spectra. This technique is advantageous for its being non-invasive, and enabling the investigator to get direct information on bond orders, electrostatic interactions, H-bonding, charge distributions, protonation states, redox states, dynamics and kinetics [53-63].

2.3.1.1 Infrared Spectroscopy

Radiations produced after the absorption result in transitions among the vibrational energy levels within a single electronic state, that can be studied by the infrared spectroscopy (Figure 4). Though the region which could be investigated covers the range between 14000 and 20 cm^{-1} , the mid-infrared region, 200 to 4000 cm^{-1} , is used for biological materials. [64]

Figure 4. Electromagnetic spectrum is shown with the characteristic transitions and the relevant spectroscopic techniques.

An infrared spectrum is the plot of radiation absorption as a function of wavenumber or frequency ($\nu \sim 1/\lambda$; where, ν is frequency and λ is wavelength). Excited molecular vibrations in a sample give unique absorption bands at almost exactly the same position in varying biological materials possessing the same group (i.e. C-O) in their structure, so called the group frequencies. Therefore, absorption spectra of the compounds are characterized by the functional group frequencies of the molecules. They are sensitive to any environmental changes or the changes in the structures and conformations of the molecules within. Those frequencies are also further characterized by the motions of the nuclei; which are mainly the twisting, bending, rotating and asymmetric/symmetric stretching modes (Figure 5). [64]

Figure 5. Normal vibrational modes for the CO₂ molecule. (by D. Kverno, Davidson College Physics, NC)

Each vibrational modes of a molecule is associated with a characteristic frequency of the vibration. For example; the characteristic frequency for the symmetric stretching mode of CO₂ is at 1288 cm⁻¹ and for the bending mode of CO₂ is at 667 cm⁻¹, but much higher for the asymmetric stretch mode of CO₂, which is at 2349 cm⁻¹. The energy levels of the vibrational modes of a molecule are quantized and approximated by the energy expression for the quantum mechanical simple harmonic oscillator, simply to the behavior of a spring. [65, 66]

The vibrational frequency, ν_{vib} , is given by the equation:

$$\nu_{vib} = \frac{1}{2\pi} \sqrt{\frac{k}{\mu}} \quad (1)$$

where k is the force constant of the bond under question and μ is the reduced mass of the molecule possessing this bond, while the reduced mass is:

$$\frac{1}{\mu} = \frac{1}{M_1} + \frac{1}{M_2} \quad (2)$$

where, M_1 and M_2 are the atomic masses of two molecules connected through this bond. [65, 66]

2.3.1.2 Instrumentation of the FTIR Spectroscopy

The source of radiation is a heated filament (or a Globar, Nernst bar...) that emits a continuous infrared spectrum. Incoming beam of radiation is directed to a beam splitter, which divides the beam into two. The beam splitter is a non-absorbing film transmitting half of the IR radiation (beam) to the mirror which is moving back and forth over a distance of about 2,5 micrometers at constant speed, whereas the other half of the radiation is reflected to the fixed mirror. Moving of one of the mirrors allows a variable change in the path length of the reflected beam to be combined with the other beam. Therefore interference occurs among the two beams during recombination and a continuum of frequencies is generated to be adsorbed by the molecular bonds in the sample. A characteristic energy difference that takes place in the beam during its passing through the sample is detected as sample transmission. The single scan of a moving mirror path length takes up to 10 milliseconds and the data is stored in the computer. A helium-neon (He-Ne) laser is simultaneously directed through the Michelson Interferometer and the interference pattern of the laser is used as a frequency reference, so that several scans can be co-added which would otherwise not be possible under the laboratory conditions with high thermal fluctuations and vibrational interventions. A schematic drawing of the interferometer of the instrument IFS66 (BrukerOptics, Germany) is shown in the Figure 6 below. [52, 64, 67, 68]

Figure 6. Schematic diagram of the optical lay-out of a Michelson interferometer, where, one of the two reflecting mirrors are movable, is used in an FTIR Spectrophotometer (IFS66, BrukerOptics, Germany).

Variation in the interference pattern intensity plotted against the mirror position is an interferogram, which is Fourier Transformed automatically to obtain a single channel spectrum with the intensity as a function of wavelength. An absorbance spectrum (A) is calculated by:

$$A = -\log \frac{I}{I_0} \quad (3)$$

where, I and I_0 are the single channel spectra of the sample and the reference, respectively. [52, 64, 67, 68]

2.3.2. Difference Spectroscopy

In order to extract the signals relevant for the reaction out of the huge background absorption of the whole protein signal, one makes use of the difference spectroscopy. The protein

spectra, which were received shortly before the initiated reaction or the forthcoming changes that is induced in the sample buffer conditions, serve as the reference. The environment of the active centre and also the volume are involved directly in the changes through the reaction. With this technique, one can study the protein interactions with the effectors, regulators, ligands; or, the protein responses to the induced environmental changes can be studied, both in the transmission and the reflection modes [69-74].

2.3.3. ATR Technology

In the ATR-technology, one gets use of the intensity losses at the boundary area through absorption after total reflection between a sample and a crystal with higher refraction index than its surrounding medium. The incident infrared beam goes under total reflection within the crystal (internal reflection element - IRE) and the emerging beam eventually enters the beam path of the spectrometer. The evanescent wave arouses at the points of reflection from the surface of the crystal through the sample; and it decays exponentially. The penetration depth (d_p) of the evanescent wave is just a few micrometers depending on the parameters given in the 4th equation, and illustrated in Figure 7.

Figure 7. Schematic drawing of the ATR-crystal (the IRE element) with the liquid sample that is shown atop on one side of the crystal, and circulating through the inlet and outlet tubing. Internally reflected IR beam is producing an evanescent wave penetrating through the liquid sample.

$$d_p = \frac{\lambda/n_1}{2\pi \sqrt{\sin^2 \theta - \left(\frac{n_2}{n_1}\right)^2}} \quad (4)$$

In the equation (4) above, d_p stands for the penetration depth. λ/n_1 denotes the wavelength in the crystal, while n_1 is the refractive index of the crystal and λ is the vacuum wavelength. θ is the angle of incidence and n_2 is refractive index of the bulk medium. Refractive index of the sample layer is omitted for the bulk medium's being the predominant determining factor in the electric field of the rarer medium. This is based on the thin film approximation by Harrick in 1967 [75]. The details of the technique can be found in different sources and the earliest, in the works of Harrick in 1967 and Fringeli, 1992 [75, 76].

By this means, when the sample is brought in close contact with the crystal surface; one can measure small amounts of samples, rare molecules in the medium, non-transparent samples and aqueous solutions. This surface sensitive technique is especially useful for us to detect the surface immobilized proteins, self assembled mono-layers, polymer coating and characterizations; and to study membrane protein adsorption on model membranes. Detailed information about the properties of the sample is obtained, which is in most cases not accessible by other vibrational spectroscopic techniques. [75, 76]

Typical materials for ATR crystals include the germanium, KRS-5, zinc selenide, and silicon. Silicon is ideal for Far-IR region of the electromagnetic spectrum; and diamond is excellent for its mechanical properties but is less preferred due its high cost.

2.3.4 Protein Structure Analysis

2.3.4.1 Hierarchical Organization of the Protein Structure

Protein structure is a hierarchic organization (Figure 8). The process resulting in the higher structures is called protein folding, and is a consequence of the primary structure. A unique polypeptide may have more than one stable folded conformation, which could have a different biological activity; but usually, only one conformation is considered to be the active or native conformation.

Figure 8. Protein structure, from 1 to 4 denotes the organizational level of the protein backbone from primary to quaternary structure, respectively. [Adapted from the National Human Genome Research Institute (NHGRI), artist Darryl Leja:

[<http://www.genome.gov/page.cfm?pageID=10000552>]

Among the four distinct aspects of a protein's structure, **primary structure** is the amino acid sequence of the polypeptide chain. It is unique and determined by the gene corresponding to

the protein. Post-transcriptional modifications (i.e. disulfide bonds, glycosylation, and phosphorylation) are part of the primary structure, and are made during the process of protein biosynthesis or translation. The two ends of the amino acid chain are referred to as the C-terminal end or carboxy terminus (C-term) and the N-terminal end or amino terminus (N-term) based on the nature of the free group on each extremity.

Secondary structure is the regular patterns of locally defined sub-structures such as α -helices, β -sheets and turns. The information about bond lengths and angles were initially defined for the major two elements of secondary structure, the α -helix and the β -sheet, by Linus Pauling and coworkers in 1951 [77]. In case of the α -helices and the β -sheets, all the H-bond donors are saturated. The patterns of hydrogen bonds between the main-chain peptide groups determine the type of secondary structure. They attain a regular geometry by this means. Besides, the hydrogen bonds are generally not that stable by themselves, since the water-amide hydrogen bond is rather favored to the amide-amide hydrogen bond. Thus, parts of the main chain buried in the protein core and form hydrogen bonds between the polar atoms [78]. There are other secondary structure elements than the α -helices and the β -sheets, such as turns, loops and other forms of helices. When the backbone is not in a regular, classified secondary structure form, then it is called to be random coil.

Tertiary structure is the three-dimensional spatial arrangement of a single protein molecule's secondary structures. The formation of molten globules and tertiary structure are held together by structurally non-specific interactions. What bring the specificity and stability to the tertiary structure are the proper packing of the side chains, which results in the burial of hydrophobic residues, and the other interactions such as ionic interactions (i.e. salt bridges) and H-bonds. The disulphide bonds may also take role in the protein structure stabilization, but in extracellular environments; because the cytosol of the cell is a reducing environment, and therefore, does not favor the disulphide bond formation in general. So the tertiary structure encompasses all the noncovalent interactions that are not considered for the secondary structure, and it defines the overall fold of the protein. Therefore it is indispensable for the function of the protein. [79-82]

Quarternary structure is the assembly of protein or polypeptide chains' units to form functional complexes. It is stabilized by the same range of interactions listed above for the tertiary structure. Not all the proteins attain a quarternary structure. The individual chains are called subunits which are not necessarily covalently connected, but might be connected by a disulfide bond. Complexes of two or more polypeptides (i.e. multiple subunits) are usually called multimers and named specifically dimer, trimer, and tetramer if they contain two, three and four subunits, respectively. Those multimers composed of identical subunits referred to with a 'homo' prefix; and if they are made up of different subunits, referred to with a 'hetero' prefix.

The proteins shift among the latter three levels of organizations detailed above, in the context of their functional rearrangements. While all inherit the 'structural change' termination upon observation of their occurrence, transitions between the tertiary or quaternary structures are usually referred to as conformational changes.

[79-82]

2.3.4.2 Domains, Motifs, and Folds

A structural domain is the part of proteins' overall structure which is common to not only a gene product or products of a whole gene family but to a variety of proteins that show the function common to the others. They are self-stabilizing, and generally folds independently of the rest of the protein chain. They are also useful genetic engineering tools by which one can get use of this highly conserved functional maintenance to make chimeras. They can be found as motifs (supersecondary structures) of secondary structural elements combinations such as helix-turn-helices. Fold is the global arrangement of the motifs, some of which are the helix-bundle, β -hairpin, or β -barrel. Those structural elements of the motifs are not necessarily sequentially encoded under the gene. Even two different genes encoding the secondary structural elements of a motif, may specify differing amino acid sequences. [83-85]

2.3.4.3 Protein Structure Determination

Mainly, the protein structures in the Protein Data Bank (pdb) are provided by the X-ray crystallography studies, which are basically the measurement and interpretation of the 3D density profiles of the electrons –*X-ray diffraction patterns*– in a crystallized protein. The resolution of the end structure (table I below) may give the 3D coordinates of the atoms in the protein. Nuclear Magnetic Resonance (NMR) is the next common technique for protein secondary structure determination, through the magnetic properties of atomic nuclei [86]. NMR allows one to work on the protein in solution. X-ray crystallography and NMR provide atomic resolution, as outlined in Table I. Resolution of small details is limited by the wave nature of light in such studies. Optical spectroscopic techniques are based on the absorption and emission properties of the molecular bond energies. They provide structural information below atomic resolution, and experimentally underdetermined. Yet, circular dichroism and FTIR can be used for secondary structure determination. These techniques are advantageous for studying the protein in differing conditions, of low amounts and without any perturbation [87-90]. Therefore, for comparison between calculated secondary structures from spectroscopy and ‘calculated’ data from X-ray crystallography, one has to recall such criterions for the protein under investigation [91]

Secondary structure can also be predicted from its sequence with a high degree of accuracy through the computational methods; while the *ab initio* prediction methods use just the sequence of the protein, threading uses the existing protein structures as well. Cryo-electron microscopy is also a means of protein structure (less than 5 angstroms or 0,5 nanometer) determination, which is preferred for large protein complexes. [92]

Table I. A short guide to the resolution of protein structures.

Resolution (Å)	Meaning
>4.0	Individual coordinates are insignificant
3.0 - 4.0	Folding can be determined with some error.
2.5 - 3.0	Folding can be determined with high accuracy.
2.0 - 2.5	Folding can be determined with high accuracy. Water molecules and small ligands become visible.
1.5 - 2.0	Folds are extremely rarely incorrect.
0.5 - 1.5	Almost with no error.

2.3.4.4 Protein Structure Determination *via* FTIR

It has been long recognized that the FTIR spectrum of a protein gives clues for the structure of a protein through the protein backbone vibrations which shows characteristic shifts upon attaining the secondary structure profiles. Several methods to estimate protein secondary structure content via the analysis of FTIR spectra have been reported. The absorption signals from individual secondary structure elements arising from a specific vibration superpose under amide regions, which are Amide I-to-V and Amide A. Amide I ($1600\text{--}1700\text{ cm}^{-1}$) is the most commonly used region for the protein structure analysis. It is governed by the C=O stretching vibrations (70-85 %) and by the C-N groups (10-20 %). Amide II ($1510\text{--}1580\text{ cm}^{-1}$) is mainly derived from the in-plane N-H bending (40-60 %), and the rest of the potential energy arises from both the C-N stretching vibrations (18-40 %) and the C-C stretching vibrations (roughly 10 %). Amide A (between $3225\text{ and }3280\text{ cm}^{-1}$) absorption is mainly, up

to 95 %, due to the N-H stretching vibration. Amide III and V also bear structural information but are very complex bands, therefore not preferred in protein structure analysis. [79, 82, 87, 88]

Curve fitting, the most common means to resolve the overlapping band volumes, was originally introduced by Byler and Susi [79], in which protein Amide I bands were analyzed by fitting with a series of Gaussian curves. The curve-fitting method compensates for band-position variation among the same secondary structure assignment by assigning all component bands found in a given region of the spectrum to a particular structure. Description of the method that we used, as an advanced application immune from some main drawbacks, is explained below.

From simpler [93-98] to more complex [99-103] multivariate statistical analysis methods were also applied and proven to be reliable for the analysis of protein secondary structure from the protein spectra. Taking the selection of the wavenumbers correlated with the particular secondary structure into account, Goormaghtigh and coworkers [104] attempted to build a model bearing the most independent information. Other approaches followed the way to combine the curve analysis for the protein secondary structure, with the isotope editing [105], hydrogen/deuterium exchange [106], or temperature [107].

2.3.4.4.1 Curve-Fitting

Curve-fitting is a mathematical modeling tool to adjust the band parameters to obtain the best agreement to the experimental data. The following equation (5) is tried to be minimized as a result of curve-fitting, where the $y(v_i)$ is the experimental data as a function of variable v , N is the total number of data and $f(v_i; a)$ is the fitted data.

$$\sum_{i=1}^N (y(v_i) - f(v_i; a))^2 \quad (5)$$

There are several parameters to find the best-fit parameters, namely the minimization of the equation (5). They are the Newton-Raphson method, the Gauss-Newton method, the Steepest Descent method, the Levenberg-Marquard method, the full Newton-type Levenberg-Marquard method and the Nelder-Mead Simplex method [108 and 109, as referred in 110].

However, curve-fitting requires an initial guess for the parameters which will eventually be the main determinant for the goodness of the fit. Those parameters can be counted as the position, width, area and band characteristics of each band; where the total number of the bands will be an additional parameter by itself. Band asymmetry can be accounted as a band parameter; yet, it is rather the outcome of under-scoring the number of bands. Usually a baseline with one or more parameters is included in the model or baseline correction is performed before curve-fitting.

By means of such a mathematical approach, curve-fitting enables the determination of the relative sub-volumes that are superposed under a main band. The process is applicable to the protein spectra Amide I band profile. It is set by the superposition of the differential absorbencies of the protein backbone vibrations, as a result of the residual interactions and the spatial arrangements, which can also be identified as the secondary structural elements. However, the deconvolution of the Amide I band does not provide an unequivocal result, because the analysis is experimentally underdetermined and one can fit an Amide I curve with varying combinations of parameters. The approach that we followed to overcome this problem is explained in the section 2.3.4.4.3, and was previously published [111].

Amide I band is overlapped with protein side chains absorbencies as well. Eventually their percent contributions overall, depend on the amino acid sequence of the protein. Therefore one needs to subtract those from the original absorbance, for the forthcoming analysis of the protein structure by curve fitting of the Amide I region. There are several approaches for side chain subtraction based on similar methodologies and the one from Vigano and coworkers (2004) [112] automated through the MATLAB[®] optimization toolbox (version R14, Mathworks, USA) R12 kinetics, is described below, for both the side chain correction and the subsequent curve-fitting approach that we followed.

2.3.4.4.2 Side Chain Subtraction

The contribution of the side chains to the spectrum of each protein is computed in the Amide I-II regions of the IR spectrum via MATLAB[®] R12 kinetics, with scaling. The scaling factor is based on both the integrated intensity and the Amide I extinction coefficients that are computed through the reported secondary structure data of the protein under investigation. Side chains and the protein backbone vibrations contribute to the overall protein absorbance as two different fractions with different molar extinction coefficients. The software is reading the files corresponding to each protein to extract the secondary structure and the amino acid composition. The individual contributions of each side chains were then rebuilt according to the data given in the literature [82, 113-115; reviewed in the given literature as references 87 and 116] and were scaled to eliminate the tyrosine ring vibration band at 1515 cm^{-1} , then subtracted.

2.3.4.4.3 Performing Curve-Fit

Amide I' bands of the spectra were utilized for curve-fitting; where, the prime (') symbol denotes that the measurement was performed in deuterated medium. Deuterated medium was preferred to eliminate the water absorbance contribution within $1600\text{-}1700\text{ cm}^{-1}$ region, which was also monitored from the O-H stretching absorbance at $3000\text{-}3600\text{ cm}^{-1}$ region. All spectra were filtered with Fourier Self Deconvolution (FSD), to remove the high frequency noise, resultantly leaving the components broader than or equal to 4 cm^{-1} full-width at half-height (FWHH). Then, contribution of the side chains to the protein spectrum was computed within the Amide I' and II' regions. It was performed by using the kinetics software (provided by E.

Goormaghtigh) running under MATLAB[®] R12 kinetics, optimization toolbox. Curve fitting was performed as initially described by Goormaghtigh and coworkers [117] and modified by Ollesch and coworkers [111]. This was a deterministic model as described by Lórenz-Fonfría and Padrós in 2004 [118].

Curve-fitting requires an initial guess for the underlying band parameters, i. e. the number, position, intensity, FWHH and shape of the bands. As of being the most predictive tools to guess among those parameters, two band narrowing techniques, namely FSD and second derivative of the smoothed spectra were inspected after performing the side chain correction, to estimate the number and positions of the bands to be fitted under Amide I'.

Least squares iterative curve-fitting was performed in order to fit a mixture of Lorentzian and Gaussian line shapes to the spectrum between 1700 and 1600 cm⁻¹. All operations were performed through the MATLAB[®] R12 kinetics, optimization toolbox. Each experimental set was repeated 3 times and the curve fitting analysis were performed with randomly selected spectra from each set. Results were showing a 3 % deviation at most for the structural component of the highest content. This value was taken as the approximate error. It was the same as reported previously [111] for the method.

Quality of the curve-fit was inspected through the standard deviation of the fit which was calculated by the MATLAB[®] R12 kinetics program automatically as the mean displacement of the curve-fit resultant spectra from the original. RMSD values were calculated according to the given formula (6):

$$RMSD = \sqrt{\frac{1}{N} \sum_{i=1}^{i=N} \delta_i^2} \quad (6)$$

Terms in the RMSD formula: N is the total number of components, namely the number of secondary structural elements, and δ_i is the deviation of the structural component percent from its reported value in the literature.

As mentioned previously, there is a multiplicity of parameter combinations which are applicable for the curve-fitting of a specific amide band. It was known that simple (single and

/ or multi) correlations exist between the percentages of secondary structure elements obtained from X-Ray crystallography and some parameters, i.e. frequency and fwhh, of the Raman Amide I band; which were giving the best fits among the Raman calculated structures and X-ray data, when applied as curve-fit parameters [91]. Thus we obtained a common parameter set which is specific for the truncated Ras protein in deuterated medium, through curve-fitting of a high quality Ras spectrum, Amide I' region [111]. The parameters of the curve-fit which was giving the best approximation to the solution was selected to be utilized during curve-fitting of the lipidated Ras protein Amide I' band [111].

2.3.5 Lipid-to-Protein Ratio

2.3.5.1 Lipids and Liposomes

Figure 9. The POPC molecule.

The ester phosphate group of a lipid molecule (Figure 9) is linking the glycerol moiety to the choline moiety, through the C-O stretch of the phosphate ester group. The $\nu_{as}(C-O[P])$ stretching vibration in the phosphate ester group is coupled with the PO and C-C stretches of the choline; while, the C-O stretching in the phosphate ester group is coupled to the PO and the C-O stretches of the glycerol. Conformation of the latter stretching source has higher rigidity. Methylene stretching absorbencies of the fatty acid chains are highly immune from

the protein stretching absorbencies and are the most representative of the lipid content on the surface. [119-121]

Hydrophobic nature of the fatty acid chains and hydrophilic nature of the phosphate head groups lead to the spontaneous vesicle formation of lipid molecules in protonated and deuterated solutions. AFM investigations of the liposome coalescence onto hydrophilic and hydrophobic solid supports were suggesting the attachment, spreading and flattening (Figure 10) as the mechanism of bilayer formation by vesicle fusion [122].

Figure 10. Transform from liposome to supported lipid multilayer. Bilayer was offered to be forming from this structure via rolling or gliding [122].

2.3.5.2 Lipid-to-Protein Ratio Calculation

Ratio of the integrated lipid (C=O) absorption at around 1750 cm^{-1} to the protein Amide I' volume, which was corrected for the side chain absorbencies, was used in this study to estimate the lipid-to-protein ratio. This ratio was divided by another ratio; which was the lipid C=O absorption per lipid molecule to the carbonyl groups per protein molecule (2 for POPC and 188 for lipidated Ras). This was a rough estimate, neglecting the differences in absorption coefficients and evanescent wave decay.

3 Results and Discussion for the Membrane-Bound Ras Protein Structure

3.1 Set-up

We presented in this study the sample preparation of the membrane-bound lipidated N-Ras protein on solid supported POPC model membranes as a tool for membrane protein interaction studies performed with the ATR-FTIR technique. With the use of horizontal multibounce ATR-FTIR set-up, we looked at the structural properties of the membrane bound Ras protein on solid supported POPC model membrane, which we have previously performed with the vertical flow-through ATR set-up. Both the horizontal and vertical ATR-FTIR set-ups are straightforward that the rapid sample exchanges were enabled (Figure 11). Material consumption with the hATR was 20 times less as the sample application surface was concentrated into a 4-mm diameter area. Hence, each measurement required only minute quantities of the sample (i.e. $\sim 2,4 \mu\text{g}$ of lipidated Ras protein) per measurement which could be modified according to different experimental designs (Figure 11).

(a)

vATR
flow-through cuvette

(b) hATR set-up & sampling

(c) hATR scheme

Figure 11. One of the ATR-FTIR set-ups for the measurements were utilizing a semiclosed flow-through vertical –v– cuvette with the germanium IRE crystal sandwiched in between (a). The other set-up was a horizontal –h– micro-sampling facility with the diamond IRE crystal surface open for direct sample application (b). The inner schematic drawing of hATR is shown below (c).

3.2 Measurements

For the membrane-bound lipidated Ras measurements, initially the lipid layer was formed on the IRE surface. Lipid self assembly was directly monitored from the methylene stretching absorbencies' (Figure 12a) evolution in the sample absorbance spectra with the buffer spectrum as the reference. Stability of the constructed layer was attained in 10-15 mins (Figure 12b).

Figure 12. ATR-FTIR absorbance spectrum of POPC methylene stretching vibrations are shown in a. Model membrane adsorption kinetics on the IRE surface is shown in b (data from J. Güldenhaupt).

Lipid layer durability was assured *via* the remaining lipid signals. Specific binding of the double lipid anchored N-Ras protein on solid supported POPC model membranes was attained and measured with the ATR-FTIR technique (Figure 13).

Figure 13. Membrane binding and specificity of the lipidated Ras protein are shown through the protein adsorption kinetics on the model membrane, in H₂O buffer. (Data from J. Güldenhaupt)

Lipid-to-protein ratio was calculated as described within the methods section and found to be 150 ± 30 lipid molecules per lipidated Ras protein. The error value was estimated from the approximate volume changes arising from the selected upper and lower frequency limits for the volume calculation, and the baseline. The volumes were approximated from a rough curve-fitting of the band under question, in order to eliminate the contribution of the neighboring bands. Amide I' band was treated to be arising completely from the C=O stretching vibration, though the actual C=O absorption percentage was 70 to 85 %; and the extinction coefficients, or the integrated molar absorbencies (ϵ_j), of the lipid C=O and the protein backbone C=O absorptions were accounted equal for the sake of simplicity during calculations.

Secondary structure outcomes and comparisons were performed on the truncated protein in solution (no lipid in the medium), full-length double lipid anchored protein in solution (no lipid in the medium) and the full-length double lipid anchored protein bound to the membrane (in solution). Comparison of the Amide I' absorbencies of these three measurements are shown in Figure 14.

Figure 14. Amide I' of the protein spectra in three comparable states are shown. Bold grey spectrum on top is the truncated Ras protein; bold black spectrum in the middle is the membrane-bound

lipidated Ras protein; and intersected black spectrum at the bottom is the lipidated Ras protein. Spectra were normalized to the highest Amide I' absorption and compared with stacking.

Amide I' bands were highly comparable and showed no unusual broadening which would be indicative of protein denaturation, upon observation. Those spectra shown in Figure 14 were normalized to the highest Amide I' absorption signal, which was arising from the lipidated Ras protein because the mass of the lipidated Ras protein was roughly 15 % higher than the truncated form of the protein that was measured. The signal of the membrane-bound lipidated Ras protein was the least due to the membrane layer on the surface which was making the protein signal weaker as the protein got further away from the surface. The washing step which was described within the methods section was necessitated only in case of the membrane bound lipidated Ras measurements, in order to separate the unbound absorbing species from the bound analyte. Rate of incorporation was depending on the concentration of the protein and also the rate that the protein was brought in contact with the surface. These two factors were differing among the hATR and the vATR set-ups which were eventually introducing a change in the adsorption kinetics of both the POPC and the protein in case of hATR *versus* vATR (data not shown). Yet the curve-fit analysis outcomes of the measurements with both set-ups were the same which provided the reproducibility of the results, also among the two set-ups.

3.3 Curve-Fit Analysis

For the sake of simplicity in calculations and the tedious implementation during data interpretation, it was assumed that the extinction coefficients, or integrated molar absorbencies (ϵ_j), were equal for all the secondary structural components of the protein.

Side chain absorbencies were needed first to be removed from the Amide I' region as they were also contributing to the protein backbone vibrations in the same region. It was performed as described in the methods section. Side chain spectrum depends on unique amino acid sequence of the protein (Figure 15a). An exemplary figure with the side chain contribution spectra underlying the original spectra Amide I' and II' regions are shown in Figure 15b.

N-Ras (1-189) unprocessed protein sequence.

Colouring (1-168) according to the X-Ray data (pdb 4Q21).

Colour coding: **Turns**, **α -Helices**, **Random coils**, **β -Sheets**.

Figure 15. N-Ras (1-189) unprocessed protein sequence is shown with one letter coding and the M1-M168 region is coloured according to the respective secondary structures of the residues, based on the pdb source 4Q21 (a). Side chain correction of the Amide I' and II' regions of the original spectrum is in black, with its side-chain spectrum below in red, and the sum of side-chains spectra in blue (b).

As stated previously, curve-fitting requires an initial guess for the underlying band parameters, i. e. the number, position, intensity, FWHH and shape of the bands. While the FWHH, area and band characteristics of each band can be approximated to a much lesser extent, the number and positions of the component bands under the Amide I' curve can be estimated using the local minima of the second derivative and the local maxima of the FSD (Figure 16).

Figure 16. Local maxima and minima tracing *via* FSD (intersected), and the second derivative (red). FSD and the second derivative of Amide I' region (black) are shown overlaid in full scale, with the original curve.

The single components as shown in Figure 16, were assigned to turns, α -helices, random coils and β -sheets as described before [111]. Namely, the high frequency bands from 1690 to 1665 cm^{-1} were assigned to turns, bands from 1662 to 1645 cm^{-1} to α -helices, bands from 1644 to 1635 cm^{-1} to random coils and bands from 1630 to 1610 cm^{-1} to β -sheets. After optimizing the parameters by fitting the predefined state spectrum, namely the truncated wild-type H-Ras

transmission spectrum, the same parameter set was applied as the initialization parameters to a minimization algorithm for the curve-fitting of the ATR-FTIR measurements' data. Curve-fitting was performed with the truncated protein Amide I' and the full-length protein Amide I' bands in both the protein's membrane-bound and not membrane-bound states' data. The exemplary curve-fitting results are shown in Figure 17.

Figure 17. Lipidated Ras protein Amide I' region with the underlying backbone absorbencies of the secondary structure elements is shown in a. Truncated Ras crystallography-based structure in its GDP

complexed form (pdb source 4Q21) is shown in b with the magnesium ion (blue dot). The α -helices and β -sheets are labelled according to their order in the primary structure.

As can be seen in Figure 17, we utilized the minimum number of bands representing the four main structural components. The number of local minima, observed from the second derivative of the Amide I' band (Figure 16), was exceeding the number of main components. This multiplicity represents the variation within the individual structural components. Therefore, compensating changes within the individual secondary structural elements themselves could have occurred. Those changes could only be resolved if the number of component bands to be fitted under the main curve were increased. However, using the minimum possible number of components during curve-fitting was preferred to avoid curve-fitting of the noise. Also, overlap among the individual structural component volumes were highest when low number of components were utilized to be fitted under the Amide I' band. Hence, those components were better correlated to the others; namely, the connectivity was increasing. This was eventually improving the solution.

3.4 Structural and Functional Interpretations

3.4.1 Curve-Fit Results

High quality ATR-FTIR spectrum of membrane-bound Ras protein, lipidated Ras protein in a membrane free environment and truncated Ras protein were obtained in a reproducible manner. Curve-fit analyses were performed on those spectra. Secondary structure results from the curve-fit analysis are summarized in the Tables II, III and IV and Figure 18, below.

Table II. X-ray (pdb 4Q21) based secondary structure of the truncated H-Ras protein is shown in comparison to the truncated H-Ras protein spectra curve-fit results, respectively.

	Truncated Ras from X-ray (pdb 4Q21), 1-166	Truncated Ras, 1-166
β -sheets	25,9 %	$21,4 \pm 3,00$ %
Random coils	12,6 %	$16,0 \pm 3,00$ %
α -helices	38,0 %	$39,3 \pm 3,00$ %
Turns	23,5 %	$23,3 \pm 3,00$ %
RMSD to X-ray and Standard deviation (StDev)		RMSD: 2,90 % StDev: $2,90 \times 10^{-6}$

The last column in Table II gives the outcome of the curve-fitting analysis for the truncated H-Ras protein, which was performed by using the previously generated curve-fit parameters. The results of the truncated Ras measurement of our experiment were in good agreement with the crystallography data (pdb source 4Q21) with minor deviations (RMSD less than 3 %). The results were also in good agreement with the truncated Ras NMR results when we utilized the average of secondary structures from pdb 1CRP, 1CRQ and 1CRR for comparison. The RMSD deviation was again less than 3 %. This was justifying the applicability of the parameters for the forthcoming measurements. Commonly, the NMR studies on the Ras protein revealed higher unstructured contents which were counterbalanced by roughly equal amounts of diminishes in other structural elements, while the overall structures were mainly consistent with the crystal structures [123, 124]. This information was pointing at the contribution of the protein dynamics enhancement in solution. Also our observation of a higher random content could be attributed to the same reason (Table II). However, a decrease

in the β -sheet content was also observed (Table II), which could be attributed to a specific orientation of the protein on the diamond IRE surface.

Truncated H-Ras protein curve-fit analysis results are shown in comparison to the full-length H-Ras protein in solution (Table III and Figure 18), in membrane-free environment and in membrane-bound states, respectively. Truncated H-Ras protein and the double lipid anchored full length protein that we have measured comprised 166 and 188 residues, respectively. So, the lipidated protein had extra 22 residues which needed to be structurally defined for proper interpretation of our data.

Table III. Truncated H-Ras protein spectra curve-fit results are shown in comparison to the secondary structure results of the lipidated Ras and membrane-bound lipidated Ras spectra.

	Truncated Ras 1-166	Lipidated Ras, 1-190	Membrane-Bound lipidated Ras, 1-190
β -sheets	$21,4 \pm 3,00 \%$	$25,8 \pm 3,00 \%$	$20,5 \pm 3,00 \%$
Random Coils	$16,0 \pm 3,00 \%$	$14,7 \pm 3,00 \%$	$20,2 \pm 3,00 \%$
α -helices	$39,3 \pm 3,00 \%$	$36,9 \pm 3,00 \%$	$37,4 \pm 3,00 \%$
Turns	$23,3 \pm 3,00 \%$	$22,6 \pm 3,00 \%$	$21,9 \pm 3,00 \%$
Standard deviation (StDev)	StDev: $2,90 \times 10^{-6}$	StDev: $1,97 \times 10^{-6}$	StDev: $7,89 \times 10^{-6}$

A recent NMR study [36, 125] on the C-term peptide of the farnesylated and hexadecylated N-Ras protein revealed that the C-term did not have any regular α -helix and β -sheet motifs, and was showing a versatile dynamics on the membrane surface. Also the NMR study [43] on the full-length H-Ras protein revealed that the C-terminal α -helix was extending to the 172th residue and the rest of the C-term was conformationally averaged in solution. Therefore we

expected that the extra 22 residues of the lipidated protein with respect to the truncated form, would be leading our observations to be in the direction of an increase in unstructured content as the major change in the structure of the lipidated protein. This was only the case for the membrane-bound lipidated Ras protein, but not in case of the lipidated protein in a membrane free environment (Table III, IV and Figure 18). Lipidated protein in a membrane free environment was showing a 4,4 % increase in the β -sheet content with respect to the truncated protein (Table III). However, membrane-bound lipidated protein was showing a 4,2 % increase for the random coils content (Table III). Deviation of the lipidated Ras protein structure results from the membrane-bound form, in a membrane free environment, could have been due to structural changes or a discrete orientation of the protein on the diamond IRE. Below (Table IV and Figure 18) is the comparison of our results with the information reported by the NMR investigation of the full-length H-Ras protein [43].

Table IV. Literature based [43 and pdb 4Q21] secondary structure of the full-length H-Ras protein is shown in comparison to the curve-fit results of the membrane-bound lipidated Ras and lipidated Ras spectra. GC insertion at residues 182 and 183 were accounted as random.

	Full-Length Ras from NMR [43] and X-ray (pdb 4Q21)	Membrane-Bound lipidated Ras, 1-188	Lipidated Ras, 1-188
β -sheets	22,9 %	$20,5 \pm 3,00$ %	$25,8 \pm 3,00$ %
Random Coils	19,7 %	$20,2 \pm 3,00$ %	$14,7 \pm 3,00$ %
α -helices	36,7 %	$37,4 \pm 3,00$ %	$36,9 \pm 3,00$ %
Turns	20,7 %	$21,9 \pm 3,00$ %	$22,6 \pm 3,00$ %
RMSD to NMR and Standard deviation (StDev)		RMSD: 1,30 % StDev: $1,97 \times 10^{-6}$	RMSD: 3,90 % StDev: $7,89 \times 10^{-6}$

Figure 18. NMR* [43] and crystallography* (pdb 4Q21) based secondary structure of the full-length H-Ras protein is shown in comparison to the secondary structure results of membrane-bound lipidated Ras and lipidated Ras spectra, respectively. GC insertions at the 182th and 183th residues were accounted as random.

The C-terminal α -helix was reported to be extending to the 172th residue and the rest of the C-term was conformationally averaged in solution, namely unstructured [43]. This information was supported by the previous NMR and crystallographic studies which were performed with the truncated form of the protein possessing 171 residues [123, 126]. Secondary structure values were recalculated from X-ray (pdb 4Q21), along with the information provided by the NMR measurement of the full-length Ras [43]. Accounting the recalculated values as a reference; membrane bound lipidated protein came to have significantly close values to that reference (Table IV). Whereas, a decrease in the random coils content accompanied with an increase in the β -sheet content was observed for the lipidated Ras protein in a membrane free environment (Table IV).

We also guessed the number of residues corresponding to the secondary structure percents of the membrane-bound N-Ras protein that were obtained from the curve-fit analysis. NMR data

[43] and the membrane bound lipidated N-Ras protein were in agreement, when the two residues at positions 181 and 182 were accounted as random for a proper comparison. However, if there were no membrane available for binding, a decrease in random coils up to 10 residues was accompanying an increase in the β -sheets and also in turns. Therefore we claim that doubly lipidated full-length protein was maintaining the native conformation when the lipidated protein was bound to the membrane. So, our results could be pointing to a novel contribution of the second anchoring moiety in the protein structure; which was already known to be necessary for the plasma membrane targeting [28, 29], and was also known to be serving distinct roles in trafficking, microlocalisation and signaling, as individual residues [127]. The full-length Ras proteins which were utilized by the former studies comparable to ours were commonly lacking the second anchoring moiety. Hence, we suggest that the second anchoring moieties can also be preventing the regular structure of the protein when the protein is in a lipid free environment. This is detailed below.

Role of the anchoring moieties;

Most of the former investigations were carried on with the full-length protein lacking the membrane-anchoring moieties, or with the non-palmitoylated protein possessing only the farnesyl moiety [43]. Otherwise the studies were carried out with the membrane-bound protein. There was no specific investigation dedicated to the fate of the doubly lipidated protein, namely the fully processed protein for plasma membrane targeting in a membrane free environment. It was observed as a result of our curve-fitting analysis that the doubly lipidated protein was deviating similarly from the previously established NMR [43] data and from our own data of the membrane-bound form, when a membrane was not available to the protein for binding (Table IV and Figure 18).

The study of Meister and coworkers [40] measured the IRRAS spectra and they were utilizing the same protein as in our measurements, bound to a lipid monolayer. However, the mentioned study was not comparable to our investigation for several reasons. First, the measurements of the mentioned study were performed in 100 mM deuterated NaCl solution;

but our measurements were performed in deuterated Tris buffer, pD 7.5. Also, there was a discrepancy among the lipid layers which were utilized. Ras protein is inserting normally to one leaflet of the membrane with its anchoring lipid moieties at the C-terminus, yet the properties of a lipid monolayer, bilayer or a multilayer are not the same for the lipid order and dynamics that is sensed by the protein directly *via* the anchoring moieties and also through the residues in contact with the membrane. Additionally, part of the observations reported by the IRRAS study was originating from the protein located at the air-D₂O interface, rather than the membrane-bound protein [40].

Further, two doubly lipidated N-Ras proteins were measured by Meister and coworkers. The hexadecylated and farnesylated N-Ras protein was the same as the one that was utilized in our measurements; and the farnesyl anchoring moiety was replaced with a hexadecyl moiety in case of the other protein. So, one N-Ras was doubly hexadecylated, and the other was hexadecylated and farnesylated. Several differences were observed by Meister and coworkers, among the two N-Ras proteins under the same conditions but with differences in their anchoring moieties. This was somehow supporting our conclusion regarding the significance of the second anchoring moieties, in terms of the protein structure.

3.4.2 Second Derivative Analysis

3.4.2.1 Second Derivative Results

3.4.2.1.1 Tyrosine Side Chain

Differences among the second derivatives of the lipidated Ras protein spectra, with regard to the membrane availability for binding, were also investigated to abstract information. The first clue came from the Tyr side chain vibration (Figure 19).

Figure 19. Effect of membrane binding on the lipidated Ras protein Tyrosine side-chain C-C stretching vibration. The dotted lines belong to the second derivative spectra.

Tyrosine side-chain is indicative for membrane-interaction upon shifts and disturbances such that we have observed as a splitting of the band at around 1515 cm^{-1} (Figure 19). This was supported with the previous modeling studies which were suggesting that there were localised perturbations of DMPC in the Ras-DMPC complex [128].

3.4.2.1.2 Membrane-Bound Ras Structure in Detail

Membrane-bound lipidated Ras spectra were normalized and compared to the lipidated Ras protein spectra, which was measured in a membrane free environment (Figure 20); along with their respective spectra, which were measured with parallel and perpendicular polarized IR beams (Figures 22 and 23). Significant changes were observed in both low and high frequency β -sheet regions (Figures 20b, 22 and 23). There were variations which could be

observed at different times of absorption, with regard to the availability of a membrane for binding (Figure 20). Yet, the band maxima were kept around $1637\text{--}1640\text{ cm}^{-1}$ during the measurements (Figure 20).

Figure 20. Lipidated Ras (red) and the membrane-bound lipidated Ras spectra (blue) are shown in comparison to each other for the spectra recorded at the 8th and 16th minutes (a). Lipidated Ras (red)

and the membrane-bound lipidated Ras spectra (blue) derivatives are shown in comparison to each other for the spectra recorded at the 8th and the 16th minutes (b).

Membrane-bound Ras absorbencies at around 1627 and 1695 cm^{-1} were representing mainly the intermolecular antiparallel β -strands. Latter was observed at 1691-1693 cm^{-1} (Figure 20b). Band at around 1668 cm^{-1} is a feature arising from reverse turns [129] (Figure 20b); and could be indicative of a nucleation site for hairpin formation [83]. If we simply comment on the data (Figure 20b), bands which are representative of strongly coupled systems at around 1618 and 1683 cm^{-1} (at 1616 and 1680 cm^{-1}) were observed in the 8th minute spectrum of the membrane-bound Ras; but the band at around 1616 cm^{-1} was observed only as a weak shoulder. Yet, it is known that broad absorbance bands cannot be observed as sharp, distinct features in the second derivative spectra. Later in the 16th minute spectrum, the band at 1680 cm^{-1} was nearly vanished. Features of the weakly coupled systems, namely the bands at around 1615, 1646 and 1660 cm^{-1} were observed rather constant. The assignment of the weak shoulder at 1615 cm^{-1} was ambiguous because both side chains [82] and β -sheets can exhibit vibrational transitions around this frequency; and also the exact positioning of a shoulder is hard to distinguish. These features all together could be attributed to β -hairpin motif. The bands were also differentiating in intensities and positions with respect to the differences in the data measurement times (Figure 20b). This could be attributed to the dynamic nature of the protein on the surface.

From a visual inspection, we may suggest that the higher splitting between the low and high frequency components were observed in case of the membrane-bound Ras (Figure 20b). This could be indicative of a β -hairpin at 8th min, which would be a tightly packed β -hairpin with a relatively strong coupling. While, the minor splitting between the low and high frequency components at the 16th min was implying that the coupling was weakening, which would then be indicative of either a twisted and/or a loosely packed β -hairpin conformation. The band at around 1648 cm^{-1} could be assigned to fully unfolded structures or random coils [130]. However, it was more likely to be assigned to α -helices, which had shifted from the higher

frequencies in deuterated medium. Finally, changes within the region from 1662 to 1645 cm^{-1} were assigned to α -helices (Figures 20b, 22 and 23).

Control;

The use of second derivative spectra interpretation in case of original spectra with a low signal-to-noise ratio is commonly criticized due to the enhancement of noise during the derivation process. In order to guess the contribution of noise in the second derivative spectra that we have used (Figure 20); random noise spectra were calculated and subtracted from the original spectra before derivation, to observe directly the contribution of noise variation in our derivative spectra (Figure 21).

Figure 21. Lipidated Ras (red) and the membrane-bound lipidated Ras spectra (blue) derivatives with varying noise contributions are shown in comparison to each other for the spectra recorded at the 8th and the 16th minutes.

Random noise spectra were calculated from the water vapour spectra which were measured with the same set-up, by subtracting two consecutively measured spectra from each other. Eventually, three different ‘noise’ spectra were calculated in the same manner; and subtracted

from the original spectra before taking the derivative of the original spectra. The resulting second derivative spectra of the original spectra with varying noise contents are shown overlapped (Figure 21). Slight variation in the original spectra was attributed to the contribution of noise, which was negligible for our interpretations (Figure 21).

3.4.2.1.3 Measurements with Polarized Light

Within this and the coming sections, ‘polarized measurements’ denotes that the measurements were performed with the polarized IR beam. Features related to the orientation of the membrane-bound Ras and lipidated Ras in a lipid free environment could be guessed from polarized measurements (Figure 22 and 23). Quantitative analysis of the spectra was not straightforward congestion with overlapping features (Figure 22 and 23). Still, it was observed that the changes were rather significant for the membrane-bound Ras, among the parallel and perpendicular polarized measurements, upon visual inspection (Figure 22).

Figure 22. Perpendicular ($\perp$) and parallel ($\parallel$) polarised measurements’ spectra second derivatives are shown overlaid for the lipidated Ras and the membrane-bound lipidated Ras spectra derivatives, in a stacked manner, respectively.

In case of the lipidated Ras in a membrane free environment; there was a redshift of the band at around 1670 cm^{-1} and blueshifts of the bands at around 1610 and 1640 cm^{-1} , for the parallel polarized measurements (Figure 22). Yet, second derivatives of the membrane-bound Ras spectra were revealing significant variations among the parallel ($\parallel$) and perpendicular ($\perp$) polarized measurements (Figure 22). In case of the membrane-bound Ras; there was a blueshift of the shoulder at around 1628 cm^{-1} and redshifts of the bands around 1675 and 1690 cm^{-1} , for the parallel polarized measurement data (Figure 22). Further, there were new bands at around 1697 , 1667 and 1617 cm^{-1} , again in case of the parallel polarized measurement data (Figure 22). The band at around 1667 cm^{-1} could be the blueshift of a feature observed as a weak shoulder in the perpendicular polarized measurement data (Figure 22).

Figure 23. Lipidated Ras and the membrane-bound lipidated Ras spectra derivatives are shown overlaid, in comparison to each other for the polarized measurements. Parallel ($\parallel$) and perpendicular ($\perp$) polarized measurements' spectra second derivatives are shown in a stacked manner.

In Figure 23; membrane-bound Ras and lipidated Ras in a lipid free environment are compared for their respective second derivative spectra of those measured with the same

polarization. Changes in the perpendicular polarized measurement data for the lipidated Ras were less prominent in comparison to the parallel polarized measurement data, when the protein was bound to the membrane (Figure 23). Still, there was a new band at around 1664 cm^{-1} in case of the membrane-bound Ras; and at around 1601 cm^{-1} in case of the lipidated Ras in a membrane free environment (Figure 23). Further, there were blueshifts at around 1676 and 1638 cm^{-1} ; and redshifts of the bands around 1649 and 1609 cm^{-1} , when the protein was bound to the membrane (Figure 23).

If we summarise the changes in the lipidated Ras from the parallel polarized measurement data on top of the Figure 23, when the protein was bound to the membrane; low frequency components at 1617 and 1609 cm^{-1} were becoming prominent, along with the high frequency components at 1648, 1667 and 1697 cm^{-1} . Besides, slight shifts in the frequencies of the components at 1658 and 1673 cm^{-1} were observed as a 1 cm^{-1} redshift and a 2 cm^{-1} blueshift, respectively, for the membrane-bound Ras protein. Accordingly, it is suggested that the characteristic features that were observed through the polarized measurement data of the membrane-bound Ras (Figures 22 and 23) were indicative of a β -hairpin motif. β -hairpin consists of several bands [130]. Three well resolved peaks at around 1615, 1637, and 1674 cm^{-1} ; and two less resolved peaks at around 1648 and 1660 cm^{-1} were observed in case of a 16-residue β -hairpin [130]. In our case (Figures 22 and 23), the absorbencies at around 1615, 1674 and 1648 cm^{-1} were the ones observed only for the membrane-bound lipidated Ras but the bands around 1637 and 1660 cm^{-1} were observed in both forms of the protein. The latter case was expected, as those bands overwhelmingly represent coils and α -helices contents in deuterated medium, respectively for the 1637 and 1660 cm^{-1} . Rigorous assignments of Amide I' spectral features were difficult, but the bands at around 1637 cm^{-1} (the low frequency component) and 1674 cm^{-1} (the high frequency component) were also attributed previously [130] to amides associated with antiparallel β -sheet conformations. The weak band at 1697 cm^{-1} (Figure 23) was previously assigned as a spectroscopic signature for the structural perturbation of a glutamine residue [131].

3.4.2.1.4 Curve-Fit Results

Curve-fit results of the lipidated Ras and membrane-bound lipidated Ras spectra for the perpendicular ($\perp$) and parallel ($\parallel$) polarized measurements' data are summarized in Table V. Curve-fit results of the polarized measurements' spectra α -helix and β -sheet contents were revealing significant deviations for the vertical polarized measurement of the lipidated Ras (Table V). Hence, those results of the lipidated Ras (Table V) were pointing at a transition associated with the conformational alterations among the components perpendicularly oriented to the IR surface. Conversely to our expectations of a random orientation, this result was suggesting that the lipidated Ras protein was also oriented directly on the IRE surface.

Table V. Curve-fit results of the lipidated Ras and membrane-bound lipidated Ras spectra are shown in comparison to the perpendicular ($\perp$) and parallel ($\parallel$) polarized measurements data, respectively.

		α -helices	β -sheets	Standard deviation (StDev)
Lipidated Ras	No polarisation	$36,9 \pm 3,00 \%$	$25,8 \pm 3,00 \%$	StDev: $7,89 \times 10^{-6}$
	Vertical pol. $\perp$	$33,7 \pm 3,00 \%$	$29,4 \pm 3,00 \%$	StDev: $7,96 \times 10^{-6}$
	Parallel pol. $\parallel$	$38,2 \pm 3,00 \%$	$20,2 \pm 3,00 \%$	StDev: $11,1 \times 10^{-6}$
Membrane-bound lipidated Ras	No polarisation	$37,4 \pm 3,00 \%$	$20,5 \pm 3,00 \%$	StDev: $1,97 \times 10^{-6}$
	Vertical pol. $\perp$	$37,2 \pm 3,00 \%$	$20,8 \pm 3,00 \%$	StDev: $2,87 \times 10^{-6}$
	Parallel pol. $\parallel$	$39,6 \pm 3,00 \%$	$18,0 \pm 3,00 \%$	StDev: $4,34 \times 10^{-6}$

Contrary to the lipidated Ras on the IRE surface, second derivatives of the spectra with the polarized measurements were varying more in case of the membrane-bound Ras protein (Figure 22). However, the changes which were observed among the parallel and vertical polarized measurements of the membrane-bound Ras (Figure 22) were not significant in case of the curve-fit results (Table V). This outcome was probably pointing at a modification which was observed mainly in the β -sheet contents of the membrane-bound Ras protein (Figure 17). Still, it was possible that the variation of the structural components oriented parallel to the membrane surface were subtle but significant, and this was only revealed in the second derivative spectra (Figure 22). Hence, curve-fit analysis might have not been able to identify changes in the membrane-bound Ras structure with regard to the parallel versus perpendicular polarized measurements (Table V). Additionally, compensating transformations among the individual structural contents cannot be detected by the curve-fit technique. Further, oligomerization might have occurred without any shift in the overall structural contents of the protein; yet, the indicative bands could have recognized in the second derivative spectra (Figure 22 and 22) with a narrow and intense IR band centered at around 1616 cm^{-1} [88].

3.4.2.2 Discussion

β -Hairpin Motif;

Membrane interaction of the protein was detected through the Tyr side chain band splitting section (3.4.2.1). Also, characteristic features which were observed through the polarized measurements' data of the membrane-bound Ras (Figures 22 and 23) were indicative of a β -hairpin motif (sections 3.4.2.2 to 3.4.2.4). Highly dynamic character of the less residue spanning β -hairpins were reported [83, 84] with special emphasis on the turn residues' and the role of pH in defining the type of β -hairpins and their coexistence [85]. Previously, strand to 3_{10} -helix transitions at residues A146, K147 and T148 were observed concomitantly among

the total 40 structural models provided by the pdb sources 1CRP and 1CRQ. The residues from F141 to Q150 were showing kind of a similar dynamicity to our interpretations. We claim that formation of a β -hairpin in the region spanning the residues P140-Q150 was probable. The region along with the nucleotide binding pocket is shown below (Figure 24).

Figure 24. F141-Q150 region is shown within the membrane-bound Ras protein according to the X-ray (pdb 4Q21) and NMR structures [36, 43]. The residues are labeled with their respective colors with the model of truncated (1-166) Ras*GDP (pdb 4Q21). The nucleotide in the middle has purple oxygen and blue nitrogen coloring.

According to the structural models provided by the pdb sources 1CRP and 1CRQ, a strand-bridge to strand transitions at F141 and strand-bridge to turns transitions at S145 and Q150

were observed. The latter two transitions were linked with each other. Further, strand to coil transitions were observed at residues I142, E143 and T144. Less frequently, strand to 3_{10} -helix transitions were observed at residues A146, K147 and T148, and in a fashion that the residues were consistent with each other. Strand-bridge to strand transitions were observed also at other strands but to a lesser extent, within the structural model contents of the pdb sources 1CRP and 1CRQ.

The possibility of β -hairpin occurrence in the F141-Q150 region depends on the response of the turns residues at positions 146 to 149, and of the residue Q150. Residues from 143 to 146, with one letter codes TSAK, are intrinsically similar to the NPGD motif which was generally selected and investigated as the segment that shows the highest type I β -turn probability in crystalline proteins [132]. Both have polar-polar-unpolar-charged residues in the given order. Additionally, the band which was observed in our data at around 1668 cm^{-1} (Figure 20b) was a feature arising from reverse turns [129], or β -turns, which were known to be the nucleation sites for the hairpin formation [83].

Driving Conditions;

We could also speculate on the significance of β -hairpin formation upon membrane-binding of the doubly lipidated N-Ras protein and the decrease in the protein stability otherwise. Solvent dependent switching between helical and hairpin conformations was shown by Rai and coworkers [83, 133]. Intramolecular H-bonding was favored by the peptides in a weakly interacting solvent; and exposed H-bonding bearing peptides in a β -hairpin conformation were favoring H-bonding solvents [83, 133]. The working conditions of Ras protein also dissected in a similar manner in terms of the intrinsically differing H-bonding capacities of the cytosol and the membrane. When the lipidated protein is not interacting with the membrane, water molecules in the cytosol would be effectively competing for the hydrogen bonding sites [134] of the protein which is exposed. The region under question, spanning from ~P140 to ~Q150, is a solvent exposed region in the vicinity of the membrane (Figure 24).

The P140-Q150 region also interacts with the guanine base in a direct and / or indirect manner. Therefore, a net effect on the GTPase activity of such a transition is also expected. The region along with the nucleotide binding pocket is shown below, with the side-chains (Figure 25).

Figure 25. F141-Q150 region is shown with sticks, along with the nucleotide binding pocket. The residues are labeled with their respective colors through the model of truncated (1-166) Ras*GDP (pdb 4Q21). The nucleotide in the middle has purple oxygen and blue nitrogen coloring.

The region encompassing the residues 141 to 150, is including part of the ExSA motif (residues 143-146), or the guanine binding region G3 [135], which has indirect interactions with the guanine base (Figure 25). Main chain NH of A146 is strongly H-bonded to O6 of the base and the side chain of S145 is H-bonded to D119, which is in contact with the guanine base [135]. Side chain of K147 makes strong aliphatic interaction with F28, which is also in contact with the guanine base [135]. E143 has several contacts with the residues in the NKxD

(residues 115-118) and ExSA regions [135]. A146 and K147 were reported to be H-bonded to the guanine base. Latter case was confirmed by another NMR study [124]. Also, perturbations for backbone amides of the full-length H-Ras in comparison to truncated H-Ras were detected [43] at residues I142, T144, S185 and T-148.

Oligomerization;

Last but not the least, it is known [134] that β -hairpin aggregate formation was judged by the appearance of a narrow and intense IR band centered at around 1616 cm^{-1} [88]. Though it was not a prominent observation of all instances, this spectral feature was observed in our second derivative spectra as well (Figures 20b, 22 and 23). Hence, such a dynamic β -hairpin motif could be taking active role in the mechanism which was triggering oligomerization of the protein on the membrane surface, in the form of nanoclusters. The dynamic nature of the protein structure was evolving in time, which was observed as the variation in the second derivative spectra of the membrane-bound Ras protein, with respect to different times of membrane adsorption (Figure 20b). Protein adsorption on the surface takes longer than the times of the data which were shown in Figure 20; therefore, the driving factor of the mentioned variation might have been the change in the protein concentration on the surface.

It was previously reported [123] that the H-bonds involving the residues D38, D57 and T58 were not strong. This was making part of the β -sheet structure flexible and the information was contrasting to the rigidity in crystalline states [126]. In relation, it was suggested [123] that the salt bridge between D33 of one Ras protein molecule and R135 of another symmetry-related molecule in the crystalline state [126] was stabilizing the loop L2 conformation and the end of antiparallel β -strand β_2 . Besides, the residue N26 which was found to be essential for the signal transducing activity of Ras [136], was exhibiting a flexible nature in solution [123]. This was also suggested to have the potential to be ascribed to the intermolecular contact of loop L2 in the crystal structure [126]. Therefore we may claim that the protein could be oligomerizing and / or stabilized with intermolecular interactions under certain

conditions which needs to be studied in further detail for the effect of membrane recruitment. The residues mentioned above, and within the rest of the text, can be seen through Figure 26.

Figure 26. Stereo view of two GDP complexed Ras proteins (pdb 4Q21). The residues are labeled with their respective colors through the model of truncated (1-166) Ras*GDP (pdb 4Q21). The nucleotide has purple oxygen and blue nitrogen coloring. Residues from 169 to 171 adapted from the NMR structure of Ras (pdb 1AA9).

Additional Structural Features;

Full-length H-Ras in a DMPC bilayer was studied through modeling [42], which was simulating the protein in the Mg^{2+} and GDP *versus* Mg^{2+} and GTP complexed forms. In both of the forms, major interaction between the G-domain and the linker was involving a salt bridge between the E49 (initial residue of $\beta 3$) and K170 side chains. This salt bridge would be prevented in case of the truncated protein with 166 residues. The other end of the $\beta 3$ possessing the residue E49 was reported to be flexible in solution [123], in contrast to the rigidity in crystalline states [126]. It was also stated [42] that the K170 and R169 had the

potential to form H-bonds with the charged residues of the lipid phosphate head groups. Recent MD-simulations [137] were suggesting for the GTP- and GDP-bound H-Ras that; D47, E49 in the $\beta 2$ - $\beta 3$ loops and R161, R164 in the 5th α -helix were arranging differently with respect to the 4th α -helix, which were in a region adjacent to the hypervariable region. Also, alanine substitution of the GTP-bound Ras residues R161/R164, D47/E49 and R128/R135 were highly correlated [137].

3.5 Summary and Outlook for the Membrane-Bound Ras Structure Investigation

This study demonstrates an application of the previously established principle of the self-assembled planar lipid bilayers on solid or gel supports [76, 138-144]. hATR and vATR set-ups were both successfully utilized for the measurements of the truncated H-Ras and lipidated N-Ras proteins. Adsorption kinetics of both the POPC and the protein were slightly differing in case of hATR *versus* vATR, due to the inherent differences of the two set-ups; otherwise the kinetics of the measurements with the two set-ups were consistent. Yet the curve-fit analysis outcomes of the measurements were the same among hATR and vATR; as they were based on the same phenomenon and straightforward, that the rapid sample exchanges were feasible. However, hATR enabled more direct handling of the system which brought the set-up to a superior level in the sense that the reduction in time, energy and material usage during the measurements increased the efficiency and reproducibility of the experiments in return. As already mentioned, material consumption with the hATR was 20 times less which lead to a requirement of only minute quantities of the sample (i.e. $\sim 2,4 \mu\text{g}$ of lipidated Ras protein) for our measurements. This could well be modified accordingly to less material demanding experimental designs. Nevertheless, increasing number of active internal reflections provided better signal quality to the vATR measurements. For specific experimental purposes, one needs to think of the special IRE properties that are available to the ATR accessories in hand.

The first high quality ATR-FTIR spectrum of membrane-bound Ras protein was obtained with the set-ups described above. A multitude of previous structural studies on Ras protein was confirmed by utilizing the instrumental facilities along with the curve-fitting of the truncated Ras protein (1-166) results, and the results of lipidated Ras protein (1-188) in membrane-bound form. There were some deviations of lipidated Ras structure from the membrane-bound form. It was concluded through comparison with the previous studies that the second anchoring moiety of the protein was not only critical for membrane targeting but was critical for maintaining the protein stability as well. Membrane interaction of the protein was detected through the Tyr side chain band splitting. Further, polarized measurements were performed and the outcomes were pointing at a modification of orientation which was observed mainly in the β -sheet contents of the membrane-bound Ras protein. Moreover, inspection of the second derivative spectra revealed indicative bands of β -hairpin motif. It could be relevant to the protein clustering on the membrane.

In the future, the details and dynamic interactions of the membrane-bound N-Ras protein with its effectors and regulators (i. e. Raf-RBD, GEFs and GAPs) will be studied, along with the Ras intrinsic activity, and activity modulation by the environmental factors and the membrane. Also, significant crosstalk among the membrane and the Ras protein [41-43] can be studied in detail, regarding the anchoring moieties and the structural perturbations, with the technique presented here. Other than elucidating the details of protein-membrane interaction phenomenon with such complex natures; this approach may pave new grounds for therapeutically significant solutions to cancer and to other pathologically relevant conditions.

4 Ras Protein Nitric Oxide Interaction

4.1 Nitric Oxide Overview

Nitric oxide (NO) is an extremely diffusible inorganic radical in gas form and well-known as a hazardous contaminant from car exhausts, fossil remnants and cigarette smokes. Its biological functions were discovered quite recently in the 1980s [145]; but not earlier, because of its low levels of physiological concentrations and short half times. Not later, it was called as the molecule of the year in 1992 [146]. It is also called as the endothelium derived relaxing factor (EDRF) [147-149].

NO is generated as shown in Figure 27, along with the citrulline, by the reaction of arginine with the O_2 via the enzymatic action of nitric oxide synthases (NOS) [150].

Figure 27. NO production by means of NOS. The NOSs catalyze an NADPH- and O_2 -dependent oxidation of Arg to generate NO and citrulline, with formation of N-hydroxyarginine as an intermediate. [Figure adapted from Wang, Z-Q. *et al. J. Biol.Chem.* **282** (2007) pp. 2196-2202.]

It can be released by several different cell types in mammalian systems [145, 149, 150]. The human (and mouse) genome contains 3 different genes encoding NOSs; those are the neuronal (n)NOS, inducible (i)NOS which are found in macrophages, and the endothelial (e)NOS that

line the lumens of the blood vessels. iNOS genes are expressed in the cell upon stimuli [151]; and its response to the changes in the calcium levels is suppressed with the activity, which is sustained at nmolar range. However, production of NO by the nNOS and eNOS are at pmolar ranges. [150, 152]

NO mediates a wide array of signaling pathways of the target cells, both intra- and extracellularly. Its roles came to be known in many physiological processes; some are the neural signaling, immune response, inflammatory response, ion channels' regulation, penile erection, phagocytic defense mechanism, and cardiovascular homeostasis along with the atherogenesis, or the cardiac decompensation after long-standing vascular diseases [153-156]. NO acts as a neuromediator including the formation of memory in neurons of the central nervous system and coordinates among the neuronal action and blood flow, and pain modulation [157]. NO also takes place in the pathogenesis of several diseases in a concentration dependent manner; where, NO deficiency can lead hypertension, hyperglycemia, atherosclerosis, Parkinson's disease, Alzheimer's disease, but increased levels of physiological NO concentrations may take part in cancer, arthritis and reperfusion injury. Reactivity of NO is highest with the superoxide and the other active radicals; moderate level with the transition metals ions; and the least with the molecular oxygen [158, 159]. Complexity in the biological media needs to be resolved with the combined model systems; therefore, several NO detection methods have been proposed [160-172, 147-149]. Its production in vast amounts by white blood cells to combat infectious bacteria and parasites, lead to the initial realization of controlled NO release as a prospective strategy against cancer.

4.1.1 Physical, Chemical Properties and Reactions of NO

Physical and Chemical Properties;

NO is a colorless gas with a molecular weight of 30,01 g/mol. Its boiling point is 151,7 °C at sea level. It is the simplest identified paramagnetic molecule. The nitrogen and the oxygen atoms are polarized with a partial specific negative charge on the oxygen and a partial specific positive charge on the nitrogen atom ($\cdot\ddot{\text{N}}\equiv\ddot{\text{O}}\cdot$) [166]. Its linear molecular shape enables the trigonal planar geometry of the electron pair around both nitrogen and oxygen atoms. The bond distance and bond energy for NO are 1,2 Å and 149,9 kcal/mol [166]. The electrostatic field of NO's repelling the hydrogen ions account for its hydrophobic properties that allow the molecule to freely diffuse through biological membranes [166]. Biological membranes and the other hydrophobic tissue compartments are the main regions of the cellular environment for the NO metabolism and for the NO-derived reactive species development [167]. While the solubility of NO in water is approximately $1,7 \times 10^{-3}$ mol/l, it is nine times higher in a hydrophobic solvent such as hexane [167]. Likewise, NO is rather soluble in hydrophobic membrane cores than in aqueous environments.

NO is thermodynamically unstable, as a result, NO synthesis from its elements N_2 and O_2 occur to an extent at high temperatures [168]. Despite the thermodynamic instability of NO, its decomposition is kinetically delayed; the gas can be stored indefinitely at 1 atm pressure and ordinary temperatures without significant decomposition [168]. NO can rapidly and directly react with the unpaired electron on both organic and oxygen-centered radicals to yield a variety of highly reactive intermediates [162]. NO can be oxidized by one electron to yield the nitrosonium ion (NO^+) or reduced by one electron to form the nitroxyl anions (NO^-) which are the central intermediates of the NO chemistry [166]. The physical properties of the three species are summarized in Table IV [168].

Table IV Characteristics of NO, NO⁺, NO⁻.

	d_{N-O}	v_{NO} (cm⁻¹)	Bond order
NO⁺	0,95	2300	3,0
NO	1,15	1840	2,5
NO⁻	1,26	1290	2,0

Reactions;

NO is highly reactive but still less reactive than many free radicals for the reason that it cannot interact with itself. In oxygenated solutions, its half-life could be long as 4 minutes [168]. However in biological systems, stability weakens to a half-life of less than a half minute, which is indicative of a high demand and fast consumption of the molecule in biological media [168]. Still, it's being small in size and an uncharged molecule enables the NO to diffuse rapidly among the cells, across the membranes, and through the distances of more than several hundred microns [168].

The reaction of NO with O₂ in the gas phase results in the nitrogen dioxide (NO₂) end product formation. The kinetics of this reaction is second order in NO (eqn. 7) in aqueous solution, however, the reaction of NO with O₂ occurs with the overall stoichiometry of eqn. 8 [169, 170].

The reaction of NO with the superoxide (O₂⁻) results in the peroxynitrite which is stable, as shown in eqn. 9.

When NO and O₂ are produced simultaneously and in close proximity, they react to form the product peroxynitrite (OONO⁻). When certain reactive centers are available, HOONO may undergo homolytic cleavage to a hydroxyl free radical (•OH) and nitrogen dioxide free radical (•NO₂), or may undergo heterolytic cleavage to a nitronium cation (NO₂⁺) and hydroxide anion (OH⁻). Three of these cleavage products (•OH, •NO₂ radicals and NO₂⁺) are among the most reactive and detrimental species for the organisms, biological systems. (Figure 28)

Figure 28. Decomposition of NO by the superoxide anions; and the sources of ROS and RNS that are generated endogeneously by cardiovascular cells. Endothelial cells produce reactive oxygene species such as superoxide anions ($\cdot\text{O}_2^-$), through the mitochondrial oxidative metabolism or the other enzymatic activities (i. e. oxidase components of NADH/NADPH, cyclooxygenase, cytochrome P450 reductase, uncoupled NOS, and during the oxidative enzymes, hemoglobin, and mitochondrial metabolisms), like the other components of the vascular peripheries. NO, which is produced primarily by 1 of the 3 forms of NOS, reacts with oxygen to generate potent S nitrosylating agents, $\cdot\text{NO}_2$ and N_2O_3 ; and can interact with $\text{O}_2^{\cdot-}$ to form the highly reactive peroxynitrite. Modified from Zimmet and Hare, 2006 [171].

4.1.2 NO Detection and Interaction Studies

Tracking of NO in living tissues possesses some challenges such as its high reactivity and low concentration in tissues [173]. The frequently used NO detection techniques are the chemiluminescence, Griess assay, fluorometry, electrochemistry, and spectroscopy [168]. All these methods except ESR and electrochemical techniques are indirect for endogenous NO estimations which are relying on the measurements of NO derivatives such as nitrite and nitrate [168].

The chemiluminescence technique [174] is based on the reaction of NO with ozone (O₃) to produce nitrogen dioxide in the excited state, as given in the equations 10 and 11 below:

The reaction with O₃ is known for its chemiluminescence that produces a deep red glow and the emission spans the range of 600-3000 nm [175]. Trace amounts of NO can be resolved through reacting with an external source of ozone. The technique has been applied to measuring NO in exhaled breath [176-182]. The major limitation is the requirement of real-time measurements for following the NO dynamics.

Griess assay is a common procedure [183] which is based on measuring the NO₂⁻ and NO₃⁻ ions after reduction of the NO₃⁻ to NO₂⁻ [184], as the biological metabolites of NO or as the solution content of NO. The Griess assay is a well-known two-step diazotization reaction in which NO₂⁻ in the sample reacts with sulfanilic acid to produce the diazonium ion, which will

then couple to N-(naphthyl) ethylenediamine to form the chromophoric azo derivative [185]. The Griess reaction is simple, reproducible and fast, but has limited detection for NO_2^- , in between the range 0,1-1,0 μM [186, 187].

Fluorometric techniques are used in bio-imaging of NO [188]. The batch fluorometric assay is based on the reaction of NO with 2, 3-diaminonaphthalene as an indicator of NO formation, and is conducted under acidic conditions to yield the fluorescent product 2, 3-naphthotriazole [189]. The major drawbacks of its indicator are the time consuming reagent preparation process and the lack of selectivity at low pH due to the interference of NO_2^- [168].

Electrochemical techniques offer advantages such as direct, continuous *in vivo* detection of NO, with minimal reagent requirement and selectivity, along with the stability and sensitivity [168]. Direct detection of NO is performed through the oxidation of NO on the surface of a working electrode producing Faradaic redox current [168]. Metallophthalocyanine and metalloporphyrins are preferred in electrochemistry among the widely used catalyst metal complexes of porphyrins [190-194] and phthalocyanines [195-198], due to their similarity and to the metalloporphyrins' mimicking the properties of the naturally occurring metalloporphyrin complexes. They enable NO detection even at concentrations in the nM range [168].

Electrochemical detection is powerful in the sense of sensor applications and for the NO release detection from the biologically relevant media; whereas, investigations for the structural and functional details of the NO-reacting media can be performed better with the spectroscopic techniques. Since the 1960s, NO has been studied with the ESR for the structural environment of metals in heme and nonheme proteins, as it binds strongly to iron to form iron-nitrosyl complexes [199]; such as the HbNO (hemoglobin nitric oxide), MbNO (myoglobin nitric oxide), and the enzymes nitrosyl cytochrome c oxidase and nitrosylguanylate cyclase. NO presence is usually detected through the formation of metal complexes, due to the difficulty of detecting NO directly by ESR [200]. Yet, continuous-real time monitoring of NO with concentrations higher than 0.5 mM is possible with ESR, but has the drawbacks which are the lack of commercially available ion traps, specialized operators requirement, high cost and interference of the other redox species [168]. Spectroscopic

techniques such as FTIR and Raman are also used for the same purpose and are prone to similar limitations and advantages. As an example of their use, 5- and 6-coordinate nitrosyl hemes were compared with their corresponding spectra for the nitrosyl hemoglobin A (HbA-NO), *via* their infrared, electron paramagnetic resonance, and visible-Soret spectra, in the presence and absence of inositol hexaphosphate [201]. Likewise, NO binding mode to the heme of myoglobin (Mb) was investigated *via* FTIR; and the environmental, mutational effects were revealed through the FTIR photolysis difference spectra of Mb¹⁴NO and Mb¹⁵NO for several Mb variants [202].

4.2 NO Studies of Ras GTPases

Through the previous cell-based studies, it was demonstrated that NO can react with the Ras protein and promote the Ras activation through the guanine nucleotide exchange [203-207]. It has been shown that nNOS induced endogenous NO release promoted the Ras activation in primary rat cortical cells [208]; while in human embryonic kidney cells, the nNOS sourced endogenous NO was contrarily shown [209] to block calcium-induced ERK activation, through the mechanism involving S-nitrosylation of Ras. Thus, NO-based Ras activity modulation can lead to controversial conditions.

As mentioned, redox active agents are among those modulators of the Ras protein signaling pathway, including the GEFs, the GTPase activating proteins and the effector proteins. Low oxidizing potential and pKa of Cysteines bring the thiols as the chief targets in the biological oxidizing media. Cell-culture studies [210-221] has proven that the NO acts as a GEF of the Ras protein, which results in the activation or the deactivation of the protein. By Campbell and coworkers [204, 222], a redox inactive variant to Ras (Ras C118S) was generated and recognized as insensitive to the NO modification. This variant was used for elucidating the effects of redox agents on Ras GTPases both *in vitro* and *in cells*.

All the three Ras isoforms, H-, K- and N-Ras [223] contain redox-active residue(s) which are labile to NO, NO derivatives, and nitrogen dioxide ($\text{NO}_2^\bullet$). Also through the studies of Campbell and coworkers [224], the S-nitrosylated Ras (Ras S¹¹⁸-NO) has been generated, and characterized both structurally and kinetically [172, 181, 182, 187, 225]. The details of the mechanism revealed that the S-nitrosylation was not altering the structure or the biochemical activity of the Ras protein [224]. Initially the Ras Cys¹¹⁸ is modified by NO when the $\text{NO}_2^\bullet$ or the O_2 are also present, in association with the the NO concentration and the reducing potential of the micro-environment of the cell. The Ras radical intermediate formation, Ras thiyl radical, is leading to the GDP dissociation from Ras [222, 226]; and resulting in the activation of the Ras protein. Ras radical intermediate was proposed to interact electronically with the Ras-bound GDP probably through the Phe²⁸ side-chain (Figure 29). Relative to that of the wild-type Ras protein, the Ras F28L mutant was shown [227] to have higher intrinsic rate of guanine nucleotide dissociation but was not effected by the presence of $\text{O}_2^{\bullet-}$, another redox modulator.

Figure 29. Ras redox active NKCD motif. Modified Ras Cys118 residue forms an electrical conduit to the Guanine base through the Phe28 side-chain.

NO regulate the Ras activity indirectly, *via* the radical propagation to the guanine nucleotide base through electron transfer (Figure 29). The Cys¹¹⁸ in Ras does not directly interact with

the GDP substrate or the other residues in Ras; rather, the Ras protein nucleotide interactions are perturbed (Figure 30) upon the Ras-thiyl radical formation and the release of the nucleotide is accelerated by this means [228, 229]. Exposure to higher concentrations of redox agents under lower reducing potential can lead to Ras inactivation [228, 229] due fast nucleotide release and apo-protein formation which is less stable than the nucleotide-bound form of the protein, with ‘molten-globule like’ characteristics [230].

Figure 30. The amino acid residues of Ras, which were shown [218] to be affected by S-nitrosylation. The residues are labeled with their respective colors with the model of truncated (1-166) Ras*GDP (pdb 4Q21) nucleotide binding pocket. The nucleotide in the middle has pink oxygen and cyan nitrogen coloring.

4.3 ATR-FTIR Studies with Membrane-Bound Ras Protein and NO

Ras protein NO interaction has been studied with a host of biochemical and biophysical techniques, but most of these studies were conducted using soluble/truncated forms of Ras. However, Ras function requires peripheral membrane association via post-translational lipid modification. FT-IR studies of membrane associated Ras require specific adsorption of lipidated Ras protein on solid supported POPC model membranes. *Via* ATR-FTIR, we started to study the reaction mechanism of membrane associated Ras and its interaction with the effector/regulator molecules. Then we initiated the NO-mediated guanine nucleotide exchange as an application of the ATR set-up that we built for the membrane-bound Ras protein investigation. This molecule enabled us to start our Ras protein GEF interaction studies immune from the overlapping bands of any intervening protein molecules. Also, NO reacts with the C-terminal cysteines involved in the membrane association; and it was unclear whether NO could also alter the Ras activity through interacting with cysteine residues involved in palmitoylation and farnesylation, which would interfere the membrane association of Ras. Results from this work suggest that the primary effect of NO lies in modulation of Ras activity concomitant with the lipid dynamics and subsequent membrane association. The relevance of this concept to the Ras signaling will be discussed in detail (section 5.3.2) under the guidance of our results.

5 Experimental Results and Data Analysis

Flow chart of the overall experimental procedure, along with the respective data analysis:

5.1 Experimental Results

If we shortly summarize the experimental procedure; model membranes on the IRE surface were attained by bringing the POPC SUVs (0,3 mM) into contact with the clean, hydrophilic surface of the germanium IRE. The system was flushed with deoxygenated buffer (pH 7.4) for 30 mins, followed by spontaneous protein incorporation *via* adding the protein up to a bulk concentration of 2,0 μ M. The flow rate of 1 ml/min was maintained in a closed circuit through the system. 1 mM GDP solution was circulated in the system for 2 hrs, to ensure that all the protein was GDP loaded. Then the system was flushed with deoxygenated buffer (pH 7.4) for 20 mins. Concentrated GTP solution was injected to an end concentration of 2 mM, and left in circulation for 10 mins. The NO saturated buffer (2 mM, pH 7.4) was subsequently injected to an end concentration of 1 mM and the spectra were measured for 2,5 hrs at room temperature. The results of nucleotide exchange obtained through this measurement are given here and in the following sections, along with the data interpretation. All spectra were corrected for the water vapour absorbance manually by subtracting a typical water vapour spectrum from the original spectrum; and were corrected for the baseline shifts through setting the absorbance at around 1800 cm^{-1} region to zero level.

Evaluation of the biological relevance of the results will be discussed in a separate section in the end. One sample single channel spectrum of the data presented here is shown below in Figure 31.

Figure 31. 930-3070 cm^{-1} region of the sample single channel spectrum.

Figure 31 was revealing that the absorbance data could safely be assessed for interpretation because the transmission was above 0.1. The absorbance spectra were calculated according to the 3rd equation given in section 2.3.1.2, with the reference single channel spectrum measured after the lipid adsorption on the crystal surface. The term 'system' is used in the coming sections, to indicate the actual sample volume on the IRE surface which was covered by the penetration depth of the evanescent wave.

5.1.1 Stabilization Period – GDP Circulation –

Figure 32. $900\text{--}1750 \text{ cm}^{-1}$ region of the spectra are shown in comparison for the system at the end of the lipidated Ras membrane adsorption and at the end of the GDP nucleotide circulation, after protein adsorption to the membrane (a). Background of the spectra was collected after lipid adsorption on the

crystal surface. Amide I absorbance and the O-H stretching at 3065 cm^{-1} are shown in comparison for about 2 hrs period (b). Absorbance intensity maxima were plotted instead of the Amide I band volumes for attaining proper comparability and also due to their similar profiles, which is not shown here.

Amide I and II bands ($1500\text{-}1700\text{ cm}^{-1}$) are generally immune from the lipid contributions, but the Amide I regions are overlapped by the water absorption. So, the $900\text{-}1500\text{ cm}^{-1}$ region of the spectra in Figure 32 are representative of the lipid content which were overlapped with the protein bound nucleotide and the other protein inborn signals, to a much less extent. The negative absorbencies, which are observed in Figure 32, were mainly due to the removal of lipid molecules from the surface and to a less extent, due to the changes in the protein itself and the protein-bound nucleotide. This was supported by the linear decay in the lipid absorbance as shown in Figure 33b.

While the GDP solution was circulating through the system, the protein Amide I intensity was restored within the initial 10-15 mins and there were not much prominent changes in the following period, except for a slight increase in absorbance (Figure 32b). O-H stretching absorption signals are mainly arising from the water content in the system. O-H absorption intensities were responding similar to the Amide I intensities (Figure 32b). Hence, the decrease in the lipid signal (Figure 33) was interpreted as the replacement of lipid molecules by the protein and the water molecules. This was deduced from the accompanying slight increases in the intensities of both Amide I and O-H stretching absorbencies at 3065 cm^{-1} (Figure 32b).

Figure 33. Methylene symmetric and asymmetric stretching vibrations' absorption signals, which are mainly arising from the lipid content in the system, are shown in comparison for the system at the end of the lipidated Ras membrane adsorption and for the system at the end of the GDP nucleotide circulation after protein adsorption to the membrane (a). Background of the spectra was collected after lipid adsorption on the crystal surface. Methylene stretching at 2923 cm^{-1} is shown for about a 2 hrs period (b).

This region is highly representative of the lipid content. The negative absorbance in Figure 33 was interpreted as the removal of lipid molecules from the surface because the reference was

the spectrum measured after lipid adsorption on the crystal surface. Ordering of the lipid acyl chains also increases the thickness of the membranes and would be observed as a decrease in the signal intensity, but it need to be accompanied with a shift of the methylene stretching vibrations to lower frequencies. This was not the case (Figure 33a).

5.1.2 GTP Nucleotide Circulation

At the end of the GDP circulation, buffer solution was flushed through the system for about 20 mins and then the concentrated GTP solution was added. Spectra with the GTP nucleotide in the system, shown in Figures 34a and 35a, were taken 10 mins after the addition of the GTP nucleotide. This time was allowed to attain a homogeneous distribution of the GTP nucleotide in the system. The observation which is shown in Figure 34a is more complicated for interpretation.

Figure 34. 900-1750 cm⁻¹ region of the spectra are shown in comparison for the system at the end of the GDP nucleotide circulation and after the GTP nucleotide addition to the system – 0,5 hrs after the

GDP nucleotide circulation data – (a). Background of the spectra was collected after lipid adsorption on the crystal surface. Amide I absorbance and the O-H stretching at 3065 cm^{-1} are shown in comparison for the GDP nucleotide circulation – first data point – and after the GTP nucleotide addition to the system – rest 4 data points – (b). Absorbance intensity maxima were plotted instead of the Amide I band volumes for attaining proper comparability and also due to their similar profiles, which is not shown here.

In case of the GTP addition (Figure 34a), increase in the Amide I volume was observed; which could be interpreted as an increase in the water content or a change in orientation of the protein with respect to the crystal surface, so to the membrane. There was also a decrease in the lipid content (Figure 35) which was expected to be leading to an increase in the water content. However, the increase in the O-H stretching intensity at 3065 cm^{-1} (Figure 34b) was significantly less than expected, lower than the absolute increase in the Amide I band intensity. Therefore the latter interpretation was probably the case, which was suggesting a change in orientation of the protein with respect to the membrane. Additionally, such a change in orientation could be bringing the protein slightly closer to the surface; because, increases in the Amide I band volumes and the peak intensities were observed (Figure 34) at the same time with a decrease in the methylene stretching of mainly the lipid signal (Figure 35). It was previously stated in the literature based on theoretical calculations [42] that the Ras protein was inserting to the membrane with different orientations in its GDP- *versus* GTP-bound forms. Yet, our observation was not the same. In the beginning, there were no nucleotide exchange factors of the Ras protein available in the medium. Therefore, GTP nucleotide could not have been exchanged with the protein-bound GDP nucleotide within the initial 10 mins. Hence, a change in the orientation of the protein upon addition of GTP to the system could not be attributed to the nucleotide which was bound to the protein. However, the effect of environmental GTP concentration on the orientation of the membrane-bound Ras protein would only be speculative at this level.

Figure 35. Methylene symmetric and asymmetric stretching vibrations' absorption signals, which are mainly arising from the lipid content in the system, are shown in comparison for the system at the end of the GDP nucleotide circulation and for the system after the GTP nucleotide addition to the system, about 0,5 hrs later (a). Background of the spectra was collected after lipid adsorption on the crystal surface. Methylene stretching at 2923 cm^{-1} is shown in comparison for the GDP nucleotide circulation – first data point – and after the GTP nucleotide addition to the system – rest 4 data points – (b).

5.1.3 NO Solution Injection

The NO saturated buffer solution was injected to the system 10 mins after the addition of GTP nucleotide. Changes in the system started to be monitored through measuring the spectra for a period of about 2,5-3 hrs, which would allow one to monitor the whole intrinsic GTPase cycle when the protein-bound GDP nucleotide was exchanged with the GTP nucleotide at the initial state of this period.

Figure 36. 900-1750 cm^{-1} region of the spectra are shown in comparison for the system after the GTP nucleotide addition to the system and at the end of the NO circulation, about 2,5 hrs after NO loaded buffer injection to the system (a). Background of the spectra was collected after lipid adsorption on the crystal surface. Amide I absorbance and the O-H stretching at 3065 cm^{-1} are shown in comparison for about a period of 2,5-3 hrs after NO loaded buffer injection to the system (b). Absorbance intensity

maxima were plotted instead of the Amide I band volumes for attaining proper comparability and also due to their similar profiles, which is not shown here.

The observation which is shown in Figure 36 was indicative for distinct aspects of the Ras protein membrane interaction. There was a prominent increase in the protein Amide I and II bands, which can be observed in case of the ATR-FTIR measurements. This observation could have been due to an increase in the surface protein concentration or might have been the outcome of protein delocalization which was bringing the protein closer to the surface. There were no extra protein injection during the measurements; therefore, the latter situation was probably the case. Similar results were obtained during previous measurements when there were no nucleotide exchange factors available in the medium, but the observations were prevalent in much longer time scales (data not shown). Such a situation might have been arising from increasing affinity of the protein for the membrane. This will be discussed later in the end. Yet, considering the fact that there were no additional protein introduction to the system; one could suggest that the increase in absorbance was due to the Ras proteins' getting closer to the IRE surface.

Figure 37. Methylene symmetric and asymmetric stretching absorption signals, which are mainly arising from the lipid content in the system, are shown in comparison for the system after the GTP nucleotide addition to the system and for the system at the end of the NO circulation, 2,5-3 hrs after NO loaded buffer injection to the system (a). Background of the spectra was collected after lipid adsorption on the crystal surface. Methylene stretching at 2923 cm^{-1} is shown for about a period of 2,5-3 hrs (b).

A decrease in the absorption intensities of mainly lipid sourced methylene stretching vibrations (Figure 37) and an increase in the water O-H stretching absorption intensity at 3065 cm^{-1} (Figure 36b) were observed. This was accompanied to a significant change in volumes and absorption intensities of both Amide I and II regions (Figure 36). Thus, we interpret the data as the lipid removal with accompanying changes, which were inherently related to the Ras protein membrane interaction. Support of this interpretation is given along with the data in the next section (6.2).

5.2 Data Analysis

In case of the data presented in this section, difference spectra of the membrane-bound Ras protein were calculated manually for both the GDP nucleotide circulation period and

afterwards, for the NO-mediated GTP exchange. All the difference spectra were calculated with respect to one reference spectrum for the sake of comparability; and that reference was the last absorbance spectrum measured before the NO loaded buffer injection to the GTP circulating system (Figure 38).

Figure 38. The spectra that were corrected for water vapor contributions, and baseline shifts at 1800 cm^{-1} are shown. The Amide I intensity of the reference spectrum (lipidated Ras during GTP circulation) was the criterion for normalization before subtracting the reference from the original.

The absorbance spectra were corrected for water vapor contribution manually by subtracting the water vapor spectrum from the original; and then, corrections for the baseline shifts were performed by setting the absorbance at around 1800 cm^{-1} to zero level. The resultant sample spectra are shown in Figure 38. After corrections, the spectra were normalized to the Amide I intensity of the reference spectrum before subtracting the reference spectrum from the original (Figure 39). Amide I intensities were favored rather than the volumes, in order to follow the structural and orientation changes of the protein in a better way. Volume normalization may lead to slight perturbations of the changes which could otherwise be better observed in the difference spectra. As a control, same calculations were performed with the spectra which

were normalized to the Amide II intensity of the same reference spectrum (section 6.2.2). Hence, the contribution of water in the Amide I region and the changes that are observed in the difference spectra will be discussed in section 6.2.2.

Figure 39. Amide I normalized spectra which were previously processed for water vapor contributions, and baseline shifts at 1800 cm^{-1} are shown. Normalizations were performed with respect to the Amide I intensity of the reference (lipidated Ras spectrum during GTP circulation). Next step would be subtraction of the reference from the normalized original spectra.

Manual lipid subtraction from the original spectra was avoided because the resultant difference spectra would then be the protein spectra with the variations in the lipid absorption. Accordingly, attribution of the features in the difference spectra merely to the protein would be misleading for interpretation.

The system was under continuous forces which were driving it away from the initial state. There was a permanent lipid desorption from the surface (Figures 33, 35 and 37). Moreover, significant increase in the overall absorption intensity was observed (Figure 36), which was pointing at protein delocalization and getting closer to the IRE surface. This was bringing

further technical difficulties. In ATR-FTIR spectroscopy, absorption peak position and intensities do not change linearly with the penetration depth of the evanescent wave. Yet, such contributions in the difference spectra were treated as negligible due to their being relatively small. There were obstacles for obtaining quantitative information. However, the information was highly qualitative. Difference spectra were revealing changes with respect to each other and to the reference state (Figure 41). The correspondence of those changes to the possible mechanisms in biologically relevant media will be discussed in the end.

Before starting the GTP nucleotide circulation and the NO loaded buffer injection to the system, GDP nucleotide was circulating and the spectra were measured for about 2 hrs, in order to attain stability and to track changes in spectra during this time. The difference spectra will be shown in the coming section.

5.2.1 Difference of Amide I Normalized Spectra with respect to the GTP spectrum

5.2.1.1 GDP Nucleotide Circulation

The GDP nucleotide circulation period was lasted for about 2 hrs, with the initial aim to attain stability; and to assure that all the protein in the system were GDP loaded. The term ‘stability’ is not representing the stability which is meant for the FTIR transmission measurements. Rather, changes in the system occurring in time were followed through monitoring the system, in such conditions that the Ras protein GTPase activity was not possible. GDP nucleotide circulation period was a control in this sense. NO-mediated GTP exchange of the protein was the actual measurement period for data collection (section 5.2.3.1).

Figure 40. Stacked view of the 900-1750 cm^{-1} region difference spectra are shown for the membrane-bound Ras protein after the GDP nucleotide addition to the system. Difference spectra calculations were performed manually after point normalization with respect to the Amide I peak intensity at around 1640 cm^{-1} . The difference spectra calculations were performed by subtracting the last spectrum collected before the NO loaded buffer injection, in order to attain comparability with the data of the NO-aided GTP exchange and activity of the protein. *Time of measurement* indicates that the time of the first data collected after the GDP nucleotide addition to the system, was taken as 0' 00".

It can be seen in Figure 40 that the difference spectra were bearing conserved spectral characteristics in case of membrane-bound Ras*GDP. Changes within the amide regions and the C=O stretching vibrations could have been due to random fluctuations in orientations of both the protein and the lipids, regardless of any concerted behavior.

5.2.1.2 NO Solution Injection after the GTP Addition

Though there were fluctuations during the GDP nucleotide circulation (Figure 41), such fluctuations were negligible with respect to the changes which were under regulation upon

NO aided GTP exchange (Figure 41). Characteristic features which are observed in Figure 41 are highlighting the lipid and protein modulation by each other, in terms of changes in orientation, or so, of the two components with respect to each other. The difference spectra in Figure 41 are revealing characteristic features of two distinct groups, one with the upper three spectra and the other with the rest two spectra. Yet, the spectrum at about 40th min was actually bearing features of both, which was revealed by visual inspection.

Figure 41. Stacked view of the 900-1750 cm^{-1} region difference spectra are shown for the membrane-bound Ras protein, after the NO-mediated GTP exchange. Difference spectra calculations were performed manually after normalization with respect to the Amide I peak intensity at around 1640 cm^{-1} , by subtracting the reference (spectrum collected before the NO loaded buffer injection) from the original. *Time of measurement* indicates that the measurement time of the last spectrum before NO injection was taken as 0' 00" (0 min 00 sec).

The changes that are observed in Figure 41 were attributed to the activity of the protein and its interaction with the membrane. The difference spectra of the GDP nucleotide circulation phase (Figure 40) were not revealing such a correlated behavior of the changes in the difference spectra.

5.2.1.2.1 GTP Hydrolysis

Roughly 1mM end concentration of GTP nucleotide was in circulation after the NO loaded buffer injection. NO facilitates nucleotide exchange of the Ras protein; otherwise, the GDP-to-GTP nucleotide exchange would not have been favored by the Ras protein, because the GDP nucleotide was tightly bound to the protein and Ras*GDP was energetically favored. Fingerprint region of the same difference spectra, which was shown in Figure 41, is shown in Figure 42 for about a 3 hrs period. This period was sufficient for monitoring the full intrinsic reaction cycle of the Ras GTPase activity. The red difference spectrum on top is representing mainly the free GTP in the system which was calculated manually by subtracting Ras*GDP spectrum measured before the GTP nucleotide addition to the system. The sequential changes in the difference spectra were indicative of the GTPase activity with the changes in the phosphate stretching vibrations and significant decay in the bands (indicated by arrows) at around 1140 cm^{-1} (Figure 42), which were assigned to the γ -phosphate group vibrations of the protein-bound GTP nucleotide (231). Yet, the lipid head group and protein-bound nucleotide phosphate stretching absorbencies were overlapping. The difference spectra characteristics arising from the changes in the protein-bound nucleotide may be judged healthier by comparing the difference spectra with an exemplary Ras GTP hydrolysis spectrum (Figure 44).

Figure 42. Stacked view of the 900-1350 cm^{-1} region difference spectra are shown for membrane-bound Ras protein, after the NO-mediated GTP exchange. The red spectrum on top (GTP addition initial response) is a difference spectrum, which was calculated by subtracting Ras*GDP spectrum measured before the GTP nucleotide addition. The latter difference spectra calculations were performed manually after point normalization with respect to the Amide I peak at around 1640 cm^{-1} , by subtracting the reference (spectrum collected before the NO loaded buffer injection) from the original. *Time of measurement* indicates that the measurement time of the last spectrum before NO injection was taken as 0' 00" (0 min 00 sec).

Fingerprint region of the difference spectra is shown in Figure 42 with the region which is generally examined for the changes in the Ras protein-bound nucleotide. In order to recognize the Ras protein GTPase activity through the FTIR absorbance spectra, one needs to observe the characteristic changes in the phosphate region for the protein bound GTP and the GDP nucleotides. Characteristic frequencies of the phosphate bands of GTP, $\text{GTP} \cdot \text{Mg}^{2+}$, and Ras-bound $\text{GTP} \cdot \text{Mg}^{2+}$; and of GDP, $\text{GDP} \cdot \text{Mg}^{2+}$, and Ras-bound $\text{GDP} \cdot \text{Mg}^{2+}$ were determined by the previous studies [231-233]. The GTP and GDP nucleotides' phosphate group vibrations

absorb at lower frequencies than that of the Mg^{2+} bound nucleotides; and the latter has still lower absorption frequencies than the protein-bound nucleotides (Figure 43). Accordingly, the phosphate bands at 1263 cm^{-1} and 1219 cm^{-1} of Ras-bound $\text{GTP}^*\text{Mg}^{2+}$ are assigned to the two nondegenerated $\nu_a(\text{PO}_3^{2-})$ vibrations of the α - and β -phosphate groups; while, the bands at 1157 cm^{-1} and 1143 cm^{-1} are assigned to the two nondegenerated $\nu_a(\text{PO}_3^{2-})$ vibrations of the γ -phosphate group [231]. The α -phosphate band frequency of the Ras-bound $\text{GDP}^*\text{Mg}^{2+}$ is 1230 cm^{-1} [231]. The bands at 1139 cm^{-1} and 1104 cm^{-1} are assigned to the two nondegenerated $\nu_a(\text{PO}_3^{2-})$ vibrations of the β -phosphate group [231].

Figure 43. Comparison of the frequencies of the phosphate bands of GTP [232], $\text{GTP}^*\text{Mg}^{2+}$ [232], Ras-bound $\text{GTP}^*\text{Mg}^{2+}$ [231], GDP [232], $\text{GDP}^*\text{Mg}^{2+}$ [232], Ras-bound $\text{GDP}^*\text{Mg}^{2+}$ [231], and HPO_4^{2-} [233]. The listed wavenumbers of Ras*GTP, Ras*GDP, and Pi correspond to the band positions in the hydrolysis double-difference spectra [231].

Evaluation of the relation among the difference spectra absorbencies with the characteristic protein-bound nucleotide phosphate vibrations (Figure 43) were done by direct comparison (Figure 44) of an inverse Ras*GTP hydrolysis spectrum in transmission [231]. Therefore, positive bands of the spectrum used for comparison in Figure 44 were indicative of the

protein-bound GTP nucleotide vibrations; and negative bands were indicating the protein-bound GDP nucleotide. The changes were relevant at first sight; yet, those were not identical (Figure 44). The degree of overlap with the phosphate head group vibrations of the lipids was quite high. For example, the peak at around 1070 cm^{-1} was attributed in the literature [234, 235], to the asymmetric single bond C-O stretch of the ester phosphate group $\nu(\text{C-O[P]})$ which is coupled with a single PO vibration. Free phosphate vibrations were not expected to be observed in case of the flow-through ATR-FTIR measurements, cause the free nucleotide which was released to the whole circulation would not be detected under such low concentrations; but $\nu(\text{C-O[P]})$ could still be mixed up with the degenerated $\nu(\text{PO}_3^{2-})$ vibrations (Figure 43). Additionally, the set-ups in the transmission and the ATR modes of the FTIR were dramatically different. The outcomes may not be similar; especially when we consider that our ATR-FTIR measurements were carried out in solution.

Figure 44. Stacked view of the $900\text{-}1750\text{ cm}^{-1}$ region difference spectra after the NO-mediated GTP exchange of the membrane-bound Ras protein are shown in comparison to the inverse difference spectra (middle) in transmission mode, of the Ras GTPase reaction with catalytic amounts of NF1-333

at 283 K [231]. Ras*GTP hydrolysis spectrum was taken from the work of Allin and Gerwert, 2001 [231]. ATR-FTIR difference spectra calculations were performed manually after point normalization with respect to the Amide I peak intensity at about 1640 cm^{-1} , by subtracting the reference (spectrum collected before the NO loaded buffer injection) from the original. *Time of measurement* indicates that the measurement time of the last spectrum before NO injection was taken as 0' 00" (0 min 00 sec).

5.2.1.2.2 Membrane Modulation

As mentioned previously, lipid head group and phosphate stretching absorbencies of the protein-bound nucleotide were overlapping; and the protein molecules were overcrowded by the lipids with a rough estimate of 150 lipids per protein, which was calculated previously with the use of data in deuterated medium. Therefore the difference spectra absorbencies were dominated by the lipid sourced spectral changes. For this reason, comparison of a roughly normalized (divided by a factor of 20) lipid spectrum was performed with the difference spectra under inspection (Figure 45). The methylene stretching vibrations were characterized mainly by the lipid acyl chain vibrations. Accordingly, the steepest signals of the difference spectra were considerably similar to the POPC spectrum on top of the Figure 45a, which was measured in the same buffer content excluding the GTP and the NO. This situation was preventing one to abstract more information on the protein and the GTPase activity. Yet, the spectra were quiet informative on the protein-membrane interactions during the Ras GTPase activity. This issue will be discussed in detail.

Figure 45. Stacked view of the 900-3050 cm^{-1} region difference spectra after NO-mediated GTP exchange of the membrane-bound Ras protein are shown in comparison to a roughly normalized (divided by a factor of 20) POPC spectrum on top (a). Difference spectra calculations were performed manually after point normalization with respect to the Amide I peak at about 1640 cm^{-1} , by subtracting the reference (spectrum collected before the NO loaded buffer injection) from the original. *Time of measurement* indicates that the measurement time of the last spectrum before NO injection

was taken as 0' 00" (0 min 00 sec). Overlapped view of the 1300-1770 cm^{-1} region difference spectra after the NO-mediated GTP exchange of the membrane-bound Ras protein are shown in (b).

Membrane Modulation;

As revealed from the data (Figure 45), not only the protein but the membrane was also responding to the nucleotide exchange of the Ras protein. This response could be termed as the membrane activity which denotes the dynamic membrane states such as the membrane snorkeling and ruffling. The sequential changes in the lipid signal (Figure 45a) which was followed along with the protein activity could actually be a modulation. These changes in the lipid signal were varying in time without significant shift in the positions of the methylene stretching vibrations (Figure 37a); and the difference spectra profile was nearly restored in the end of the measurement period, with regard to the $\sim 3^{\text{rd}}$ and the $\sim 174^{\text{th}}$ min spectra (Figure 45a).

Dynamic β -Sheet Structure;

In support to the previous analysis (section 3.4.2), which was suggesting a dynamic β -hairpin motif close to the C-term of the Ras protein; the prominent bands at around 1680 cm^{-1} and 1612 cm^{-1} in the Figure 45b, were also pointing at a dynamic β -sheet structure which could be related. A wide band at around 1680 cm^{-1} was arising with accompanying decrease in the random structure, indicated by the negative peak at around 1635 cm^{-1} (Figure 45b). Towards the end of the measurement period, the difference spectra was restoring its previous profile with an additional band at around 1612 cm^{-1} , which was also indicative of a β -sheet structure (Figure 45b).

Perturbation of Lipid Acyl Chains' Networking;

The CH_2 scissoring band at around the 1468 cm^{-1} absorption signal (Figure 45b) is indicative of the networking among the membrane [121]. Methylene scissoring modes, which occur at

around 1462-1474 cm^{-1} were found to split into doublets if the acyl chains are packed in orthorhombic perpendicular subcells [236]. The effect is of short range and perpendicular to the chain direction [236]. Additionally, the magnitude of splitting reflects the number of interacting chains [236].

In our case, cessation of the splitting was observed as a negative shoulder at $\sim 1490 \text{ cm}^{-1}$ (Figure 45b). Magnitude of the band separation around 1468 cm^{-1} is indicative for the degree of interchain interaction, in correlation to the packing of the lipid acyl chains [121]. This separation was observed to be lost in the $\sim 14^{\text{th}}$ and 16^{th} min spectra (Figure 45b). Thus, our observation was implying a decrease in the number of interacting chains and loss of the networking among the membrane, at around 14^{th} and 16^{th} mins.

The group splitting phenomenon is explained in the literature [121] as the degree of interaction over the full chain length which is observed as a group splitting upon increase in the chain length. So, the weakening and breaking of the connective interaction among the lipid molecules would not be thermodynamically favored, and would hardly be interrupted by the molecules solvated in water. Therefore, if the packing was gradually loosened by the NO interaction; it would have been easier to overcome such a thermodynamically unfavorable condition. This is supported by the NO-membrane interaction data which will be presented in the section 5.3. It is concluding from the increasing intensities of the $\nu_{\text{s}}\text{CH}_3$ and $\nu_{\text{as}}\text{CH}_3$ that the NO was localizing in the hydrophobic core of the membrane (Figures 50 and 51). Also, the peak of the symmetrical methyl deformation mode [237] at 1378 cm^{-1} (Figure 45b) was detected previously [238] as the bound-nitrate region. This band was perturbed, which can also be seen in the $\sim 14^{\text{th}}$ and 16^{th} min spectra of the Figure 45b.

Perturbation of Lipid C=O stretching;

Additional information for the dynamic membrane interactions was provided by the shift in the C=O stretching vibrations (Figure 45b). Increase in the C=O stretching vibration up to the 1736 cm^{-1} from 1733 cm^{-1} was observed as a negative peak (Figure 45b). Similar frequency shifts normally point at higher dynamics due to perturbation of the stabilizing interactions of

the C=O region between the glycerol and the fatty acid chains of the POPC. Observation of such a shift as a negative peak can thus be interpreted, in support with the previous information, as the stabilizing interactions of the C=O region which were occurring along with the NO-aided loosening of the membrane networking. A dynamic random to sheet transition in the membrane-bound Ras protein was accompanying to the perturbation of lipid C=O stretching.

Membrane Snorkeling and Ruffling;

These observations listed above, were done only in conditions that the protein was bound to the membrane and the concerted GTP exchange was attained by the aid of NO. The variation in the lipid absorbance intensities could be interpreted as dynamic membrane fluctuations and unstable membrane thickness, which could be leading to the membrane snorkeling. Such cases are generally driven by the lipid-protein interactions. Hydrophobic mismatch and charge of the residues at or in the vicinity of the insertion sites causes phase separations and snorkeling [119]. Aromatic residues also take role in lipid snorkeling *via* direct interaction [119]. The Lys 185 snorkeling of H-Ras was previously reported to be observed during membrane anchor insertion [239]. This was supported by the Tyr band splitting in case of our measurements (Figure 19).

It was suggested [42] by the theoretical studies that the lipid modified H-Ras inserted DMPC bilayer membrane thickness was not uniform, but was varying in space and time with an increase in the membrane peristaltic motion upon protein insertion. In case of the GDP complexed full-length Ras protein, each Ras lipid tail was crossing the bilayer center and interacting with lipids of the opposite leaflet to achieve hydrophobic matching [128]. There were localised perturbations of DMPC in the Ras-DMPC complex; and those perturbations were suggested to be drawn by the insertion depth and backbone orientation of the anchoring region, which was in turn modulated by the catalytic G-domain and the linker region of the protein [128]. Thus, activity related protein snorkeling can have further direct biological relevance to previous theoretical studies and to our experimental results; maybe, in terms of

the cell surface membrane ruffling which is observed during the Ras signal transduction [240].

5.2.2 Difference with Amide II Normalized Spectra

As mentioned before, original absorbance spectra were corrected for water vapor contribution manually by subtracting water vapor spectrum from the original; then, corrections for the baseline shifts were performed by setting the absorbance at around 1800 cm^{-1} to zero level. The resultant sample spectra before normalizations were already shown (Figure 38), along with the Amide I normalizations to the reference, lipidated Ras spectrum at GTP circulation (Figure 39). Here, the resultant sample spectra after Amide II normalizations to the same reference are shown in Figure 46.

Figure 46. The spectra after normalization to the Amide II intensity of lipidated Ras spectrum under GTP circulation are shown. They were corrected for the water vapor contributions and the baseline shifts at 1800 cm^{-1} . Following step will be the subtraction of reference spectrum (lipidated Ras spectrum under GTP circulation) from the normalized original spectra.

The reference (lipidated Ras spectrum under GTP circulation) was previously subtracted from the Amide I normalized spectra (Figure 39). Here, the same reference was subtracted from the Amide II normalized spectra (Figure 46). Two resultant spectra from each of the calculations are shown in comparison below (Figure 47).

Figure 47. Overlapped view of the 1240-3070 cm^{-1} region difference spectra after the NO-mediated GTP exchange of the membrane-bound Ras protein are shown. Difference spectra calculations were performed manually after point normalization with respect to the Amide I peak at about 1640 cm^{-1} and also with respect to the Amide II peak at about 1540 cm^{-1} , by subtracting the reference (lipidated Ras spectrum under GTP circulation) from the original. *Time of measurement* indicates that the measurement time of the last spectrum before NO injection was taken as 0' 00" (0 min 00 sec).

According to the comparison, we observe that there was a distinct negative peak at Amide I, which was initially observed in case of the difference for the Amide II normalized spectrum at time 16' 32" (Figure 47). This negative peak was diminishing later in the respective spectrum at time 173' 52" (Figure 47). Yet, the interesting feature was the positive absorbance of the water O-H stretching which was observed for the Amide II normalized spectra difference at

time 16' 32" (Figure 47). So, the negative Amide I peak was associated with a conformational change rather than a change in the water content. This is supported by the plot of the absorbencies at 3065 cm^{-1} for the difference spectra (Figure 48).

Figure 48. Absorbencies at 3065 cm^{-1} are plotted for the differences of the Amide I and the Amide II normalized spectra, with the reference spectrum as the data measured during GTP circulation. Absorbencies at 1640 cm^{-1} are plotted only for the difference spectra of the Amide II normalized spectra, with the reference spectrum as the data measured during GTP circulation. Time is shown in the logarithmic scale.

In Figure 48 above, we observe that the O-H stretching absorbencies were directly correlated among the difference spectra calculated from the Amide I normalized spectra and the Amide II normalized spectra. Whereas, the Amide I intensity differences of the Amide II normalized spectra were inversely correlated with the O-H stretching absorbencies of their respective spectra (Figure 48). This observation was supporting the previous suggestion that such a

correlation was pointing at a conformational change; probably further in association with a change in the water content.

Figure 49. Absorbencies at 1730 and 2930 cm^{-1} are plotted for the differences of the Amide I and the Amide II normalized spectra, with the reference spectrum as the data measured during GTP circulation. Time is shown in the logarithmic scale.

A similar comparison was done for the difference spectra absorbencies at 1730 and 2930 cm^{-1} (Figure 49). Variation in the lipid sourced absorbance intensities were compared with regard to the Amide I *versus* Amide II normalizations for the difference spectra calculations. Accordingly, lipid methylene stretch at 2930 cm^{-1} and the lipid C=O stretch at 1730 cm^{-1} were coherent among the differences of the Amide I normalized spectra, but modulated in a distinct manner among the differences of the Amide II normalized spectra (Figure 49).

In Conclusion;

The water O-H stretching absorbencies were behaving similar with regard to the Amide I *versus* Amide II normalized difference spectra calculations (Figure 48), but not the lipid methylene stretches at 2930 cm^{-1} and the lipid C=O stretches at 1730 cm^{-1} . Therefore, the changes that we have observed in lipid absorbance intensities were not due to the water O-H stretching absorbencies. This was supporting that the lipid modulation was possibly in relation to the conformation of the Ras protein, and thus, to the water content of the system. Yet, change in the water content of the system was not the source of lipid modulation. Performing the same analysis with a different reference spectrum was only shifting the graphs along the absorbance axis (data is not shown); so, choice of the reference spectrum was equal for the nature of variations. Our initial aim was to check if the lipid absorbance intensities were modulated by the water content of the spectra. However, choosing the Amide II for its being immune from the water absorbance intensity came out to be underestimating the conformational changes in the protein. As a result, water absorbance intensities were seemingly in association with the conformational changes of the protein (Figure 48). Hence the lipids, which were suggested to be modulated by the protein activity, were in a rather more complex interaction with the protein.

5.3 Control for the NO effect on POPC model membrane

These measurements were carried with the diamond IRE bearing micro-sampling ATR set-up. The POPC membrane preparation on the surface was performed with deoxygenated sample material as before; by following the procedure described in the sections 3.1 and 3.2. The system was closed, and an inlet was used for mild Argon flow during the incubation periods. The flow was stopped after the membrane preparation and 50 μl NO loaded buffer (1 mM) was injected from the inlet to an end concentration of about 0,7 mM. The measurements were started in a correlated fashion and continued for 4-5 hrs.

Methylene stretching vibrations are sensitive for ordering of the lipid acyl chains. While the frequency of the νCH_2 provides information about the static order of the lipid acyl chains, the bandwidths are a measure of the acyl chain librational and torsional motions. Both the order and dynamics are qualitative information [241]. Therefore, absorbance spectra were normalized to the 2923 cm^{-1} and compared in the methylene stretching regions for the NO interaction with the membrane alone (Figures 50) and for the NO interaction with the membrane, when Ras protein was bound to the membrane and active on the surface (Figures 51). Lipid desorption from the surface was not observed in case of the NO interaction with the membrane alone. The system was stable.

Figure 50. Maximized view of the $2840\text{--}2960\text{ cm}^{-1}$ region POPC spectrum (red) overlapped with the spectrum (blue) which was measured for approximately 4.5th hrs after the NO loaded buffer injection. Measurement was performed with the diamond IRE bearing micro-sampling ATR set-up.

The effect on the methylene stretching vibrations was negligible when there was only membrane on the surface, except for a slight increase in the CH_3 symmetric stretching (Figure 50). This increase might have been due to NO localization at the hydrophobic core of the membrane. In support, such an increase in the CH_3 symmetric stretching of the Ras protein-

bound membrane under the NO circulation was observed along with an increase in the CH₃ asymmetric stretching as well (Figure 51).

Figure 51. Maximized view of the 2840-2960 cm⁻¹ region POPC spectrum (red) overlapped with the Ras-bound POPC spectrum which was measured 2,5th hrs after the NO injection (blue). Measurement was performed with the germanium IRE bearing ATR set-up.

As can be seen from the Figure 51 above, methylene stretching vibrations were consistent in positions. Changes in the intensities of $\nu_{as}CH_3$ and ν_sCH_3 (Figure 51) were probably due to the NO localization in the hydrophobic core of the membrane and due to interaction of NO with the CH₃ ends of the lipid acyl chains.

In the end; the data suggests that the NO was causing mild perturbations to the packing, through the terminal methyl groups. Hydrophobic core regions of the membrane bilayer introduce lipid packing to the bilayer, along with the lipid head groups and the acyl chains. The electrostatic field of NO, which is repelling the hydrogen ions, account for the hydrophobic properties of NO. Therefore, NO is allowed to freely diffuse through biological membranes [166]. In agreement, ν_sCH_3 and $\nu_{as}CH_3$ were perturbed upon NO interaction (Figures 50 and 51). However, GTP exchange and activity of the membrane-bound Ras protein were required to observe the kind of membrane response which was reported in

section 5.2.1.2.2. We termed this response as the modulated excitation. The reason was the typical variations in the lipid absorbance intensity; namely, we observed changes in the lipid absorbance intensities of the difference spectra (Figure 45a), which could be attributed to the variations in the apparent lipid surface concentration.

5.4 Indirect Test of the Ras Protein Membrane Affinity

These measurements were performed with the diamond IRE bearing micro-sampling ATR set-up. Contrary to the previous experimental procedures, lipidated protein was injected before the membrane preparation on the surface. Afterwards, POPC membrane preparation on the surface was carried out in the same manner with deoxygenated sample material as before; by following the procedure which was described in sections 3.1 and 3.2. This time, GTP nucleotide (1 mM) was included in the buffer content of the POPC ULV solution. The system was closed with an inlet which was used for mild Argon flow during an incubation period 15 mins. Then the flow was stopped and 50 μ l NO loaded buffer (1 mM) was injected from the inlet to an end concentration of about 0,7 mM. The measurement was started concomitantly with the NO loaded buffer injection and continued for 4 hrs.

Figure 52. 1000-1700 cm^{-1} of lipidated Ras protein spectrum (black) with the NO-mediated nucleotide exchange (red). The protein was on the diamond IRE of the horizontal ATR set-up, with the membrane on top.

The experiment with the data shown in Figure 52 was performed to test the Ras protein insertion through the membrane as a result of the increasing affinity, which could be arousing due to the Ras protein nucleotide exchange and the GTPase activity. The Amide I region (black) in Figure 52 was characterized by a different profile than the previous measurements which were carried in the same buffer content (i.e. Figures 32a, 34a and 36a). The reason was probably the direct protein adsorption on the IRE surface. Replacement of the water on the surface was resulting in a negative water absorbance signal, as the reference spectrum was taken with the buffer on the surface. The membrane was further away from the surface than the Ras protein; thus, a decrease in the absorbance signal was expected upon Ras protein insertion and affinity to the membrane. Accordingly, a slight decrease in the absorbance signal (Figure 52) was observed after 4 hrs. This observation was accounted significant; because, if the insertion could not have been attained by the protein, then the overall intensity

need to have remained stable or, would have been highlighted by a decrease in the water O-H stretching absorbance. The latter situation is generally observed in case of protein unfolding on the surface. The increasing volume of the protein during unfolding on the IRE surface leads to a dramatic decrease in the water O-H stretching absorbance; which could even drag the Amide I intensity down to the negative values, while, intensity of the Amide II remaining the same. However, the observation in Figure 52 was an overall decrease in the protein absorbance intensity.

One might suggest that our result could have been due to the replacement of some protein molecules with the lipids; however, such a case would have been observed as an initial response after the addition of nucleotide and the lipid vesicles. Still, the experimental conditions were not optimized; hence, it could not be guaranteed that all the protein on the IRE surface was bound to the membrane vesicles. So, the optimal conditions would be expected to provide a more prominent decrease in intensity. We also need to keep in mind that the *g* force was acting in the opposite direction of the suggested affinity towards the membrane. Further indications of this measurement are discussed in the next section (5.5).

5.5 Discussion for the ATR-FTIR Studies with Membrane-Bound Ras Protein and NO

For the first time, direct effects of NO on the membrane-bound Ras protein was investigated in solution, by means of a surface sensitive biophysical technique, ATR-FTIR. The set-up was immune from the adverse effects of indirect NO donors. The lipid packing was seemingly perturbed through the terminal methyl groups (Figure 51b). Further, POPC membrane was modulated when the Ras protein was active on the surface; since, variations in the lipid sourced absorption intensities were observed in the difference spectra (Figure 45a). Namely, we observed a modulation of the lipid concentration through the changes in lipid absorbance intensities. It was a cyclic stimulation and the initial spectrum was restored at the end of the measurement. This was further supported by the characteristic similarity of the difference

spectra (Figure 45a) to the modulated excitation response [242]. The interpretation of our data was additionally leading to the conclusion that; NO-aided loosening of the membrane networking was occurring along with the stabilizing interactions of the lipid C=O region (Figure 45b). In relation, a dynamic random to sheet transition in the membrane-bound Ras protein was taking place (Figure 45b). Hence, POPC membrane was modulated when the protein was bound to the membrane. It can be speculated that the concerted exchange of the GDP nucleotide with the GTP nucleotide was starting the GTPase activity and was somehow resulting in a modulated behavior of the membrane. Further analysis (section 6.2.2) also implied that the lipids might have involved in a complex means of interaction with the protein, which could be pertinent to the conformation of the Ras protein and the surrounding water content (Figure 48 and 49).

Modulated excitation (ME) is applicable to the ATR-FTIR, provided that the sample of interest admits (periodic) excitation by the modulation of an external parameter and the weak absorption changes which are hindered due to a huge background noise are revealed by this means [242, 243]. Such a modulated response was not observed during the GDP nucleotide circulation (Figure 40), but was observed after the NO-mediated GTP exchange of the Ras protein (Figure 45a). Lipid methylene stretching vibrations were not shifting (Figure 37a and 50); so, the observation (Figure 45a) was not due to the effect of temperature [242, 243].

Such a modulation could be correlated to the analogue-digital signaling of the Ras protein; which was suggesting that the cells were building an analogue-digital-analogue circuit relay that was transmitting the signal across the plasma membrane with high fidelity [244]. Actual cellular complexity is ultimately higher. Yet, our observations might have been relevant to the membrane ruffling, which was postulated [245-247] to be accompanied to the Ras protein activity in the live cell, along with the ruffling and the fluid-phase pinocytosis. The nature of membrane ruffling which is induced by such complex sub-cellular activities has been investigated so far [248-256], but the details remain elusive.

The mechanism;

It is known that the Ras protein takes role in the signal transduction events of several cellular activities which is yet to be elucidated in terms of differentiation of the signal outputs that were generated from the same source, namely the Ras protein. So, the Ras protein involves in a multitude of signal transduction pathways. One may suggest a novel level of signaling differentiation mechanism for cellular signaling, which acts like a “multi-output DC-DC converter using a resonance circuit” [257]. So, the Ras protein may be achieving its multi-function capability by changing the switching frequency of itself according to the input stimuli; and creating a frequency of particular output circuit in the membrane, to be the input of its downstream effectors and regulators. Accordingly, changes in the frequencies would be varying the signal outputs which can be achieved by slight changes in the protein activity or the surrounding lipid configuration. The effective functioning of the lipid microdomains with distinct lipid contents may thus be involved in modulation. This was supported by a previous study [258] which was demonstrating that the G proteins reconstituted in liposomes were showing an increase in the G protein activity in response to the shear stress, when the protein receptors were lacking. The protein activity was observed [258] to have increased with the bilayers of higher rigidity due to their cholesterol contents. Therefore, proper sub-cellular localizations would be crucial, such as in case of the Ras proteins that are laterally segregating into the non-overlapping and dynamic domains of the plasma membrane microdomains [259].

Significance of the mechanism;

The proposed mechanism would thus be relating and combining the mechanical inputs to the biological outputs, in conjunction with the activity regulation of the Ras protein by the mechanical terms such as the shear force [260] and the plasma membrane viscosity [261]. The shear stress within the body, generated by the cardiac cycle and experienced by the endothelial cells, was given as a function of blood flow patterns throughout the vasculature [262]. Similarly, the IRE surface was experiencing pulsatile shear stress during our flow-through measurements. The significance of this concept is underlying the fact that

hypertension [263], diabetes [264], and hypercholesterolemia [265] could promote atherosclerosis by disrupting the responding capability of the endothelium to the shear stress. Differential coupling of the signaling mechanisms and the subsequent endothelial cellular responses to the individual ‘shear stress’ receptor sub-types were suggested to be providing the flexibility of the endothelial cellular responses to the variations in the shear stress [262].

Mechanotransduction;

Transduction of mechanical forces due to a combination of force transmission through the cytoskeleton, and the transduction of physical forces to biochemical signals at mechanotransducer sites [266] take place through a combination of the physical and the biochemical terms. The integrin-matrix interactions, membrane microdomains, ion channels and the G proteins were already proposed as the mechanotransducer candidates [262]. Hence, in order to sense and transduce the signals in response to the shear stress, those candidates need to be anchored; such as the integrins, which transduce the mechanical stimuli to biochemical signals in several cellular processes such as inflammation, migration, growth and differentiation [267].

Ras protein may also be involving in mechanotransduction. A mechanical force was previously suggested [268] to be generated by the GTP-hydrolyzing G proteins. According to the molecular dynamics simulations, Ras protein was shown [268] to be generating a force upon hydrolysis. The force was within the ranges (up to 10-pN) comparable to those produced by the ATP-hydrolyzing motor proteins such as myosin and kinesin [269]. The ‘stress-relaxation’ model [270] was offered as a force-transduction mechanism [268]. The motor proteins that were in a tense, elastically stretched state before hydrolysis, were releasing their energy while going under a structural transition [270]. A similar scenario was proposed in case of the force generation by kinesin [271]. Eventually, one significant support [272] came recently which was coupling the contractile forces of molecular motors, such as myosin, and the membrane waves. The study [272] was showing that the convex membrane proteins, through which the contractile forces were generated as a result of the myosin activity, were

also giving rise to robust transverse membrane waves that were similar to those observed on different cells.

According to our data, increase in the protein related amide (I and II) band absorbencies were observed (Figure 36). It could have been due to the increasing Ras protein affinity towards the membrane during the GTPase activity. An increase in the absorption signal during the course of an experiment could be due to the protein molecules which were getting closer to the IRE surface. Such an observation was prevalent in our experiments with the membrane-bound Ras protein, only in conditions that the GTP nucleotide was available in the medium (Figure 36). Thus the observation was related to the GTP exchange and the GTPase activity of the Ras protein. Increasing membrane affinity might have occurred due to a conformational change or a force generation during the Ras protein activity. This would eventually be sensed by the membrane.

A test for the membrane affinity was performed (section 6.4) by adding excess amount of GTP nucleotide and the lipid vesicles onto the lipidated Ras protein (HD Far MIC Ras) which was injected on the diamond IRE of the horizontal ATR set-up (Figure 55). The membrane was further away from the surface than the Ras protein. Thus, a decrease in the absorbance signal was expected during the protein insertion through the membrane. In due course, a slight but significant decrease in the absorbance signal was observed after 4 hrs (Figure 55).

Further structural features in action;

If a local displacement as a result of increasing membrane affinity of the Ras protein was taking place, such an action would probably be prevented in the live cell by the interaction partners of the Ras protein and the cytoskeletal meshwork of the cell. Tendency of the Ras protein to move towards the membrane might have been in relation to the factors such as the (Lys, Arg) bias [273-277], which is normally applied to the proteins adopting a mixture of opposite orientations in the membrane. The proteins with a (Lys, Arg) bias close to zero are sensitive to the addition and removal of a single positively charged residue from the loop regions. One may consider the possibility of a similar situation in case of the Ras protein.

Such a bias might be applicable for the Ras protein in the sense that, the acidic and basic residues in the linker domain (residues 166-178/179) were found to be necessary for stable palmitoylation and membrane localization [278] (Figure 53); and the K170 and R169 had the potential to form H-bonds with the charged residues of the lipid phosphate head groups [42].

It was previously mentioned that the major interaction between the G-domain and the linker was involving a salt bridge between the E49 (initial residue of $\beta 3$) and K170 side chains [42] (Figure 53). Also, recent MD-simulations [137] for the GTP- *versus* GDP-bound H-Ras were suggesting that the end of $\beta 2$ and the start of $\beta 3$ loops were arranging differently with respect to the 4th α -helix, along with some residues in the in the 5th α -helix (Figure 53).

Figure 53. Illustration of the membrane undulations associated with membrane-bound Ras protein. Adapted from the membrane-bound Ras illustration of the 3D-model which was built according to the X-Ray [8] and NMR structures [36, 43], by Till Rudack.

Additionally, lysine side chains were suggested [279] to be causing snorkeling of the membrane with a free energy cost between 0,007 to 0,7 kkal/mol [279]. Ras protein could be accounted as a peripheral protein in the live cell, which increases its hydrophobic part *via* snorkeling, and inserts deeper in the membrane for a stronger binding. The Lys 185 of H-Ras anchor was observed to be snorkeling during membrane insertion [239]. This situation was further supported by the Tyr band splitting in case of our measurements (Figure 19).

In the previous sections (3.4.2.2), structural transitions within the F141-Q150 region was offered to induce a β -hairpin motif; which would be linking the nucleotide binding affinity to the membrane interaction affinity (Figure 53). As an experimental support, a dynamic β -sheet structure was implied through the data of the NO-mediated nucleotide exchange measurements of the Ras protein (Figure 44b). There were also other residues which were not highlighted in section 3.4.2.2. Those were the positively charged R41, K42, K117 and R128 residues with increased tendencies in their dynamicity, according to the pdb sources 1CRP and 1CRQ.

In the end;

By means of this ATR-FTIR study on the membrane-bound Ras protein, a coherent theory on the action mechanism of the Ras protein in distinct signal transduction pathways was attempted to be hypothesized through a novel means of mechanotransduction involving Ras-mediated signal transduction. The significance of this proposal is underlying the co-ordination of multiple pathways with regard to the possible interference patterns of the signals, including those arousing from the Ras signal transduction pathways. In the future, validity and the details of the concept need to be examined. Yet the theory seems sufficient as a starting point for the theoretical studies and practical applications.

5.6 Summary of the ATR-FTIR studies with membrane-bound Ras protein and NO

NO-mediated concerted nucleotide exchange and the subsequent GTPase activity of the model membrane-bound Ras protein were investigated for the first time in solution, by means of a surface sensitive biophysical technique. The set-up was immune from the adverse effects of the indirect NO donors. Protein activity was achieved; and interpretation of our data was leading to the conclusion that, NO-aided loosening of the membrane networking was occurring along with the stabilizing interactions in the lipid C=O region. Also, a dynamic random to sheet transition within the membrane-bound Ras protein structure was occurring in a concerted fashion. Further analysis also implied that the change in the lipid absorbance intensities could be pertinent to the conformational alterations of the Ras protein.

The intriguing observation was the typical behavior of the lipids, which was a modulated excitation response. Namely, the lipid concentration was varying, as observed through the changes in the lipid absorbance intensities of the difference spectra. Also, the initial difference spectrum was restored at the end of measurement; so, could be gathered as a cyclic response. It was suggested that the concerted exchange of the GDP nucleotide with the GTP nucleotide was starting up the GTPase activity, and was somehow causing a modulated excitation of the membrane. Hence, the membrane was modulated when the protein was bound to the membrane and active on the surface.

Our observation could have biological relevance to the membrane ruffling, which was postulated to be accompanied in the live cell with the Ras protein activity, along with the fluid-phase pinocytosis [245]. Ruffling is common to distinct cellular actions and was stimulated in response to varying inherent factors of a cell, and in response to the extracellular agents. Hence, the multi-functionality of the Ras protein may be achieved through varying the switching frequency of itself according to the input stimuli; thus creating a frequency of particular output circuit. The signal, transmitted through the membrane, would be the input of downstream effectors and regulators. In the future, validity and details of this theory need to be investigated along with the possible practical applications.

6 Methods

6.1 General Methods and Procedure for the ATR-FTIR Measurement

Diamond surface of hATR was cleaned chemically by applying 6 N guanidine hydrochloride and/or 2 M sodium hydroxide solutions directly on the surface. Methyl ethyl ketone (MEK) was applied as an organic solvent to remove the lipid remnant. Cleaning solutions, which were brought into contact with the crystal surface, sucked from the surface by the aid of a micropipette tip connected to a vacuum pump through a home-made cleaning system. Double distilled water was used for rinsing the surface after each treatment. Germanium IRE of vATR was cleaned chemically and gained hydrophilic character by dipping the crystal into 90 % sulphuric acid solution for 3 mins after detaching the crystal from its semi-closed holder. Rinsing the crystal was also performed with double distilled water and the crystal surface was dried under mild nitrogen flow. Methanol chloroform mixture (1:3) was applied as an organic solvent to remove the lipid remnant, again with a 3 mins of incubation period. After cleaning, germanium crystal was mounted back to the crystal holder. The holder with the crystal was shown in Figure 11a, and the inner schematic drawing was shown in Figure 7.

For POPC small unilamellar vesicle (SUV) preparation; 12.9 μ l of POPC in chloroform was taken from a 25 mg/ml stock solution in an eppendorf tube and two volumes of chloroform was added. Then, chloroform was evaporated under mild nitrogen flow. More concentrated lipid solutions were additionally kept under vacuum for 2-3 hrs for the complete removal of the chloroform remnant. 50 μ l deuterated buffer solution was added onto the multilayered dry lipid film and incubated while shaking with MS1 minishaker (IKA, Brazil) at 1200/min for one hour at room temperature. Multilamellar vesicle (MLV) solution was the outcome after incubation. SUVs were prepared from the MLV solution by sonification in ice cold water bath for 7 mins. Clearance of the opaque lipid solution was indicating the formation of

vesicles with less than a 100 nm radius; which was immediately observed after sonification and vesicles' size was measured using the High-Performance Particle Sizer with NIBS™ Technology from Malvern Instruments (Malvern, Worcs., UK).

POPC SUVs ($\sim 0,3$ mM, 60 μ l for hATR; $\sim 0,3$ mM, 1,2ml for vATR) were brought into contact with the clean, hydrophilic surface of the solid support for initiating the vesicle fusion process on the IRE [139, 280]. Spectra collection was started immediately after the sample application. An incubation period of 30 mins was allowed for the lipid layer assembly. Then the system was flushed with vast amount of buffer. In case of hATR, this was attained by 10 successive steps with a two factor dilution of the solute volume atop the crystal, resulting in $\sim 10^3$ times dilution. Simply, 60 μ l deuterated buffer was added on top, mixed and removed repeatedly for 10 times. In case of vATR, a similar result was attained by allowing 10 ml deuterated buffer flow through the semi-closed system by means of a peristaltic pump induced flow.

Protein incorporation followed the membrane adsorption. It was initiated by mixing the protein into the sample solution on the surface. Starting bulk concentration of the protein was $\sim 2,0$ μ M. Protein adsorption on the membrane was followed from the evolution of the 1500-1700 cm^{-1} regions in the sample absorbance spectra with the reference spectrum recorded before the protein addition. Measurements were performed with the protein in deuterated buffer for the structural investigation which was presented in the 3rd section. Besides, specificity of the protein binding to the membrane was tested by the membrane affinity of the protein lacking the hypervariable C-term with the lipid anchors. Resultantly, negligible or no surface binding of the protein was observed. Measurements were carried out at room temperature and performed at an instrument resolution of 2 cm^{-1} with 4 times zero filling. Polarized spectra were recorded with the incident light polarized parallel or perpendicular (vertical) to the incident plane. The three-term Blackman-Harris apodization was applied prior to Fourier Transformation. 256 scans were averaged for each spectrum.

6.2 Procedure for the ATR-FTIR studies with the membrane-bound Ras and NO

These measurements were performed with the peristaltic pump induced flow of the vertical, germanium IRE bearing flow-through experimental set-up, if not indicated otherwise. In case of the NO mediated nucleotide exchange measurements, the buffer solution to be consumed during the experiment was deoxygenated by bubbling with Argon for at least 30 mins per 50 ml buffer solution. The surface preparation steps, including the POPC SUV vesicle fusion and the protein incorporation on the surface were performed as described in the methods (7th) section. The system was maintained as a closed circuit only except for the buffer flushing steps, to prevent any extreme oxygenation in the system. Sample material injections were performed with steel needle syringes.

Before starting the GTP exchange by the aid of NO saturated buffer solution; approximately 1 mM GDP solution was circulated through the system for 2 hrs in order to attain stability and to ensure the protein-bound nucleotide state of the membrane-bound Ras protein. Buffer solution was flushed for 20 mins with a flow rate of 1 ml/min before the addition of GTP nucleotide to the system for the exchange. Concentrated GTP solution was injected afterwards to an end concentration of about 2 mM and left for circulation for 10 mins in order to attain a homogeneous dissipation through the whole system. Then 2 mM NO saturated buffer solution was injected to an end concentration of about 1 mM; thus, lowered the circulating GTP concentration down to approximately 1 mM. Data collection was performed for about 2,5 hrs, in the same manner as described above in section 6.1.

7 Chemicals and Materials

POPC was purchased from Lipoid (Lipoid GmbH, Ludwigshafen, Germany). Lipid solutions of a concentration of about 32 mM were prepared in chloroform, and lipid vesicle solutions were prepared in D₂O (Deutero GmbH, Germany) buffer (20 mM Tris/HCl pH 7.4, 5 mM MgCl₂, 2 mM DTE). The protein stock solution (40,8 g/l) was used for the injection of N-Ras protein onto the buffer solution of the adsorbed POPC model membrane. Measurements of the 3rd section were carried out in the same deuterated buffer medium given above, and at room temperature.

Both a horizontal (h) ATR multireflection unit (DuraSampIR[®], Resultec Analytic Equipment, Germany) mounted in an IFS88 spectrometer (BrukerOptics, Germany) and a vertical (v) ATR multireflection unit, (Specac, Germany) mounted in an IFS66 spectrometer (BrukerOptics, Germany) were used for the measurements. Instruments were equipped with a KBr beam polarizer. Diamond crystal surface, namely the internal reflection element (IRE) of hATR was 4 mm with an aperture angle of 45° yielding 7 active internal reflections. IRE of vATR was a 52 x 20 x 2 mm trapezoidal germanium ATR plate with an aperture angle of 45° yielding 25 internal reflections among which 21 was active.

The expression and purification of truncated H-Ras was carried out in our laboratory by Angela Kallenbach and the detailed procedure is described elsewhere [281]. Shortly, truncated (residues 1-166) wildtype (without any oncogenic mutation) H-Ras was overexpressed in *E. coli* CK600K strain with transformed ptac c-h-ras (A. Wittinghofer, MPI Dortmund) by using full media, to which 0,834 mM of each amino acid was added. Purification of the protein was performed by using DEAE ion exchange chromatography and gel filtration, as described previously [281].

The synthesis of the farnesylated and hexadecylated N-Ras lipopeptide was performed in MPI-Dortmund, by the department of chemical biology and the department of Structural biology. If we summarize the procedure [44-49]; truncated (residues 1–181) wildtype N-Ras was expressed in *E. coli* CK600K strain, and then purified using DEAE ion exchange

chromatography and gel filtration. Chemically synthesized N-Ras lipopeptide was coupled to the protein in 20 mM Tris/HCl, pH 7.4, 5 mM MgCl₂, saturated with the detergent Triton X114. The detergent removed by another DEAE ion exchange chromatography and the lipoprotein concentrated in 20 mM Tris/HCl, pH 7.4, 5 mM MgCl₂, 2 mM DTE by size exclusion filtration, using Amicon[®] concentrators. All protein batches were analyzed by SDS-PAGE and MALDI-TOF mass spectrometry.

NO loading to the buffer was performed by Sonnur Isik Uppenkamp as described elsewhere [168], in the electrochemistry laboratory of Prof. Wolfgang Schuhmann, Ruhr-Uni-Bochum. The NO stock solution was prepared in a closed system consisting of 10 % diluted H₂SO₄, concentrated NaNO₂, and saturated KOH solutions. All the O₂ was removed from the system by flushing with argon; then, the reaction was started with the slow addition of H₂SO₄ to the concentrated NaNO₂ to produce the NO gas. NO gas was passed through saturated KOH to remove any NO₂⁻ which was produced during the reaction, and bubbled into deoxygenated buffer solution for at least 30 minutes. This was sufficient to ensure the saturation concentration of NO.

REFERENCES

- 1- W. P. Parks and E. M. Scolnick. In vitro translation of Harvey murine sarcoma virus RNA. *J. Virol.* **22** (1977) pp. 711-719.
- 2- J. J. Harvey. An unidentified virus which causes the rapid production of tumors in mice. *Nature* **204** (1964) pp. 1104-1105.
- 3- W. H. Kirsten and L. A. Mayer. Morphologic responses to a murine erythroblastosis virus. *J. Nat. Cancer Inst.* **39** (1967) pp. 311-319.
- 4- E. M. Scolnick, A. G. Papageorge and T. Y. Shih. Guanine nucleotide-binding activity as an assay for src protein of rat-derived murine sarcoma viruses. *PNAS*, **76** (1979) issue 10, pp. 5355-5359.
- 5- E. F. Pai, W. Kabsch, U. Krengel, K. C. Holmes, J. John and A. Wittinghofer . Structure of the guanine-nucleotide-binding domain of the Ha-*ras* oncogene product p21 in the triphosphate conformation. *Nature* **341** (1989) pp. 209-214.
- 6- I. R. Vetter and A. Wittinghofer. The Guanine nucleotide-binding switch in three dimensions. *Science*, **294** (2001), no. 5545, pp. 1299–1304.
- 7- R. Newbold. Mutant *ras* proteins and cell transformation. *Nature* **310** (1984) no. 5979, pp. 628–629.
- 8- M. V. Milburn, L. Tong, A. M. Devos, A. Brunger, Z. Yamaizumi, S. Nishimura and S. H. Kim. Molecular switch for signal transduction – Structural differences between active and inactive forms of protooncogenic Ras proteins. *Science* **247** (1990) pp. 939-945.
- 9- J. P. McGrath, D. J. Capon, D. V. Goeddel and A. D. Levinson. Comparative biochemical properties of normal and activated human *ras* p21 protein. *Nature* **310** (1984) pp. 644 – 649.
- 10- X. Du, H. Frei and S.-H. Kim. The mechanism of GTP hydrolysis by Ras probed by Fourier Transform Infrared Spectroscopy. *J. Biol. Chem.* **275** (2000) issue 12, pp. 8492-8500.

- 11- C. Koetting, M. Blessenohl, Y. Suveyzdis, R. S. Goody, A. Wittinghofer and K. Gerwert. Characterisation of a phosphoryl transfer intermediate in the GAP-catalysed GTPase reaction of Ras by FTIR-difference spectroscopy, *PNAS*, **103** (2006) pp. 13911-13916.
- 12- T. Finkel, C. J. Der and G. M. Cooper. Activation of *ras* genes in human tumors does not affect localization, modification, or nucleotide binding properties of p21. *Cell*, **37** (1984) issue 1, pp. 151-158.
- 13- D. R. Lowy and B. M. Willumsen. Function and regulation of Ras. *Annu. Rev. Biochem.* **62** (1993) pp. 851-891.
- 14- J. Heo and S. L. Campbell. Mechanism of Redox-mediated Guanine Nucleotide Exchange on Redox-active Rho GTPases. *J. Biol. Chem.* **280** (2005) pp. 31003-31010.
- 15- F. McCormick. Activators and effectors of *ras* p21 proteins. *Current Opinion in Genetics & Development*, **4** (1994) issue 1, pp. 71-76.
- 16- M. S. Boguski and F. McCormick. Proteins regulating Ras and its relatives. *Nature*, **366** (1993) pp. 643-654.
- 17- L. A. Feig and B. Schaffhausen. The hunt for Ras targets. *Nature*, **370** (1994), no. 6490, pp. 508-509.
- 18- G. Oxford and D. Theodorescu. Ras superfamily monomeric G proteins in carcinoma cell motility. *Cancer Lett.* **189** (2003) pp. 117-28.
- 19- A. Malliri and J. G. Collard. Role of Rho family proteins in cell adhesion and cancer. *Curr Opin. Cell Biol.* **15** (2003) pp. 583-589.
- 20- Y. Takai, T. Sasaki and T. Matozaki. Small GTP binding proteins. *Physiol. Rev.* **81** (2001) pp. 153-208.
- 21- J. M. Shields, K. Pruitt, A. Mcfall, A. Shaub and C. J. Der. Understanding Ras: 'it ain't over 'till it's over'. *Trends Cell Biol.* **10** (2000) pp. 147-154.
- 22- S. L. Campbell, R. Khosravi-Far, K. L. Rossman, G. J. Clark and C. J. Der. Increasing complexity of Ras signaling. *Oncogene*, **17** (1998) pp. 1395-1413.

- 23- G. W. Reuther and C. J. Der. The Ras branch of small GTPases: Ras family members don't fall from the tree. *Curr. Opin. Cell Biol.* **12** (2000) 157-165.
- 24- M. Kjeldgaard, J. Nyborg and B. F. Clark. The GTP binding motif: variations on a theme. *Faseb J.* **10** (1996) pp. 1347-1368.
- 25- T. Joneson and D. Bar-Sagi. Ras effectors and their role in mitogenesis and oncogenesis. *J. Mol. Med.* **75** (1997) pp. 7-593.
- 26- S. L. Campbell, R. Khosravi-Far, K. L. Rossman, G. J. Clark and C. J. Der. Increasing complexity of Ras signaling. *Oncogene*, **17** (1998) pp. 1395-1413.
- 27- B. M. Willumsen, A. Christensen, N. L. Hubbert, A. G. Papageorge, and D. R. Lowy. The p21 ras C-terminus is required for transformation and membrane association. *Nature*, **310** (1984) pp. 583-586.
- 28- J. H. Jackson, J. W. Li, J. E. Buss, C. J. Der and C. G. Cochrane. Polylysine domain of K-ras 4B protein is crucial for malignant transformation. *Biochemistry*, **91** (1994) pp. 12730-12734.
- 29- J. F. Hancock, H. Paterson and C. J. Marshall. A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21 ras to the plasma membrane. *Cell*, **63** (1990) pp. 133-139.
- 30- K. Walker and M. F. Olson. Targetting Ras and Rho GTPases as opportunities for cancer therapeutics. *Current Opinion in Genetics & Development*, **15** (2005) pp. 62-68.
- 31- A. Wittinghofer and H. Waldmann. Ras – A molecular switch involved in tumor formation. *Angewandte Chemie*, **39** (2000) issue 23, pp. 4192-4214.
- 32- M. Edidin. The state of lipid rafts: from model membranes to cells. *Annu. Rev. Biophys. Biomol. Struct.* **32** (2003) pp. 257-283.
- 33- I. A. Prior, A. Harding, J. Yan, J. Sluimer, R. G. Parton and J. F. Hancock JF. GTP-dependent segregation of H-ras from lipid rafts is required for biological activity. *Nat. Cell Biol.* **3** (2001) pp. 368-375.

- 34- H. Niv, O. Gutman, Y. Kloog and Y. I. Henis. Activated K-Ras and H-Ras display different interactions with saturable nonraft sites at the surface of live cells. *J. Cell Biol.* **157** (2002) pp. 865-872.
- 35- J. F. Hancock. Ras proteins: different signals from different locations. *Nat. Rev. Mol. Cell Biol.* **4** (2003) pp. 373-384.
- 36- G. Reuther, K. T. Tan, A. Vogel, C. Nowak, K. Arnold, J. Kuhlmann, H. Waldmann and D. Huster. The lipidated membrane anchor of full length N-Ras protein shows an extensive dynamics as revealed by solid-state NMR spectroscopy. *JACS* **128** (2006) pp.13840-13846.
- 37- R. G. Parton and J. F. Hancock. Lipid rafts and plasma membrane microorganization: insights from Ras. *Trends Cell Biol.* **14** (2004) pp. 141-147.
- 38- O. Rocks, A. Peyker, M. Kahms, P. J. Verveer, C. Koerner, M. Lumbierres, J. Kuhlmann, H. Waldmann, A. Wittinghofer and P. I. H. Bastiaens. An acylation cycle regulates localization and activity of palmitoylated Ras isoforms. *Science*, **307** (2006) issue 5716, pp. 1746-1752.
- 39- C. Nicolini, J. Baranski, S. Schlummer, J. Palomo, M. Lumbierres-Burgues, M. Kahms, J. Kuhlmann, S. Sanchez, E. Gratton, H. Waldmann and R. Winter. Visualizing association of N-Ras in lipid microdomains: influence of domain structure and interfacial adsorption. *JACS*, **128** (2006) issue 1, pp. 192-201.
- 40- A. Meister, C. Nikolini, H. Waldmann, J. Kuhlmann, A. Kerth, R. Winter and A. Blume, Insertion of lipidated Ras proteins studied by Infrared Reflection Absorption Spectroscopy (IRRAS). *Biophys. J.* **91** (2006) pp. 1388-1401.
- 41- F. Bringezu, M. Majerowicz, S. Wen, G. Reuther, K-T. Tan, J. Kuhlmann, H. Waldmann and D. Huster. Membrane binding of a lipidated N-Ras protein studied in lipid monolayeres. *Eur. Biophys. J.* **36(4-5)**, (2007) pp.491-498.

- 42- A. A. Gorfe, M. Hanzal-Bayer, D. Abankwa, J. F. Hancock and J. A. McCommon. Structural and dynamic studies of the full-length lipid-modified H-Ras protein in complex with a DMPC bilayer. *J. Med. Chem.* **50** (2007) issue 4, pp. 674-684.
- 43- R. Thapar, J. G. Williams and S. L. Campbell. NMR characterization of full-length farnesylated and non-farnesylated H-Ras and its implications for Raf activation. *J. Mol. Biol.* **343** (2004) pp. 1391–1408.
- 44- D. Kadereit, J. Kuhlmann and H. Waldmann. Linking the fields-the interplay of organic synthesis, biophysical chemistry, and cell biology in the chemical biology of protein lipidation. *ChemBioChem*, **1** (2000) pp. 144-169.
- 45- B. Bader, K. Kuhn, D. J. Owen, H. Waldmann, A. Wittinghofer and J. Kuhlmann. Bioorganic synthesis of lipid-modified proteins for the study of signal transduction. *Nature*, **403** (2000) pp. 223–226.
- 46- J. Kuhlmann. Protein lipidation. *Methods in Molecular Biology*, **283** (2004) pp. 217-220.
- 47- S. Sommer, T. Voigt, I. Heinemann, B. Popkirova, R. Reents and J. Kuhlmann. Lipidation of proteins and peptides: Farnesylation of the Ras protein. In: *Chemical Biology - A practical course*, Wiley-VCH, Weinheim, 2004, pp. 107-122.
- 48- M. Wagner and J. Kuhlmann. Generation and characterization of Ras lipoproteins based on chemical coupling. *Methods in Molecular Biology*, **283** (2004) pp. 245-254.
- 49- L. Brunsveld, J. Kuhlmann, K. Alexandrov, A. Wittinghofer, R. S. Goody and H. Waldmann. Lipidated Ras and Rab peptides and proteins - synthesis, structure, and function. *Angewandte Chemie International Edition*, **45** (2006) issue 40, pp. 6622-6646.
- 50- G. Brezesinski and H. Moehwald. Langmuir monolayers to study interactions at model membrane surfaces. *Adv. Colloid. Interface Sci.* **100–102** (2003) pp. 563–584.
- 51- H. McConnell. Structures and transitions in lipid monolayers at the air–water interface. *Ann. Rev. Phys. Chem.* **42** (2006) pp.171–195.

- 52- P. R. Griffiths and J. A. de Haseth. *Fourier Transform Infrared Spectroscopy*, John Wiley & Sons, NY, 1986.
- 53- M. Engelhard, B. Scharf and F. Siebert. Protonation changes during the photocycle of sensory rhodopsin II from *Natronobacterium pharaonis*. *FEBS Letters*, **395** (1996) Issues 2-3, pp. 195–198.
- 54- P. Hellwig, T. Mogi, F.L. Tomson, R.B. Gennis, J. Iwata, H. Miyoshi and W. Mantele, Vibrational modes of ubiquinone in cytochrome *bo*₃ from *Escherichia coli* identified by Fourier transform infrared difference spectroscopy and specific ¹³C labeling. *Biochemistry*, **38** (1999), pp. 14683–1468.
- 55- M. Lübben, A. Prutsch, B. Mamat and K. Gerwert, Electron transfer induces side-chain conformational changes of glutamate-286 from cytochrome *bo*₃. *Biochemistry*, **38** (1999) pp. 2048–2056.
- 56- A. Barth, Phosphoenzyme conversion of the sarcoplasmic reticulum Ca²⁺-ATPase – molecular interpretation of infrared difference spectra. *J. Biol. Chem.* **274** (1999) pp. 22170–22175.
- 57- R. Vogel and F. Siebert. Vibrational spectroscopy as a tool for probing protein function. *Current Opinion in Chemical Biology*, **4** (2000) issue 5, pp. 518–523.
- 58- C. Allin and K. Gerwert. Ras catalyzes GTP hydrolysis by shifting negative charges from gamma- to beta-Phosphate as revealed by Time-resolved FTIR difference spectroscopy. *Biochemistry*, **40** (2001) pp. 3037–3046.
- 59- R. M. Nyquist, D. Heitbrink, C. Bolwien, R. B. Gennis, and J. Heberle. Direct observation of protonation reactions during the catalytic cycle of cytochrome c oxidase. *PNAS*, **100** (2003) no. 15, pp. 8715–8720.
- 60- M. G. Friedrich, F. Giess, R. Naumann, W. Knoll, K. Ataka, J. Heberle, J. Hrabakova, D. H. Murgida and P. Hildebrandt. Active site structure and redox processes of cytochrome C oxidase immobilised in a novel biomimetic lipid membrane on an electrode. *Chem. Comm.* (2004) pp. 2376–2377.

- 61- C. Koetting and K. Gerwert. Proteins in action monitored by Time-resolved FTIR Spectroscopy. *Chem. Phys. Chem.* **6** (2005a) pp. 1–8.
- 62- M. Klaehn, J. Schlitter and K. Gerwert. Theoretical IR spectroscopy based on QM/MM calculations provides changes in charge distribution, bond lengths and bond angles of the GTP ligand induced by the Ras-protein. *Biophys. J.* **88** (2005) pp. 3829–3844.
- 63- C. Koetting and K. Gerwert. Protein reactions: Resolved with tr-FTIR. *Spectroscopy Europe* **19** (2007) vol.3, pp. 19-23.
- 64- A. Thorne, U. Litzén and S. Johansson (eds.) *Spectrophysics – Principles and Applications*. Springer-Verlag, Germany, 1999
- 65- E. B. Wilson, J. C. Decius and P. C. Cross. *Molecular Vibrations*. McGraw-Hill, NY, 1955.
- 66- L. A. Woodward. *Introduction to the theory of molecular vibrations and vibrational spectroscopy*. Oxford: Clarendon Pr. 1972.
- 67- W. Herres. FT-IR: The Renaissance of Infrared Spectrometry, *CHIMIA* **39** (1985) pp. 64-68
- 68- P. J. Hendra. How does FTIR work? *Int. J. Vibr. Spec.* [www.ijvs.com] **5**, 5, 2 (2001)
- 69- A. Barth, W. Mäntele and W. Kreutz. Infrared spectroscopic signals arising from ligand binding and conformational changes in the catalytic cycle of sarcoplasmic reticulum calcium ATPase. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, **1057** (1991) issue 1, pp. 115-123.
- 70- C. Roedig and F. Siebert. Monitoring fast reactions of slow cycling systems with time-resolved FTIR spectroscopy. *Vib. Spectrosc.* **19** (1999) issue 2, pp. 271-276.
- 71- R. M. Nyquist, K. Ataka, and J. Heberle. The Molecular Mechanism of Membrane Proteins Probed by Evanescent Infrared Waves. *ChemBioChem* **3** (2004) pp. 431-436.
- 72- M. G. Fiedrich, F. Giess, R. Naumann, W. Knoll, K. Ataka, J. Heberle, J. Hrabakova, D. H. Murgida and P. Hildebrandt. Active site structure and redox processes of cytochrome C

oxidise immobilised in a novel biomimetic lipid membrane on an electrode. *Chem. Comm.* (2004) pp. 2376-2377.

73- C. Koetting and K. Gerwert. Monitoring protein-ligand interactions by time-resolved FTIR difference spectroscopy. *In: Protein-Ligand Interactions; Methods in Molecular Biology*, Editor: Nienhaus, U. The Humana Press Inc., NJ, USA, 2005.

74- K. Ataka and J. Heberle. Biochemical applications of surface-enhanced infrared absorption spectroscopy. *Anal Bioanal Chem.* **388** (2007) issue 1, pp. 47–54.

75- N. J. Harrick. *Internal Reflection Spectroscopy*, Inter-Science, NY, 1967.

76- U. P. Fringeli, in F. M. Mirabella (ed.), *Internal Reflection Spectroscopy, Theory and Applications*, Marcel Dekker, NY, 1992.

77- L. Pauling, R. B. Corey and H. R. Branson. The structure of proteins; two hydrogen-bonded helical configurations of the polypeptide chain. *PNAS*, **37** (1951) pp. 205-11.

78- J. Heringa. Predicting secondary structure from protein sequences. In *Bioinformatics: Sequence, Structure and Databanks* (eds. D. Higgins and W. Taylor) Oxford University Press Inc. USA, 2000.

79- D. M. Byler and H. Susi. Examination of the secondary structure of proteins by deconvolved FTIR spectra. *Biopolymers.* **25** (1986) pp. 469–487.

80- L. Pauling, R. B. Corey, H. R. Branson. The structure of proteins; two hydrogen-bonded helical configurations of the polypeptide chain. *PNAS*, **37** (1951) pp. 205-11.

81- J. Bandekar. Amide modes and protein conformation. *Biochim. Biophys. Acta* **1120** (1992) pp. 123–143.

82- A. Barth. The infrared absorption of amino acid side chains. *Prog. Biophys. Mol. Biol.* **74**, (2000) pp. 141–173.

83- R. Rai, S. Raghothama, R. Sridharan and P. Balaram. Tuning the β -turn segment in designed peptide β -hairpins: Construction of a stable type I' β -turn nucleus and hairpin-helix promoting transition sites. *Peptide Science*, **88** (2006) pp.350-361.

- 84- E. de Alba, M. Rico and M. A. Jiménez. The turn sequence directs β -strand alignment in desined β -hairpins. *Protein Science*, **8** (1999) pp. 2234-2244.
- 85- J. Higo, N. Ito, M. Kuroda, S. Ono, N. Nakajima and H. Nakamura. Energy landscape of a peptide consisting of α -helix, 3_{10} -helix, β -turn, β -hairpin, and other disordered conformations. *Protein Science*, **10** (2001) pp. 1160-1171.
- 86- G. Rontó and I. Tarján (eds.) An Introduction to Biophysics with medical orientation, Chapter 3, Akadémiai Kiadó és Nyomda Vallalat, Hungary, 1984.
- 87- E. Goormaghtigh, V. Cabiaux, and J. M. Ruyschaert. Determination of soluble and membrane protein structure by Fourier transform infrared spectroscopy. *Subcell. Biochem.* **23**, (1994) pp.329–450.
- 88- S. Krimm and J. Bandekar. Vibrational spectroscopy and conformation of peptides, polypeptides, and proteins. *Adv. Protein Chem.* **38** (1986) pp. 181-363.
- 89- J. L. Arrondo, A. Muga, J. Castresana and F. M. Goñi. Quantitative studies of the structure of proteins in solution by Fourier-transform infrared spectroscopy. *Prog. Biophys. Mol. Biol.* **59** (1993), pp. 23-56.
- 90- W. K. Surewicz, H. H. Mantsch and D. Chapman. Determination of protein secondary structure by Fourier transform infrared spectroscopy: a critical assessment. *Biochemistry*, **32** (1993) pp. 389-394.
- 91- A. J. P. Alix, M. Berjot, J. Marx and G. Pedanou. Determination of the seceondary structure of proteins: From X-Ray crystallography to raman spectroscopy. In *Spectroscopy of Biological Molecules New Advances* (eds. E. D. Schmid, F. W. Schneider and F. Siebert) John Wiley and Sons Ltd. Great Britain, 1988.
- 92- M. Habeck, M. Nilges and W. Rieping. Bayesian inference applied to macromolecular structure determination. *Physical review. E, Statistical, nonlinear, and soft matter physics* **72** (2005) pp. 031912.

- 93- V. Baumruk, P. Pancoska and T. A. Keiderling. Predictions of secondary structure using statistical analyses of electronic and vibrational circular dichroism and Fourier transform infrared spectra of proteins in H₂O. *J. Mol. Biol.* **259** (1996) pp. 774–791.
- 94- D. C. Lee, P. I. Haris, D. Chapman and R. C. Mitchell. Determination of protein secondary structure using factor analysis of infrared spectra. *Biochemistry*, **29** (1990) pp. 9185–9193.
- 95- R. Pribic, I. H. van Stokkum, D. Chapman, P. I. Haris and M. Bloemendal. Protein secondary structure from Fourier transform infrared and/or circular dichroism spectra. *Anal. Biochem.* **214** (1993) pp. 366–378.
- 96- R. W. Sarver, Jr. and W. C. Krueger. An infrared and circular dichroism combined approach to the analysis of protein secondary structure. *Anal. Biochem.* **199** (1991) pp. 61–67.
- 97- F. Dousseau, and M. Pezolet. Determination of the secondary structure content of proteins in aqueous solutions from their amide I and amide II infrared bands. Comparison between classical and partial least-squares methods. *Biochemistry*, **29** (1990) pp. 8771–8779.
- 98- K. Rahmelow, and W. Hubner. Secondary structure determination of proteins in aqueous solution by infrared spectroscopy: a comparison of multivariate data analysis methods. *Anal. Biochem.* **241** (1991) pp. 5–13.
- 99- G. Vedantham, H. G. Sparks, S. U. Sane, S. Tzannis, and T. M. Przybycien. A holistic approach for protein secondary structure estimation from infrared spectra in H₂O solutions. *Anal. Biochem.* **285** (2000) pp. 33–49.
- 100- J. A. Hering, P. R. Innocent, and P. I. Haris. Automatic amide I frequency selection for rapid quantification of protein secondary structure from Fourier transform infrared spectra of proteins. *Proteomics*, **2** (2002) pp. 839–849.
- 101- J. A. Hering, P. R. Innocent, and P. I. Haris. An alternative method for rapid quantification of protein secondary structure from FTIR spectra using neural networks. *Spectrosc. Int. J.* **16** (2002) pp. 53–69.

- 102- M. Severcan, P. I. Haris, and F. Severcan. Using artificially generated spectral data to improve protein secondary structure prediction from Fourier transform infrared spectra of proteins. *Anal. Biochem.* **332** (2004) pp. 238–244.
- 103- J. A. Hering, P. R. Innocent, and P. I. Haris. Towards developing a protein infrared spectra databank (PISD) for proteomics research. *Proteomics*, **4** (2004) pp. 2310–2319.
- 104- E. Goormaghtigh, J.-M. Ruyschaert and V. Raussens, Evaluation of the information content in infrared spectra for protein secondary structure determination, *Biophys. J.* **90**, (2006) pp. 2046-2057.
- 105- S. Y. Venyaminov, J. F. Hedstrom, and F. G. Prendergast. Analysis of the segmental stability of helical peptides by isotope-edited infrared spectroscopy. *Proteins*, **45** (2001) pp. 81–89.
- 106- B. I. Baello, P. Pancoska, and T. A. Keiderling. Enhanced prediction accuracy of protein secondary structure using hydrogen exchange Fourier transform infrared spectroscopy. *Anal. Biochem.* **280** (2000) pp. 46–57.
- 107- J. L. Arrondo, J. Castresana, J. M. Valpuesta, and F. M. Goni. Structure and thermal denaturation of crystalline and noncrystalline cytochrome oxidase as studied by infrared spectroscopy. *Biochemistry*. **33** (1994) pp. 11650–11655.
- 108- W. H. Press, S. A. Teukolsky, W. T. Vetterling, B. P. Flannery. Numerical recipes, in: *C: The Art of Scientific Computing*, 2nd edn. Cambridge University Press, Cambridge, 1996.
- 109- M. L. Johnson and L. M. Faunt. Parameter estimations by least-squares methods. *Methods Enzymol.* **210** (1992) pp. 1-37.
- 110- V. A. Lórenz-Fonfría and E. Padrós. Curve-fitting of Fourier manipulated spectra comprising apodisation, smoothing, derivation and deconvolution. *Spectrochim. Acta Part A*, **60** (2004) pp. 2703-2710.
- 111- J. Ollesch, E. Kuennemann, R. Glockshuber and K. Gerwert. Prion protein α -to- β transition monitored by Time-resolved Fourier Transform Infrared Spectroscopy. *Applied spectroscopy*, **61** (2007) pp. 1025-1031.

- 112- C. Vigano, M. Smeyers, V. Raussens, F. Scheirlinckx, J. M. Ruyschaert, and E. Goormaghtigh. Hydrogen-deuterium exchange in membrane proteins monitored by IR spectroscopy: a new tool to resolve protein structure and dynamics. *Biopolymers*, **74**, (2004) pp.19–26.
- 113- Y. N. Chirgadze, O. V. Fedorov, and N. P. Trushina. Estimation of amino acid residue side-chain absorption in the infrared spectra of protein solutions in heavy water. *Biopolymers*, **14**, (1975) pp. 679–694.
- 114- S. Y. Venyaminov and N. N. Kalnin. Quantitative IR spectrophotometry of peptides compounds in water (H₂O) solutions. I. Spectral parameters of amino acid residue absorption band. *Biopolymers*, **30**, (1991) pp. 1243–1257.
- 115- K. Rahmelow, W. Hubner, and T. Ackermann. Infrared absorbancies of protein side chains. *Anal. Biochem.* **257**, (1998) pp. 1–11.
- 116- A. Barth and C. Zscherp. What vibrations tell us about proteins. *Quart. Rev. Biophys.* **35**, (2002) pp. 369–430.
- 117- E. Goormaghtigh, V. Cabiaux, and J. M. Ruyschaert. Secondary structure and dosage of soluble and membrane proteins by attenuated total reflection Fourier-transform infrared spectroscopy on hydrated films. *Eur. J. Biochem.* **193**, (1990) pp.409–420.
- 118- V. A. Lórenz-Fonfría and E. Padrós. Curve-fitting overlapped bands: quantification and improvement of curve-fitting robustness in the presence of errors in the model and in the data. *The Analyst*, **129** (2004) pp. 1243-1250.
- 119- Strandberg, E. Morein, S. Rijkers, D. T. S. Liskamp, R. M. J. van der Wel, P. C. A. and Killian, J. A. Lipid dependence of membrane anchoring properties and snorkeling behavior of aromatic and charged residues in transmembrane peptides. *Biochemistry*, **41** (2002) pp. 7190-7198.
- 120- U. P. Fringeli and H. H. Gunthard. Infrared membrane spectroscopy. *Mol. Biol. Biochem. Biophys.* **31** (1981) pp. 270-332.

- 121- D. G. Cameron, E. F. Gudgin and H. H. Mantsch. Dependence of Acyl Packing of Phospholipids on the Head Group and Acyl Chain Length. *Biochemistry*, **20** (1981) pp. 4496-4500.
- 122- J. Jass, T. Tjärnhage and G. Puu. From liposomes to supported, planar bilayer structures on hydrophilic and hydrophobic surfaces: An Atomic Force Microscopy study. *Biophysical J.* **79** (2000) pp. 3153-3163.
- 123- Y. Muto, K. Yamasaki, Y. Ito, S. Yajima, H. Masaki, T. Uozumi, M. Wälchli, S. Nishimura, T. Miyazawa and S. Yokoyama. Sequence-specific ^1H and ^{15}N resonance assignments and secondary structure of GDP-bound human c-Ha-Ras oritein in solution. *J. Biomol. NMR*, **3** (1993) pp. 165-184.
- 124- P. J. Kraulis, P. J. Dommelle, S. L. Campbell-Burk, T. V. Aken and E. D. Laure. Solution structure and dynamics of Ras p21*GDP determined by heteronuclear three- and four-dimensional NMR spectroscopy. *Biochemistry*, **33** (1994) pp. 3131-3135.
- 125- G. Reuther, K-T. Tan, J. Köhler, C. Nowak, A. Pampel, K. Arnold, J. Kuhlmann, H. Waldmann and D. Huster. Structural model of the membrane-bound C-terminus of lipid-modified human N-Ras protein. *Angew. Chem. Int Ed. Engl.* **45** (2006) pp. 5387–5390.
- 126- L. Tong, A. M. de Vos, M. N. Milburn and S.-H. Kim. Crystal structures at 2.2 Å resolution of the catalytic domains of normal *ras* protein and an oncogenic mutant complexed with GDP. *J. Mol. Biol.* **217** (1991) pp. 503-516.
- 127- S. Roy, S. Plowman, B. Rotblat, I. A. Prior, C. Muncke, S. Grainger, R. G. Parton, Y. I. Henis, Y. Kloog and J. F. Hancock. Individual palmitoyl residues serve distinct roles in H-Ras trafficking, microlocalisation and signalling. *Molecular and Cellular Biology*, **25** (2005) pp. 6722-6733.
- 128- A. A. Gorfe, A. Babakhani and J. A. McCammon. H-ras protein in a bilayer: Interaction and structure perturbation. *J. Am. Chem. Soc.* **129** (2007) pp. 12280-12286.

- 129- J. A. Perez-Pons, E. Padros and E. Querol. Prediction and Fourier-transform infrared-spectroscopy estimation of the secondary structure of a recombinant β -glucosidase from *Streptomyces sp.* (ATCC 111238) *Biochem. J.* **308** (1995) pp. 791-794.
- 130- Y. Xu, T. Wang and F. Gai. Strange temperature dependence of the folding rate of a 16-residue β -hairpin. *Chemical Physics*, **323** (2006) pp. 21-27.
- 131- J. J. van Thor, G. Y. Georgiev, M. Towrie and J. T. Sage. Ultrafast and low barrier motions in the photoreactions of the green fluorescent protein. *J. Biol. Chem.* **280** (2005) pp. 33652-33659.
- 132- S. H. Gellman. Minimal model systems for β -sheet secondary structure in proteins. *Curr. Opin. Chem. Biol.* **2** (1998) pp. 717-725.
- 133- S. K. Awasthi, S. C. Shankaramma, S. Raghothama and P. Balaram. Solvent-induced β -hairpin to helix conformational transition in a designed peptide. *Biopolymers*, **58** (2001) pp. 465-476.
- 134- C. S. Colley, S. R. Griffiths-Jones, M. W. George and M. S. Searle. Do interstrand hydrogen bonds contribute to β -hairpin peptide stability in solution? IR analysis of peptide folding in water. *Chem. Commun.* **7** (2000) pp. 593-594.
- 135- A. Valencia, P. Chardin, A. Wittinghofer and C. Sander. The ras protein family: Evolutionary tree and role of conserved amino acids. *Biochemistry*, **30** (1991) pp. 4637-4648.
- 136- M. S. A. Nur-E-Kamal, A. Sizeland, G. Abaco and H. Maruta. Asparagine 26, Glutamic Acid 31, Valine 45, and Tyrosine 64 of Ras proteins are required for their oncogenicity. *J. Biol. Chem.* **267** (1992) pp. 1415-1418.
- 137- D. Abankwa, M. Hanzal-Bayer, N. Ariotti, S. J. Plowman, A. A. Gorfe, R. G. Parton, J. A. McCammon and J. F. Hancock. A novel switch region regulates H-Ras membrane orientation and signal output. *The EMBO Journal* (2008) doi:10.1038/emboj.2008.10
- 138- E. Reimhult, M. Zäch, F. Höök and B. Kasemo. A multitechnique study of liposome adsorption on Au and lipid bilayer formation on SiO₂. *Langmuir*, **22** (2006) pp. 3313-3319.

- 139- A. Brian and H. M. McConnell, Allogeneic Stimulation of Cytotoxic T Cells by Supported Planar Membranes, *PNAS*, **81**, (1984) pp. 6159-6163.
- 140- X. D. Lu, A. L. Ottova, and H. T. Tien. Biophysical aspects of agar-gel supported bilayer lipid membranes: a new method for forming and studying planar bilayer lipid membranes. *Bioelectrochem. Bioenerget.* **39**, (1996) pp. 285-289.
- 141- L. K. Tamm and H. M. McConnell. Supported Phospholipid Bilayers. *Biophys. J.* **47**, (1985) pp. 105-113.
- 142- P. Wenzl, M. Fringeli, J. Goette and U. P. Fringeli. Supported Phospholipid Bilayers Prepared by the 'LB/Vesicle Method': A Fourier Transform Infrared Attenuated Total Reflection Spectroscopic Study on Structure and Stability, *Langmuir*, **10**, (1994) pp. 4253-4264.
- 143- A. L. Plant. Self-assembled phospholipid/alkanethiol biomimetic bilayers on gold, *Langmuir*, **9** (1993) pp. 2764-2767.
- 144- R. Richter, A. Mukhopadhyay and A. Brisson. Pathways of lipid vesicle deposition on solid surfaces: A combined QCM-D and AFM study. *Biophys. J.* **85**, (2003) pp. 3035-3047.
- 145- R. F. Furchgott and J. V. Zawadzki. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288** (1980) pp. 373-376.
- 146- D. E. Koshland. The molecule of the year. *Science*, **258** (1992) pp. 1861-1865.
- 147- L. J. Ignarro, G. M. Buga, K. S. Wood, R. E. Byrns and G. Chaudhuri. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci.* **84** (1987) pp. 9265-9269.
- 148- L. J. Ignarro, R. E. Byrns, G. M. Buda and K. S. Wood. Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical. *Cer. Res.* **61** (1987) pp. 866-879.
- 149- R. M. Palmer, A. G. Ferrige and S. Moncada. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327** (1987) pp. 524-526.

- 150- S. Moncada, R. M. J. Palmer and E. A. Higgs. Nitric oxide: physiology, pathophysiology and pharmacology. *Pharm. Rev.* **43** (1991) pp. 109-142.
- 151- C. Nathan. Nitric oxide as a secretory product of mammalian cells. *FASEB Journal*, **6** (1992) pp. 301-3064
- 152- C. J. Lowenstein, J. L. Dinerman and S. H. Synder. Nitric oxide: a physiologic messenger. *Ann. Intern. Med.* **120** (1994) pp. 227-237.
- 153- S. Moncada, R. M. Palmer and E. A. Higgs. The discovery of nitric oxide as the endogenous nitrovasodilation. *Hypertension*, **12** (1988) pp. 365-372.
- 154- S. Moncada, M. W. Radomski and R. M. Palmer. Endothelium-derived relaxing factor. Identification as nitric oxide and role in the control of vascular tone and platelet function. *Biochem. Pharmacol.* **37** (1988) pp. 2495-2501.
- 155- L. J. Ignarro. Nitric oxide as a unique signaling molecule in the vascular system: A historical overview. *J. Physiol. Pharmacol.* **53** (2002) pp. 503-514.
- 156- L. Gong, G. M. Pitari, S. Schulz and S. A. Waldman. Nitric oxide signaling: systems integration of oxygen balance in defense of cell integrity. *Curr. Opin. Hematol.* **11** (2004) pp. 7-14.
- 157- S. H. Snyder and D. S. Bredt. Biological roles of nitric oxide. *Sci. Am.* **266** (1992) pp. 68-71.
- 158- J. S. Beckman, J. Chen, H. Ischiropoulos and J. P. Crow. Oxidative chemistry of peroxynitrite. *Methods in Enzymol.* **233** (1994) pp. 229-240.
- 159- J. S. Stamler. Redox signaling: nitrosylation and related target interactions of nitric oxide. *Cell*, **78** (1994) pp. 931-936.
- 160- R. S. Braman and S. A. Hendrix. Nanogram nitrite and nitrate determination in environmental and biological materials by vanadium (III) reduction with chemiluminescence detection. *Anal. Chem.* **61** (1989) pp. 2715-2718.

- 161- A. Wennmalm, B. Lanne and A. S. Petersson. Detection of endothelial-derived relaxing factor in human plasma in the basal state following ischemia using electron paramagnetic resonance spectrometry. *Anal. Biochem.* **187** (1990) pp. 359-363.
- 162- G. Gabor and N. Allon. Spectrofluorometric method for NO determination. *Anal. Biochem.* **220** (1994) pp. 16-19.
- 163- O. Raveh, N. Peleg, A. Bettelheim, I. Silberman and J. Rishpon. Determination of NO production in melanoma cells using an amperometric nitric oxide sensor. *Bioelectrochem. Bioenerg.* **43** (1997) pp. 19-25.
- 164- K. T. Kwagoe and R. M. Wightman. Characterization of amperometry for in vivo measurement of dopamine dynamics in the rat brain. *Talanta*, **41** (1994) pp. 865-874.
- 165- Y. Xian, W. Zhang, J. Xue, X. Ying and L. Jin. Direct measurement of nitric oxide release from rat hippocampus. *Anal. Chim. Acta*, **415** (2002) pp. 127-133.
- 166- J. S. Beckman. The physiological and pathological chemistry of nitric oxide. In *Nitric Oxide-Principles and Actions*. J. Lancaster, ed. Academic Press, NY, pp.1-83, 1996.
- 167- L. Xiaoping, M. Miller, M. S. Joshi, D. D. Thomas and J. R. Lancaster JR. Accelerated reaction of nitric oxide with O₂ within the hydrophobic interior of biological membranes. *PNAS*, **95** (1998) pp. 2175-2179.
- 168- S. Isik. *Electrochemical detection of nitric oxide from living cells*. Dissertation, Faculty of Chemistry, Ruhr-University Bochum, Germany, 2005.
- 169- S. Venkataraman, S. M. Martin, F. Q. Schafer and G. R. Buettner. Detailed methods for the quantification of nitric oxide in aqueous solutions using either an oxygen monitor or EPR. *Free Radic. Biol. Med.* **29** (2000) pp. 580-585.
- 170- D. A. Wink, J. S. Beckman and P. C. Ford. Kinetics of nitric oxide in liquid and gas phase. In *Methods in Nitric Oxide Research*. M. Feelisch and J. S. Stamler, eds. John Wiley and Sons, NY, pp.29-39, 1996.
- 171- J. M. Zimmet and J. M. Hare. Nitrosoredox interactions in the cardiovascular system. *Circulation*, **114** (2006) pp.1531-1544.

- 172- R. M. Palmer, D. S. Ashton and S. Moncada. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333** (1988) pp. 664-666.
- 173- J. S. Stamler, D. I. Simon, O. Janaki, J. A. Osborne, S. Francis, M. Mullins, D. Singel and J. Loscalzo. S-nitrosylation of tissue-type plasminogen activator confers vasodilatory and antiplatelet properties on the enzyme. *Proc. Natl. Acad. Sci.* **89** (1992) pp. 8087-8091.
- 174- M. S. McMurtry, D. H. Kim, T. Dinh-Xuan and S. L. Archer. Measurement of nitric oxide, nitrite and nitrate using a chemiluminescence assay: an update for the year 2000. *Analysis*, **28** (2000) pp. 455-465.
- 175- T. S. Gorimar. Total nitrogen determination by chemiluminescence. In *Bioluminescence and Chemiluminescence: Instruments and applications*. K. Van Dyke, ed. CRC Press, Boca Raton, FL, pp.43-78, 1985.
- 176- R. E. Girgis, M. A. Qureshi, J. Abrams and P. Swerdlow. Decreased exhaled nitric oxide in sickle cell disease: relationship with chronic lung involvement. *Am. J. Hematol.* **72** (2003) pp. 177-184.
- 177- O. Aikio, M. L. Pokela and M. Hallman. Exhaled and nasal nitric oxide in mechanically ventilated preterm and term newborns. *Acta Paediatr.* **91** (2002) 1078-1086.
- 178- M. V. H. Pijnenburg, E. T. Lissenberg, W. Hofhuis, L. Ghio, W. C. J. Hop, W. P. Holland and J. C. De Jongste. Exhaled nitric oxide measurements with dynamic flow restriction in children aged 4-8 yrs. *Eur. Respir. J.* **20** (2002) pp. 919-924.
- 179- I. Hadjikoumi, A. Hassan and A. D. Milner. Exhaled nitric oxide measurements in childhood asthma: Comparison of two sampling techniques. *Pediatr. Res.* **52** (2002) pp. 745-749.
- 180- L. Lefevre, T. Willems, S. Lindberg and M. Jorissen. Nasal nitric oxide. *Acta Otorhinolaryngol. Belg.* **54** (2000) pp. 271-280.
- 181- I. Narang, R. Ersu, N. Wilson and A. Bush. Nitric oxide in chronic airway inflammation in children: Diagnostic use and pathophysiological significance. *Thorax*, **57** (2002) pp. 586-589.

- 182- P. G. Djupesland, W. Qian and J. S. Haight. A new method for the remote collection of nasal and exhaled nitric oxide. *Chest*, **120** (2001) pp. 1645-1650.
- 183- J. P. Griess. Coloring matters. *Ber. Dtsch. Chem. Ges.* **12** (1879) pp. 426-429.
- 184- K. M. Miranda, M. G. Espey and D. A. Wink. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. *Nitric Oxide*, **5** (2001) pp. 62-71.
- 185- A. M. Miles, D. A. Wink, J. C. Cook and M. B. Grisham. Determination of nitric oxide using fluorescence spectroscopy. *Methods Enzymol.* **268** (1996) pp. 105-120.
- 186- T. Nagano. Practical methods for detection of nitric oxide. *Luminescence*, **14** (1999) pp. 283-290.
- 187- H. Li, C. J. Meininger and G. Wu. Rapid determination of nitrite by reversed phase high-performance liquid chromatography with fluorescence detection. *J. Chromatogr. B*, **746** (2000) pp. 199-207.
- 188- H. Kojima, M. Hirotsu, N. Nakatsubo, K. Kikuchi, Y. Urano, T. Higuchi, Y. Hirata Y and T. Nagano T Bioimaging of nitric oxide with fluorescent indicators based on the rhodamine chromophore. *Anal. Chem.* **73** (2001) pp. 1967-1973.
- 189- J. H. Wiersma. 2, 3-Diaminonaphthalene as a spectrophotometric and fluorometric reagent for the determination of nitrite ion. *Anal. Lett.* **3** (1970) pp. 123-132.
- 190- T. Malinski and Z. Taha. Nitric oxide release from a single cell measured in situ by a porphyrinic-based microsensor. *Nature*, **358** (1992) pp. 676-678.
- 191- F. Lantoni, S. Trevin, F. Bedioui and J. Devynck. Selective and sensitive electrochemical measurement of nitric oxide in aqueous solution: discussion and new results. *J. Electroanal. Chem.* **392** (1995) pp. 85-89.
- 192- S. Trevin, F. Bedioui and J. Devynck. New electropolymerized nickel porphyrin films. Application to the detection of nitric oxide in aqueous solution. *J. Electroanal. Chem.* **408** (1996) pp. 261-265.
- 193- A. Ciszewski and G. Milczarek. A new Nafion-free bipolymeric sensor for selective and sensitive detection of nitric oxide. *Electroanal.* **10** (1998) pp. 791-793.

- 194- N. Diab and W. Schuhmann. Electropolymerized manganese porphyrin/polypyrrole films as catalytic surfaces for the oxidation of nitric oxide. *Electrochim. Acta*, **47** (2001) pp. 265-273.
- 195- A. V. Kashevskii, A. Y. Safronov and O. Ikeda. Behaviors of H₂TPP and CoTPP/Cl in Nafion film and the catalytic activity for nitric oxide oxidation. *J. Electroanal. Chem.*, **510** (2001) pp. 86-95.
- 196- F. Bedioui, S. Trevin, J. Devynck, F. Lantonie, A. Brunet and M. A. Devynck. Elaboration and use of nickel planar macrocyclic complex-based sensors for the direct electrochemical measurement of nitric oxide in biological media. *Biosens. Bioelectron.* **12** (1997) pp. 205-212.
- 197- M. Pontie, H. Lecture and F. Bedioui. Improvement in the performance of a nickel complex-based electrochemical sensor for the detection of nitric oxide in solution. *Sensor. Actuat. B*, **B56** (1999) pp. 1-5.
- 198- J. Jin, T. Miwa, L. Mao, H. Tu and L. Jin. Determination of nitric oxide with ultramicrosensors based on electropolymerized films of metal tetraaminophthalocyanines. *Talanta*, **48** (1999) pp. 1005-1011.
- 199- W. Blumberg. The study of hemoglobin by electron paramagnetic resonance spectroscopy. *Methods Enzymol.* **76** (1981) pp. 312-329.
- 200- T. H. Han, D. R. Hyduke, M. W. Vaughn, J. M. Fukuto and J. C. Liao. Nitric oxide reaction with red blood cells and hemoglobin under heterogeneous conditions. *PNAS*, **99** (2002) pp. 7763-7768.
- 201- J. C. Maxwell and W. S. Caughey. An infrared study of NO bonding to Heme B and Hemoglobin A. Evidence for inositol hexaphosphate induced cleavage of proximal histidine to iron bonds. *Biochemistry*, **15** (1976) pp. 388-396.
- 202- M. R. Thomas, D. Brown, S. Franzen and S. G. Boxer. FTIR and Resonance Raman studies of nitric oxide binding of H93G cavity mutants of myoglobin. *Biochemistry*, **40** (2001) pp. 15047-15056.

- 203- T. M. Dawson, M. Sasaki, M. Gonzalez-Zulueta and V. L. Dawson. Regulation of neuronal nitric oxide synthase and identification of novel nitric oxide signaling pathways. *Prog. Brain. Res.* **118** (1998) pp. 3-11.
- 204- H. M. Lander, D. P. Hajjar, B. L. Hempstead, U. A. Mirza, B. T. Chait, S. Campbell and L. A. Quilliam. A molecular redox switch on p21(ras). Structural basis for the nitric oxide-p21(ras) interaction. *J. Biol. Chem.* **272** (1997) pp. 4323-4326.
- 205- H. M. Lander, A. J. Milbank, J. M. Tauras, D. P. Hajjar, B. L. Hempstead, G. D. Schwartz, R. T. Kraemer, U. A. Mirza, B. T. Chait, S. C. Burk and L. A. Quilliam. Redox regulation of cell signalling. *Nature*, **381** (1996) pp. 380-381.
- 206- H. M. Lander, J. S. Ogiste, S. F. Pearce, R. Levi and A. Novogrodsky. Nitric oxide-stimulated guanine nucleotide exchange on p21ras. *J. Biol. Chem.* **270** (1995) pp. 7017-7020.
- 207- H. M. Lander, P. K. Sehajpal and A. Novogrodsky. Nitric oxide signaling: a possible role for G proteins. *J. Immunol.* **151** (1993) pp. 7182-7187.
- 208- H. Y. Yun, M. Gonzalez-Zulueta, V. L. Dawson and T. M. Dawson. Nitric oxide mediates N-methyl-D aspartate receptor-induced activation of p21ras. *PNAS*, **95** (1998) pp. 5773-5778.
- 209- K. W. Raines, G. L. Cao, E. K. Lee, G. M. Rosen and P. Shapiro. Neuronal nitric oxide synthase-induced S-nitrosylation of H-Ras inhibits calcium ionophore-mediated extracellular-signal-regulated kinase activity. *Biochem. J.* **397** (2006) pp. 329-336.
- 210- A. J. Gow, D. G. Buerk and H. Ischiropoulos. A novel reaction mechanism for the formation of S-nitrosothiol in vivo. *J Biol Chem.* **272** (1997) pp. 2841-5.
- 211- J. T. Groves and C. C. Wang. Nitric oxide synthase: models and mechanisms. *Curr. Opin. Chem. Biol.* **4** (2000) pp. 687-95.
- 212- H. H. Schmidt and U. Walter. NO at work. *Cell* **78** (1994) pp. 919-925.
- 213- L. Moldovan, K. Mythreye, P. J. Goldschmidt-Clermont and L. L. Satterwhite. Reactive oxygen species in vascular endothelial cell motility. Roles of NAD(P)H oxidase and Rac1. *Cardiovasc. Res.* **71** (2006) pp. 236-346.

- 214- M. Sundaresan, Z. X. Yu, V. J. Ferrans, D. J. Sulciner, J. S. Gutkind, K. Irani, P. J. Goldschmidt Clermont and T. Finkel. Regulation of reactive-oxygen-species generation in fibroblasts by Rac1. *Biochem. J.* **318** (1996) pp. 379-382.
- 215- K. K. Griendling, D. Sorescu, B. Lassegue and M. Ushio-Fukai. Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. *Arterioscler. Thromb. Vasc. Biol.* **20** (2000) pp. 2175-2183.
- 216- D. R. Pimentel, J. K. Amin, L. Xiao, T. Miller, J. Viereck, J. Oliver-Krasinski, R. Baliga, J. Wang, D. A. Siwik, K. Singh, P. Pagano, W. S. Colucci and D. B. Sawyer. Reactive oxygen species mediate amplitude-dependent hypertrophic and apoptotic responses to mechanical stretch in cardiac myocytes. *Circ. Res.* **89** (2001) pp. 453-60.
- 217- N. R. Madamanchi, A. Vendrov and M. S. Runge. Oxidative stress and vascular disease. *Arterioscler Thromb Vasc Biol* **25** (2005): 29-38.
- 218- S. W. Benson. Thermochemistry and kinetics of sulfur-containing molecules and radicals. *Chem. Rev.* **78** (1978) pp. 23-43.
- 219- D. F. McMillen and D. M. Golden. Hydrocarbon Bond Dissociation Energies. *Annu. Rev. Phys. Chem.* **33** (1982) pp. 493-532.
- 220- C. Schoneich, M. Bonifacic, U. Dillinger and K-D. Asmus. *Sulfur-centered Reactive Intermediates in Chemistry and Biology*. Plenum Press, NY, 1990.
- 221- O. Augusto, M. G. Bonini, A. M. Amanso, E. Linares, C. C. Santos and S. L. De Menezes. Nitrogen dioxide and carbonate radical anion: two emerging radicals in biology. *Free Radic. Biol. Med.* **32** (2002) pp. 841-59.
- 222- H. R. Mott, J. W. Carpenter and S. L. Campbell. Structural and functional analysis of a mutant Ras protein that is insensitive to nitric oxide activation. *Biochemistry*, **36** (1997) pp. 3640-3644.
- 223- W. K. Alderton, C. E. Cooper and R. G. Knowles. Nitric oxide synthases: structure, function and inhibition. *Biochem. J.* **357** (2001) pp. 593-615.

- 224- J. G. Williams, K. Pappu and S. L. Campbell. Structural and biochemical studies of p21Ras S nitrosylation and nitric oxide-mediated guanine nucleotide exchange. *PNAS*, **100** (2003) pp. 6376-81.
- 225- J. Heo and S. L. Campbell. Ras regulation by reactive oxygen and nitrogen species. *Biochemistry*, **45** (2006) pp. 2200-2210.
- 226- U. Forstermann and H. Kleinert. Nitric oxide synthase: expression and expressional control of the three isoforms. *Naunyn Schmiedebergs Arch. Pharmacol.* **352** (1995) pp. 351-364.
- 227- J. Reinstein, I. Schlichting, M. Frech, R. S. Goody and A. Wittinghofer. p21 with a phenylalanine 28----leucine mutation reacts normally with the GTPase activating protein GAP but nevertheless has transforming properties. *J. Biol. Chem.* **266** (1991) pp. 17700-17706.
- 228- J. Heo and S. L. Campbell. Mechanism of Redox-mediated Guanine Nucleotide Exchange on Redox-active Rho GTPases. *J. Biol. Chem.* **280** (2005) pp. 31003-31010.
- 229- J. Heo, K. C. Prutzman, V. Moocanu and S. L. Campbell. Mechanism of free radical nitric oxide-mediated Ras guanine nucleotide dissociation. *J. Mol. Biol.* **346** (2005) pp. 1423-40.
- 230- J. Zhang and C. R. Matthews. Ligand binding is the principal determinant of stability for the p21 (H-)ras protein. *Biochemistry*, **37** (1998) pp. 14881-14890.
- 231- C. Allin and K. Gerwert. Ras catalyses GTP hydrolysis by shifting negative charges from γ - to β -phosphate as revealed by time-resolved FTIR difference spectroscopy. *Biochemistry*, **40** (2001) pp. 3037-3046.
- 232- J. H. Wang, D. G. Xiao, H. Deng, R. Callender, R. and M. R. Webb. (1998) Vibrational study of phosphate modes in GDP and GTP and their interaction with magnesium in aqueous solution. *Biospectroscopy*, **4** (1998) pp. 219-227.
- 233- V. Cepus, C. Ulbrich, C. Allin, A. Troullier and K. Gerwert. Fourier transform infrared photolysis studies of caged compounds. *Methods Enzymol.* **291** (1998) pp. 223-245.

- 234- U. P. Fringeli. The structure of lipids and proteins studied by attenuated reflection (ATR) infrared spectroscopy. *Z. Naturforsch.* **32C** (1977) pp. 20-5.
- 235- I. Zawisza, A. Lachenwitzer, V. Zamlynny, S. L. Horswell, J. D. Goddard and J. Lipkowski. Electrochemical and photon polarization modulation infrared reflection absorption spectroscopy study of the electric field driven transformations of a phospholipid bilayer supported at a gold electrode surface. *Biophysical J.* **85** (2003) pp. 4055-4075.
- 236- R. Mendelsohn and C. R. Flach. Infrared Reflection-Absorption Spectroscopy of lipids, peptides, and proteins in aqueous monolayers. In *Peptide-Lipid Interactions*. S. A. Simon and T. J. McIntosh, eds. Academic Press, Elsevier Science, USA, pp. 74-75, 2002.
- 237- J. M. Chalmers, N. J. Everall, M. D. Schaeberle, I. W. Levin, E. N. Lewis, L. H. Kidder, J. Wilson and R. Crocombe. FT-IR imaging of polymers: an industrial appraisal. *Vibrational Spectroscopy*, **30** (2002) pp 43-52.
- 238- J. Guijarro, M. Engelhard and F. Siebert. Anion uptake in Halorhodopsin from *Natromonas pharaonis* studied by FTIR spectroscopy: Consequences for the anion transport mechanism. *Biochemistry*, **45** (2006) pp. 11578-11588.
- 239- A. A. Gorfe, A. Babakhani and J. A. McCammon. Free energy profile of H-ras membrane anchor upon membrane insertion. *Angew. Chem. Int. Ed.* **46** (2007) pp. 8234-8237.
- 240- Li, G. D'Souza-Schorey, C. Alehandro Barbieri, M. Cooper, J. A. and Stahl, P. D. Uncoupling of membrane ruffling and pinocytosis during Ras signal transduction. *JBC*, **272** (1997) pp. 10337-10340.
- 241- Zein, M. and Winter, R. Effect of Temperature, pressure and lipid acyl chain length on the structure and phase behaviour of phospholipids-gramicidin bilayers. *Phys. Chem. Chem. Phys.* **20** (2000) pp. 4545-4551.
- 242- D. Baurecht and U. P. Fringeli. Quantitative modulation excitation Fourier transform infrared spectroscopy. *Review of Scientific Instruments*, **72** (2001) pp. 3782-3792.

- 243- M. Schwarzott, P. Lasch, D. Bauecht, D. Naumann and U. P. Fringeli. Electric-field induced changes in lipids investigated by Modulated Excitation FTIR spectroscopy. *Biophysical J.* **86** (2004) pp. 285-295.
- 244- T. Tian, A. Harding, K. Inder, S. Plowman, R. G. Parton and J. F. Hancock. Plasma membrane nanoswitches generate high-fidelity Ras signal transduction. *Nature Cell Biology*, **9** (2007) pp. 905-914.
- 245- D. Bar-Sagi and J. R. Feramisco. Induction of membrane-ruffling and fluid phase pinocytosis in quiescent fibroblasts by ras protein. *Science*, **233** (1986) issue 4768, pp. 1061-1068.
- 246- T. Joneson, M. A. White, M. H. Wigler and D. Bar-Sagi. Stimulation of membrane ruffling and MAP kinase activation by distinct effectors of Ras. *Science*, **271** (1996) pp. 810-812.
- 247- G. Pearson, F. Roberson, T. B. Gibson, B.-E. Xu, M. Karandikar, K. Berman and M. H. Cobb. Mitogen activated protein (MAP) kinase pathways: Regulation and physiological functions. *Endocrine Reviews*, **22** (2001) pp. 153-183.
- 248- D. Bar-Sagi, F. McCormick, R. J. Milley and J. R. Feramisco. Inhibition of cell surface ruffling and fluid-phase pinocytosis by microinjection of anti-*ras* antibodies into living cells. *Journal of Cellular Physiology*, **133** (2005) pp. 69-73.
- 249- P. Rodriguez-Viciana, P. H. Warne, A. Khwaja, B. M. Marte, D. Pappin, P. Das, M. D. Waterfield, A. Ridley and J. Downward. Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. *Cell*, **89** (1997) pp. 457-467.
- 250- C. A. Heckmann, J. M. Urban, M. Cayer, Y. Li, N. Boudreau, J. Barnes, H. K. Plummer III, C. Hall, R. Kozma and L. Lim. Novel p21-activated kinase-dependent protrusions characteristically formed at the edge of transformed cells. *Exp. Cell Res.* **295** (2004) pp. 432-447.

- 251- C. A. Heckmann, K. I. Oravec, D. Schwab and J. Pontén. Ruffling and locomotion: Role in cell resistance to growth factor-induced locomotion. *Journal of Cellular Physiology*, **154** (1992) pp. 554-565.
- 252- C. A. Heckmann, H. K. Plummer III and C. S. Runyeon. Persistent effects of phorbol 12-myristate 13-acetate: Possible implications of vesicle traffic. *Journal of Cellular Physiology*, **166** (1995) pp. 217-230.
- 253- B. Borm, R. A. Requardt, V. Herzog and G. Kirfel. Membrane ruffles in cell migration: indicators of inefficient lamellipodia adhesion and compartments of actin filament reorganization. *Exp. Cell Res.* **302** (2005) pp. 83-95.
- 254- L. Cai, N. Holweck, M. D. Schaller and J. E. Bear. Phosphorylation of coronin 1 B by protein kinase C regulates interaction with Arp 2/3 and cell motility. *J Biol Chem.* **280** (2005) pp. 31913-31923.
- 255- C. Ballestrem, B. Wehrle-Haller, B. Hinz and B. A. Imhof. Actin- dependent lamellipodia formation and microtubule dependent tail retraction control-directed cell migration. *Molec. Biol. Cell*, **11** (2000) pp. 2999-3012.
- 256- C. A. Heckmann. *Method of assaying shape and structural features in cells*. No. 10109394 filed on 2002-03-28, US Patent Issued on May 23, 2006.
- 257- H. Nochi. Multi-output DC-DC converter using a resonance circuit. United States Patents – USP 5386359
- 258- J. A. Frangos and S. R. P. Gudi. Shear stress activates reconstituted G proteins in the absence of protein receptors by changes in membrane fluidity. *FASEB J.* **11** (1997) Abstract no 521.
- 259- D. Abankwa, A. A. Gorfe and J. F. Hancock. Ras nanoclusters: Molecular structure and assembly. *Semin. Cell Dev. Biol.* **18** (2007) pp. 599-607.
- 260- K. Lawler, E. Foran, G. O’Sullivan, A. Long and D. Kenny. The mobility and invasiveness of metastatic esophageal cancer is potentiated by shear stress in a ROCK and Ras dependent manner. *Am. J. Phys. Cell Phys.* **291**, (2006) C667-C668.

- 261- J. S. Goodwin, K. R. Drake, C. L. Remmert and A. K. Kenworthy. Ras diffusion is sensitive to plasma membrane viscosity *Biophys. J.* **89**, (2005) pp. 1398-1410
- 262- O. Traub and B. C. Berk. Laminar shear stress: Mechanisms by which endothelial cells transduce an atheroprotective force. *Atheroscler. Thromb. Vasc. Biol.* **18** (1998) pp. 677-685.
- 263- A. Koller and A. Huang. Shear stress-induced dilation is attenuated in skeletal muscle arterioles of hypertensive rats. *Hypertension*, **25** (1995) pp. 758-763.
- 264- M. A. Corson and S. L. MacKellar. Fibronectin advanced glycation end products (FN-AGE) impair flow-dependent endothelial cell (EC) mechanotransduction. *Circulation*, **94** (1996) Abstract of suppl I, I-703.
- 265- E. J. Smart, Y. S. Ying, P. A. Conrad and R. G. Anderson. Caveolin moves from caveolae to the Golgi apparatus in response to cholesterol oxidation. *J. Cell Biol.* **127** (1994) pp. 1185–1197.
- 266- B. C. Berk, M. A. Corson, T. E. Peterson and H. Tseng. Protein kinases as mediators of fluid shear stress stimulated signal transduction in endothelial cells: a hypothesis for calcium-dependent and calcium-independent events activated by flow. *J. Biomech.* **28** (1995) pp. 1439-1450.
- 267- N. Wang, J. P. Butler and D. E. Ingber. Mechanotransduction across the cell surface and through the cytoskeleton. *Science*, **260** (1993) pp. 1124-1127.
- 268- I. Kosztin, R. Bruinsma, P. O’Lague and K. Schulten. Mechanical force generation by G proteins. *PNAS*, **99** (2002) pp. 3575-3580.
- 269- H. Yin, M. D. Wang, K. Svoboda, R. Landick, S. M. Block and J. Gelles. Transcription against an applied force. *Science*, **270** (1995) pp. 1653-1657.
- 270- J. Howard, in *Mechanics of Motor Proteins and the Cytoskeleton*, Sinauer, Sunderland, MA. pp. 195–212, 2001.
- 271- M. J. Schnitzer, K. Visscher and S. M. Block. Force production by single kinesin motors. *Nat. Cell Biol.* **2** (2000) pp. 718-723.

- 272- R. Shlomovitz and N. S. Gov. Membrane waves driven by actin and myosin. *Physical Review Letters*, **98** (2007) pp. 168103(1)-168103(4).
- 273- J. U. Bowie. Flip-flopping membrane proteins. *Nat.Str.Molec.Biol.* **13** (2006) pp 94-96
- 274- M. Rapp, E. Granseth, S. Seppälä and G. von Heijne. Identification and evolution of dual topology membrane proteins. *Nat.Str.Molec.Biol.* **13** (2006) pp. 112-116.
- 275- G. von Heijne. Membrane protein topology. *Nature Reviews - Molec. Cell Biol.* **7** (2006) pp. 909-918.
- 276- G. von Heijne. Transcending the impenetrable: How proteins come to terms with membranes. *Biochim. Biophys. Acta.* **947** (1988) pp. 307-333.
- 277- E. London and C. L. Luongo. *Biochem. Biophys. Res. Comm.* Domain-specific bias in arginine/lysine usage by protein toxins. **160** (1989) pp. 333-339.
- 278- A. J. Laude and I. A. Prior. Palmitoylation and localization of RAS isoforms are modulated by the hypervariable linker domain. *J. Cell Sci.* **121** (2008) pp. 421-427.
- 279- E. Strandberg and J. A. Killian. Snorkeling of lysine side chains in transmembrane helices: how easy can it get? *FEBS Letters*, **544** (2003) pp. 69-73.
- 280- J. Raedler, H. Strey and E. Sackmann. Phenomenology and kinetics of lipid bilayer spreading on Hydrophilic surfaces. *Langmuir*, **11** (1995) pp. 4539-4548.
- 281- J. Tucker, G. Sczakiel, J. Feuerstein, J. John, R. S. Goody and A. Wittinghofer. Expression of p21 proteins in *Escherichia coli* and stereochemistry of the nucleotide-binding site. *The EMBO Journal*, **5** (1986) no. 6, pp. 1351-1358.

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