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In this thesis, new model systems were established to investigate the influence of the cellular environment on biomolecule folding and behaviour. Therefore, a crowding sensitive polymer was designed and tested in vitro and directly inside the cell [Gnutt et al. 2015]. To understand how other classes of biomolecules behave inside the cell, a RNA hairpin, as the easiest nucleic acid based folding motif, was used to compare folding in buffer, in crowded environments and in living cells [Gao et al. 2016]. Last, a protein folding reporter was constructed based on the monomeric SOD1 barrel variant. Introducing site-specific mutations allowed to investigate the influence of the cellular environment at different temperatures and in different in vitro and in-cell conditions. Targeting the protein to subcellular structures further allowed to probe folding with high spatial resolution. The main results of this thesis are:

1. Inside cells, excluded volume effects are overshadowed by contributions from nonspecific interactions with the environment leading to the expansion of a PEG polymer and destabilisation of SOD1.

2. RNA folding is only marginally affected by the cell but in vitro crowding agents lead to a RNA hairpin destabilisation due to changes of water activity and direct nucleobase interactions. However, RNA folding is subject to a high subcellular and as well as cell-to-cell variability.

3. Osmotic perturbations of mammalian cells lead to an imperfect adaptation response that can be fine-tuned by osmolytes.

4. Changes of intracellular physicochemical properties, due to physiological responses to osmotic stress, proteasome inhibition, or neuronal differentiation, can affect the folding properties of SOD1.
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5. Single amino acid substitutions in SOD1 can cause a significant change in thermodynamic and kinetic folding modulation by the cell and by BSA.

6. Localised folding reporters can be used to study folding with increased subcellular resolution due to restriction of diffusion or report on folding in the vicinity of complex structures, e.g. within chromosomes.

In the following, the main results are highlighted in the context of recent advances and future challenges.

5.1. The cellular environment: more than excluded volume

For many years, crowding was regarded as a phenomenon based solely on steric effects (Minton, 1981), the so called excluded volume effect. Although this could explain some of the effects observed in artificial polymeric crowding agents (Zhou et al., 2008), it was neither considering the physicochemical properties of the environment nor the properties of the protein. Using the PEG based crowding sensor (see section 4.1) it was shown that polymeric crowding agents are not able to reproduce the conformations observed in protein crowding or directly inside the cell (Gnutt et al., 2015). In addition, the folding properties of lm-4U RNA (section 4.3) were mostly dominated by effects of water activity or nucleobase interactions (Pincus et al., 2008; Knowles et al., 2011) and only a weak modulation by the cell was observed. Similarly, most disease related mutants of SOD1 are not stabilised inside the cell as expected from pure volume exclusion (Minton, 1981, 2001), but are destabilised (see section 2.1.4) in agreement with in-cell NMR data (Danielsson et al., 2015).

Thus, artificial crowding agents such as PEG or Ficoll, that were often employed as potential mimics of the crowded cell (Zhou et al., 2008) are insufficient in explaining the effects of the cell. This notion is even further supported by the fact that a pure volume exclusion mechanism, i.e. an entropic stabilisation of the folded state, is insufficient to explain recent thermodynamic observations. Senske et al. (2014) showed that typical crowding agents can lead to an enthalpic stabilisation. This is in good agreement with new models that describe crowders not only by a hard-sphere repulsive potential but add "soft" chemical and attractive interactions (Sapir and Harries, 2015a, 2014). More than that, not only attractive interactions between cosolute and protein matter but the contributions of the solvent are important (Sapir and Harries, 2016, 2015b). For example, solvent mediated mechanisms were suggested to increase or decrease the stability of proteins (Senske et al., 2014, 2016a; Knowles et al., 2015).
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Using the cell as the test tube, it became possible to directly observe the influence of such interactions and effects (Gnutt and Ebbinghaus, 2016; Wirth and Gruebele, 2013; Kuznetsova et al., 2015): PEG was shown to be elongated in low concentrations of BSA, cell lysate or the cellular environment (section 4.1). Such an elongation is rationalised by chemically attraction of the PEG to its environment (Gnutt et al., 2015). These chemical interactions are stronger for PEG than the volume exclusion effects that would favour a more compact conformation (Minton, 1981). The expanded conformations of PEG present more surface accessible area to the surrounding allowing more interactions to occur. Similarly, Groen et al. (2015) observed the compression of a FRET probe in artificial crowding and an expansion, explained by attractive interactions, in protein crowding.

The destabilisation of SOD1 through the cell environment can be rationalised similarly (see section 2.1.4). The unfolded state is able to present more SASA to the surrounding. If chemical attraction outweigh the entropic stabilisation of the compact native state, the fold is destabilised. This was observed experimentally in this thesis as well as by the Oliveberg lab (Danielsson et al., 2015). Further, Danielsson et al. (2015) observed an increased surface area of the I35A variant in-cell hinting towards direct interactions with the environment.

In summary, growing evidence in recent years suggests that, although in-cell volume exclusion is omnipresent (Zhou et al., 2008), direct interactions of the protein with the cell environment dominate the observed in-cell stability (Gnutt and Ebbinghaus, 2016; Danielsson and Oliveberg, 2017; Rivas and Minton, 2016; Kuznetsova et al., 2015).

5.2. Attractive interactions destabilise SOD1 inside the cell

Interestingly, studying the effect of single point mutations in the SOD1bar on in-cell stability revealed diverging effects for different mutations (see section 2.1.4). The most pronounced influence was observed for the H46R mutation, leading to a similar stability in-cell and in vitro. This was confirmed by the double mutant A4V/H46R which was only destabilised mildly compared to A4V alone. Other chemical substitutions (A4V/H46S or A4V/H46F) revealed a similar effect, leading to the hypothesis that H46 is crucial for destabilising non-specific interactions to occur. BSA was used as protein crowder to mimic the destabilisation in vitro. While A4V or G41D are strongly destabilised, histidine mutants of A4V were not affected anymore. Looking into the folding kinetics further revealed a change of the entropic and enthalpic contributions for the histidine mutant A4V/H46S compared to A4V. Interestingly, MD simulations by Dr. Matthias Heyden (personal communication)
revealed a decrease in hydrophobic SASA and increase of hydrophilic SASA in the histidine variant. In wt full length protein, H46 is involved in the metal binding by coordination with two other His in the full length protein (Winkler et al., 2009). The simulation revealed that for the barrel variant, this interactions prevails even without metal binding. Replacement of the histidine breaks this interactions, leading to a structural change that leads to the described changes of SASA.

The results reveal that the chemical nature of tracer protein has to be considered when the influence of the environment on folding is discussed. This could explain why in-cell experiments yield diverging effects on protein stability inside the cell (Ignatova and Gierasch, 2004; Danielsson et al., 2015; Guzman et al., 2014; Smith et al., 2016; Monteith et al., 2015; Ghaemmaghami and Oas, 2001; Ebbinghaus et al., 2010). Intriguingly, changes to a single amino acid can affect the stability mutation as shown by the SOD1 variant. In agreement with such large mechanistic effects, the Pielak lab revealed that a single point mutant can destabilise a protein more strongly inside the cell than in vitro (Monteith et al., 2015).

The experimental results are now supported by simulations which can recapitulate the complexity of the cell (Yu et al., 2016; McGuffee and Elcock, 2010; Feig et al., 2015) and agree with weak attractive interactions leading to a destabilisation of proteins inside the cell. This interplay further affects diffusion (Wang et al., 2010, 2011; Mu et al., 2017; McGuffee and Elcock, 2010) and may lead to the cytoplasmic organisation of the cytosol based on physicochemical gradients and properties (Spitzer and Poolman, 2009, 2013; Yu et al., 2016). Using in-cell NMR in combination with a large set of mutations, Mu et al. (2017) recently showed that quinary modulation probed by in-cell mobility of proteins is affected by the net charge of the protein, its hydrophobic SASA and its electric dipole-moment.

In summary, the in-cell stability results within this thesis support the growing importance of weak attractive interactions in the cell environment revealed by simulation and in-cell folding studies (Rivas and Minton, 2016; Gnut and Ebbinghaus, 2016; Yu et al., 2016; Danielsson and Oliveberg, 2017). Even slight changes to the protein can lead to big influences on the protein stability. This finding might provide a useful starting point for development of new drugs. Protein stability was shown to correlate with disease progression for ALS (Lindberg et al., 2005) (see section 2.1.4), presumably by defining the amount of unfolded species as a key factor for its aggregation (Lang et al., 2015). Thus, affecting the destabilisation by the cell could deplete the pool of unfolded species helping to slow down disease progression.
5.3. Changes of the environment modulate protein stability

So far, most studies on in-cell protein stability were performed at basal cellular conditions. However, cells experience dynamic changes based on their environment. One of the prime examples is osmotic stress (Burg et al., 2007). Using the crowding sensor it was possible to map the adaptation response of the cell (see section 4.2). It could be shown that the cellular adaptation to osmotic stress is fast (20 min) but imperfect. Such an adaptation process is in line with a proposed crowding homoeostasis (van den Berg et al., 2017). The elevated crowding levels depend on the strength of the osmotic perturbations, and can be tuned by incubation with osmolytes. Investigating the effect of this elevated crowding on SOD1 stability revealed an increased stability with increased crowding. Although one might speculate that this could be related to changes in volume exclusion (Minton, 1981; Burg, 2001), changes in nonspecific interactions or in ionic strength could be other explanations. In presence of the osmolyte TMAO, the stabilisation was even enhanced which could be rationalised by the preferential hydration mechanism of TMAO (Timasheff, 1993; Yancey, 2005). Conversely, the Pielak lab observed a destabilisation of protein in osmotically perturbed E. coli. This diverging effect again illustrates that the specific compositions of the environment, and the physicochemical interplay between protein and environment plays a major role in determining the outcome on protein stability. Recently, Sukenik et al. (2017) used perturbations of the cell volume by osmotic stress as a way to perturb the cell. Thereby, it was possible to probe and quantify weak protein-protein interactions in the native cellular context.

Other physicochemical changes of cells may occur, for example, due to changes of proteostasis (see 4.5.5) or neuronal differentiation (see 4.5.6). Inhibition of the proteasome using MG132 (Han et al., 2009; Lee and Goldberg, 1998) lead to distinct cellular morphologies and a decrease of SOD1 stability. PC12 differentiation by NGF (Greene and Tischler, 1976) lead to a small destabilisation accompanied by a large change of the folding parameter $\delta g_1$ which could indicate changes to the mechanism of folding in differentiated PC12 cells. Such results reinforce that folding is strongly dependent on the environment. Besides the physicochemical properties, changes in chaperone content could play a major role in determining the intracellular folding stability (Mayer and Bukau, 2005; Buchberger et al., 2010).
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5.4. New localised probes to study folding with subcellular resolution

Until now, protein folding was studied inside cells using freely diffusing probes, both in FRel (Ebbinghaus et al., 2010) Dhar et al. (2010a) or in-cell NMR (Danielsson et al. 2015; Smith et al. 2016). As pointed out recently by Rivas and Minton (2016), such freely diffusing probes might not be sufficiently suited to report on the general behaviour of a protein in specific microdomains. Therefore, the SOD1 based folding reporters were coupled to specific targeting sequences to test folding modulation in structurally well defined spaces. First, cytoskeletal variants were designed binding towards the actin network or the vimentin intermediate filaments. Using FRAP as a tool to assess diffusion properties (Lippincott-Schwartz et al., 2001), it could be shown that vimentin species were strongly restricted in diffusion whereas the actin variant remained highly mobile. Interestingly, using subcellular folding analysis (Gao et al. 2016; Ebbinghaus and Gruebele 2011), it was shown that vimentin targeted species sample a much larger heterogeneity of melting temperatures. This could relate towards the decrease of spatial averaging that freely diffusing species might experience.

Another target was the histone H4-23 (see 4.6.3) which could experience different physicochemical properties during mitosis, where genomic DNA has to be heavily condensed into chromosomes (Khorasanizadeh 2004; Walczak et al., 2010). During mitosis, a significant stabilisation of the folding reporter was observed. Interestingly, such results might reflect on the different chromatin volume concentrations between interphase and mitotic cells (Ou et al. 2017). Thereby, SOD1 might be shielded against destabilising nonspecific interactions with the environment. A stabilisation during mitosis was further observed for freely diffusing PGK (Wirth et al., 2013). A direct comparison, however, is difficult as both proteins show a different behaviour when in vitro and in-cell folding are compared.

The results illustrate that targeting of folding reporters to subcellular structures is feasible and allows to probe folding (and crowding) in the specific vicinity of certain macromolecular structures.

5.5. Concluding remarks and future challenges

In-cell protein folding has developed rapidly in the recent years by the development of techniques such as in-cell NMR (Sakakibara et al., 2009; Pastore and Temussi 2017) or FRel (Ebbinghaus et al., 2010; Gruebele et al. 2016). Further, the development of crowding sensitive macromolecules (Gnutt et al. 2015; Boersma et al. 2015).
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Groen et al., 2015 now allows to investigate the physicochemical properties of the cell in more detail. Accompanied by whole-cell simulations (Yu et al., 2016; McGuffee and Elcock, 2010) the importance of nonspecific chemical interactions on protein stability was revealed (Danielsson and Oliveberg, 2017; Gnutt and Ebbinghaus, 2016; Yu et al., 2016; Kuznetsova et al., 2015; Rivas and Minton, 2016; Smith et al., 2016). The interactions of proteins with the cell environment are highly specific depending on the properties of the protein, e.g. its potential to interact via electrostatic or hydrophobic interactions (Mu et al., 2017), the physicochemical properties of the cell, which could change depending on different environmental influences, and, last, on the specific subcellular context in which the protein functions (Rivas and Minton, 2016). Further, inside cells, quality control machineries play a major role in targeting misfolded and unfolded species (Mayer and Bukau, 2005; Buchberger et al., 2010). Finally, all such factors acting together will determine the stability of the protein in a specific context.

The tools introduced in this thesis will help to disentangle some of those effects. Gradually varying the physicochemical properties of SOD1 by mutations (Mu et al., 2017), e.g. increasing the hydrophobic surface area or changing electrostatic surfaces, will help to understand nonspecific interactions in the cell. Such studies should be accompanied by studies in vitro, where the experimentalist has complete control over the composition of the environment. As shown by different groups, cosolute influences can be decomposed into thermodynamic parameters to explain the molecular origin of stability modulation (Sukenik et al., 2013b; Sapir and Harries, 2015a; Senske et al., 2014, 2016a). Further, in vitro reconstituted systems of different cellular features (Rivas et al., 2014; Schwille, 2011), minimal cells, i.e. cells with the minimum amount of different genes (Hutchison et al., 2016) or cells with a dramatically different proteome and therefore different physicochemical environment (Malinovska and Alberti, 2015) might be interesting targets for folding reporters and crowding sensors.

Using cellular perturbations as osmotic stress in combination with recent crowding sensors (Gnutt et al., 2015; Boersma et al., 2015) one can further tune the cell to a state with different physicochemical properties. Combination with gradually mutated proteins (Mu et al., 2017) could reveal the influence of specific contributions, e.g. reveal the balance of volume exclusion and nonspecific interactions based on properties like hydrophobicity. More than that, the crowding and folding reporters could be directly studied in more physiological settings, e.g. ageing cells. Such cells were proposed to dehydrate which could favour aggregation of disease related proteins (Minton, 2014). Further, ageing induced changes of proteostasis (Labbadia and Morimoto, 2015; Yerbury et al., 2016; Hartl et al., 2011) could influence the
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folding and aggregation behaviour of disease related proteins such as SOD1.

The SOD1 based folding reporters will further be useful to screen for stabilising
drugs or conditions. As demonstrated by the binding of CLR01 to SOD1 (see section
4.5.7), supramolecular ligands can affect its stability (Fokkens et al., 2005). Also the
osmolyte TMAO increased its stability (Yancey, 2005; Arakawa and Timasheff, 1985).
Designing drugs that could specifically affect the interplay of SOD1 with the cell
environment could overcome the destabilisation by the cell. Thereby, the generation
of toxic species could be delayed due to a decreased fraction of misfolded/unfolded
protein (Lang et al., 2015; Cohen et al., 2011).

Finally, targeting of the folding reporter to specific structures has been pre-
sented as a feasible method to probe folding with subcellular precision (see section
4.6). Use of cytoskeletal targeted proteins might be helpful to uncover the role
of intracellular gradients, e.g. during differentiation of PC12 cells as a model for
differentiation (Greene and Tischler, 1976) or in tumour cell migration (Stroka
et al., 2014). A lot of proteins by now have further been shown to undergo phase
separations forming non-membranous compartments (Hyman et al., 2014; Banani
et al., 2017), e.g. the ALS-associated protein FUS which forms liquid compartments
in vivo (Patel et al., 2015). As such compartments are sequestered from the rest of
the cytosol and present a diffusive barrier to other proteins, it will be intriguing
to test crowding and protein folding directly within such structures. Therefore,
the targeting approach of SOD1 or genetic crowding sensors could be extended
to various proteins undergoing liquid-liquid phase separations. Also, probing
crowding and folding at membrane/cytosol interfaces would become possible by
linking it to membrane anchors. This is intriguing as 2-D crowding at a membrane
has recently been shown to induce α-synuclein conformational changes (Banerjee
et al., 2016).

All in all, combining rational changes to folding and crowding reporters (e.g.
changing their hydrophobicity or electrostatic properties) with perturbations
of the cell and specific subcellular targeting will allow to disentangle the individual
contributions of folding and crowding in the native context of different proteins.