Thermodynamics of protein destabilization in live cells

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Although protein folding and stability have been well explored under simplified conditions in vitro, it is yet unclear how these basic self-organization events are modulated by the crowded interior of live cells. To find out, we use here in-cell NMR to follow at atomic resolution the thermal unfolding of a β-barrel protein inside mammalian and bacterial cells. Challenging the view from in vitro crowding effects, we find that the cells destabilize the protein at 37 °C but with a conspicuous twist: While the melting temperature goes down the cold unfolding moves into the physiological regime, coupled to an augmented heat-capacity change. The effect seems induced by transient, sequence-specific, interactions with the cellular components, acting preferentially on the unfolded ensemble. This points to a model where the in vivo influence on protein behavior is case specific, determined by the individual protein’s interplay with the functionally optimized “interaction landscape” of the cellular interior.

thermodynamics | protein stability | crowding | in vivo | NMR

Unlike their static impression in X-ray structures and text-book illustrations, some proteins are tuned to work at marginal structural stability. The advantage of such tuning is that it enables the protein to easily switch from one conformation to another, providing sensitive functional control. A well-known example is the tumor suppressor P53 whose function in gene regulation relies on a complex interplay of local folding-unfolding transitions (1). Likewise, the maturation pathway of the radical scavenger Cu/Zn superoxide dismutase (SOD1) involves a marginally stable apo species that seems required for interorganelle trafficking (2) and effective chaperone-assisted metal loading (3). As an inevitable consequence of such near-equilibrium action, however, the proteins become critically sensitive to perturbations (1): Mutation of SOD1 triggers with full penetrance late-onset neurodegenerative disease even though the causative mutations shift the structural equilibrium only by less than a factor of 3 (4). In the latter case, it is not the loss of native function that poses the acute problem, but rather the promotion of competing disordered SOD1 conformations that eventually exhaust the cellular proteostasis system and end up in pathologic deposits (5). Uncovering the rules, capacity and limitations of this delicate interplay between individual proteins and the cellular components (9, 10) requires not only information about the in vivo response to molecular perturbations, but also precise quantification of the structural equilibria at play. The question is then, to what extent are existing data obtained under simplified conditions in vitro transferable to the complex environment in live cells (11)? The answer is not clear cut. Defying predictions from steric crowding effects, experimental data have shown that cells in some cases stabilize and in other cases destabilize the native protein structures. In this study, we shed light on these seemingly conflicting results by mapping out the thermodynamic behavior of a marginally stable β-barrel protein (SOD1βbarrel), using in-cell NMR. Our results show that mammalian and bacterial cells not only destabilize SOD1βbarrel, but also render its structure essentially disordered at 37 °C. The effect is assigned to transient interactions with the cellular interior, which counterbalance the crowding pressure, narrow the width of the thermal unfolding transitions, and move both cold and heat unfolding into the physiological regime. Moreover, these transient interactions are seen to be sequence and context dependent, reconciling the previous observations that different proteins yield different results. The emerging picture is thus that proteins are optimized not only for structure and function but also for their interplay with the host-cell environment, raising interesting questions about the physiological manifestation of marginal stability, as well as the constraints on protein behavior across evolutionary diverse organisms.

Results

In-Cell Effects on the Folded State. Our model protein is the 110-residue β-barrel scaffold [Protein Data Bank (PDB) code 4B6U] of the ubiquitous radical scavenger Cu/Zn superoxide dismutase (PDB code 1HLS). This SOD1 variant (SOD1βbarrel) was constructed by truncating the metal-binding loop IV and the electrostatic loop VII of the mother protein (26), which obliterates the native dimerization and leaves a catalytically inactive, well-behaved monomer that presents several advantages for in-cell analysis (Fig. S1). The SOD1βbarrel displays a simplistic two-state folding transition (26); lacks complexity in form of native metal-binding ligands (27) and cysteine moieties (28); and is extensively characterized with respect to mutational response (27, 29, 30), structural dynamics (26, 31), and aggregation behavior (6). Also, SOD1βbarrel displays fully resolved NMR spectra in mammalian cells (32). For the mammalian-cell experiments, we used the

Significance

A key question in structural biology is how protein properties mapped out under simplified conditions in vitro transfer to the complex environment in live cells. The answer, it appears, varies. Defying predictions from steric crowding effects, experimental data have shown that cells in some cases stabilize and in other cases destabilize the native protein structures. In this study, we reconcile these seemingly conflicting results by showing that the in-cell effect on protein thermodynamics is sequence specific: The outcome depends both on the individual target protein and on its detailed host-cell environment.

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB code 4XCR).

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human ovary adenocarcinoma cell line A2780 (33), which was found to have good properties for protein delivery and sustainability in the NMR tubes. 15N-labeled protein was delivered into the cytosol of mammalian cells by electroporation (SI Materials and Methods) and after recovery and washing, the treated cells were gently packed in an NMR tube (SI Materials and Methods). Intracellular SOD1 barrel concentrations were 20–30 μM, matching those in transgenic ALS mice (34, 35), and substantially higher than the 1- 5-μM endogenous concentration of SOD1 in mammalian cells (36). Controls of efficiency and yield of internalization are described in SI Controls and Fig. S2A. The results show high-resolution in-cell heteronuclear multiple quantum coherence (HMQC) spectra of folded and freely tumbling SOD1 barrel molecules, matching closely those obtained in vitro (SI Controls and Fig. S2B). A notable effect of the internalization, however, is an increased degree of protonation of the protein’s histidine side chains. By using SOD1I35A itself as a pH probe, we determine the intracellular pH to 6.5 (SI Controls and Fig. S2C). This cytosolic acidification is expected and arises from the hypoxic conditions in tightly packed NMR tubes: The cultured cancer cells redirect their metabolism to glycolytic pathways with little effect on viability (32). The NMR cross peaks show that the acidification commences early in the experiment, is uniform across the protein population, and remains stable for more than 5 h.

**Thermodynamic Analysis.** Although the in-cell spectrum of folded SOD1 barrel can be used for establishing the cytosolic pH and basic molecular mobility, it cannot be used on its own for measurement of the folding equilibrium. Such an analysis requires simultaneous detection of both the folded (N) and denatured (D) states in free equilibrium, i.e., an unfolding titration curve (37), where the folding equilibrium (K\textsubscript{D-N}) and stability (ΔG\textsubscript{D-N}) are given by

\[
\Delta G\textsubscript{D-N} = -RT \ln K\textsubscript{D-N} = \frac{-RT \ln [N]}{[D]}. \tag{1}
\]

To establish such balanced equilibrium, we destabilized SOD1 barrel by the core mutation I35A (SOD1\textsuperscript{I35A}). The mutation leaves the structure and surface unchanged (SI Controls, Fig. S3 A-F, and Table S1) but renders the protein partly unfolded under physiological conditions. As proof of principle, the NMR spectrum of SOD1\textsuperscript{I35A} shows mixed populations of D and N in PBS buffer at pH 6.5 and 37 °C (Fig. 1). For quantification of K\textsubscript{D-N} = [N]/[D] we use the volumes of the C-terminal Q153 cross peaks, which are well separated and insensitive to temperature/viscosity-induced relaxation effects (SI Controls and Fig. S3 G-L). Upon lowering the temperature, the SOD1\textsuperscript{I35A} equilibrium shifts progressively toward N, displaying a thermal unfolding midpoint of T\textsubscript{m} = 35 °C in the in vitro control (Fig. 1). At 17 °C, N reaches a maximum occupancy of 85% to finally decrease again as the temperature becomes lower still. This curved temperature dependence of K\textsubscript{D-N} is a generic effect of the heat-capacity increase upon unfolding (ΔC\textsubscript{p}) according to refs. 37 and 38,

\[
\Delta G\textsubscript{D-N} (T) = \Delta H\textsubscript{D-N} (T_0) - T \Delta S\textsubscript{D-N} (T_0) + \Delta C\textsubscript{p} \left[ T - T_0 - T \ln \left( \frac{T}{T_0} \right) \right], \tag{2}
\]

where ΔH\textsubscript{D-N} and ΔS\textsubscript{D-N} are the enthalpy and entropy of unfolding, respectively. The ΔC\textsubscript{p} change is due to an increase in the hydrophobic hydration and provides a useful measure of the increase in solvent-accessible surface area of the N to D transition (39). Because the hydration grows “stronger” at lower temperatures, the larger surface area of D promotes cold unfolding and curved ΔG\textsubscript{D-N}(T) profiles (38). For SOD1\textsuperscript{I35A}, the cold-unfolding midpoint is determined to T\textsubscript{C} = −3 °C, in good agreement with independent controls based on CD data (SI Controls and Fig. S4 A and B).

**Cells Promote Global Unfolding of SOD1\textsuperscript{I35A}**. Upon transfer into the mammalian A2780 cells the protein SOD1\textsuperscript{I35A} is clearly destabilized: At 37 °C, the folding equilibrium shifts fourfold toward the denatured state (Fig. 2 and Table 1). Notably, this effect is opposite to that expected from steric crowding (11–13) and points to the presence of attractive interactions between SOD1\textsuperscript{I35A} and the intracellular medium. The nature of these interactions is indicated by the temperature dependence of the in-cell stability. Inside cells, the D ⇄ N transition shows a 37% increase of ΔC\textsubscript{p} (Table S2), resulting in a narrowing of the thermal unfolding transitions (Fig. 2). Also, the D and N species remain in dynamic equilibrium during the 4-h experiments, without significant drift of populations or loss of protein material (SI Controls and Fig. S4C). Because the NMR chemical shifts and line broadening suggest that the structure of internalized N remains unchanged and free of specific interactions (Fig. S3), it is reasonable to conclude that the ΔC\textsubscript{p} increase is mainly due to in-cell modulation of D. For controls of data skewing by temperature-induced pH shifts and ionic strength, see SI Controls and Fig. S4 D–H. As an additional test, we performed in-cell experiments on SOD1\textsuperscript{I35A} overexpressed in *Escherichia coli* (Fig. 2). The results show that *E. coli* decreases T\textsubscript{m} to a smaller extent than A2780 cells, but lifts the ΔG\textsubscript{D-N}(T) profile to overall lower stability (Fig. 2). Coupled to this lift is a substantial increase in the cold-unfolding midpoint, which moves into the physiological regime at T\textsubscript{C} = 8.4 ± 1.7 °C (Table 1), and the temperature for maximum SOD1\textsuperscript{I35A} stability shifts from 14 °C in mammalian cells to 20 °C in *E. coli* (Fig. 2, Table 1, SI Data, and Fig. S5 A, H, and I). Thus, judging by T\textsubscript{m}, the mammalian cells would deceptively appear to have a smaller destabilizing effect than *E. coli*, emphasizing the importance of characterizing the whole ΔG\textsubscript{D-N}(T) profile in this type of experiment. Because of higher line broadening in *E. coli* cells (Fig. S5 H and I), we are currently unable to accurately determine K\textsubscript{D-N} = [N]/[D] below 10 °C and, hence, the precise effect on ΔC\textsubscript{p}. From the ΔC\textsubscript{p} increase in mammalian cells, however, it is indicated that the SOD1\textsuperscript{I35A} destabilization is here accompanied by increased surface area of the
Table 1. Thermodynamic parameters of the in-cell data and in vitro controls

| Protein/conditions | $\Delta G_{\text{D-N}}$, kJ/mol | $T_{\text{ref}}$, °C | $T_C$, °C |
|---------------------|---------------------------------|--------------------|----------|
| SOD$^\text{I35A}/$PBS | $-18.6 \pm 0.3$ | $61.0 \pm 0.3$ | $-33.1 \pm 1.8$ |
| SOD$^\text{I35A}/$PBS$^\text{SI}$ | $0.64 \pm 0.12$ | $35.6 \pm 0.3$ | $-2.1 \pm 1.4$ |
| SOD$^\text{I35A}/$in A2780 cells | $4.49 \pm 0.50$ | $28.0 \pm 0.5$ | $1.1 \pm 0.6$ |
| SOD$^\text{I35A}/$in E. coli cells | $2.25 \pm 0.30$ | $31.0 \pm 0.7$ | $8.4 \pm 1.7$ |
| SOD$^\text{I35A}/$ficoll 70$^\text{b}$ | $-0.62 \pm 0.14$ | $38.5 \pm 0.4$ | $-7.8 \pm 1.7$ |
| SOD$^\text{I35A}/$PEG 40$^\text{d}$ | $-0.39 \pm 0.15$ | $37.6 \pm 0.5$ | $-8.3 \pm 7.2$ |
| SOD$^\text{I35A}/$holoSOD$^\text{I35A}$ (dimer$^\text{f}$) | $0.53 \pm 0.14$ | $35.6 \pm 0.4$ | $-4.0 \pm 1.8$ |
| SOD$^\text{I35A}$/BSA$^\text{g}$ | $0.94 \pm 0.14$ | $34.6 \pm 0.4$ | $-6.1 \pm 1.8$ |
| SOD$^\text{I35A}$/TTHA$^\text{h}$ | $1.02 \pm 0.13$ | $34.0 \pm 0.4$ | $-14.8 \pm 3.3$ |
| SOD$^\text{I35A}$/lysozyme$^\text{e}$ | $5.72 \pm 0.29$ | $21.2 \pm 1.0$ | $13.5 \pm 2.6$ |

For a complete set of thermodynamic parameters, see Table S2.

*At 37 °C (SI Materials and Methods).

$^\text{a}$Negative values extrapolated from thermodynamic parameters (SI Materials and Methods).

$^\text{b}$Derived from CD data (SI Controls).

$^\text{c}$Calculated at 100 mg/mL crowder concentration (SI Controls).

$^\text{d}$Parameters extrapolated to 100 mg/mL (SI Controls).

denatured state. The canonical structures of D, which are observed to be relatively collapsed in pure water (40), seem to expand upon interaction with the intracellular components. Such expansion is also consistent with previous observations of increased unfolding m values in E. coli (21) and increased temperature sensitivity of the protein refolding kinetics in mammalian cells (18).

### Formal Description of In-Cell Interactions

Provided that the interactions between SOD$^\text{I35A}$ and the intracellular environment are overall weak, as is suggested by the NMR data, it is possible to formally describe their effect on the D $\rightarrow$ N equilibrium as follows. Assume that one has a number of cellular components ($j$) of concentration $C_j$. For each component the interaction potential with SOD$^\text{I35A}$ is given by $U_j(r_j, \tau_j)$, where $i$ denotes either N or D, $r_j$ is the relative position of $i$ and $j$, and $\tau_j$ denotes all other coordinates needed to describe the potential. The effect on the D $\rightarrow$ N equilibrium of the unspecific interactions $U(r_j)$ can then be quantified using a virial expansion of the osmotic pressure and the second virial coefficient is

$$B_i = -\frac{1}{N(\tau)} \int \exp \left\{ -\frac{U_j(F, \tau)}{kT} - 1 \right\} d\tau_i,$$

where $N(\tau)$ is a normalization integral over the variables ($\tau_i$). The integral over the center of mass separation $d\tau_i$ implies that $B_i$ has the dimension of a volume. It follows from the Gibbs–Duhem relation that the chemical potential of SOD$^\text{I35A}$ in conformation $i$ is

$$\mu_i = \mu_i^{(0)} + kT \ln C_i + kT \sum_j B_{ij} C_j.$$

When we neglect higher-order terms in the virial expansion, it follows from Eq. 4 that the in-cell equilibrium constant is

$$K_{\text{D-N}}^\text{cell} = K_{\text{D-N}}^\text{ref} \exp \left\{ \sum_j (B_{Nj} - B_{Dj}) C_j \right\},$$

where $K_{\text{D-N}}^\text{ref}$ is the in vitro reference. This, depending on the difference between the virial coefficients in the cell environment, either N or D can be favored. It is furthermore likely that the sum over cell components $j$ contains both negative and positive terms, where the value of the virial coefficient $B_{ij}$ is determined by the intermolecular potential $U_{ij}$ (Eq. 3). The main repulsive contribution to the potential $U_{ij}$ is due to the excluded volume interaction. Excluded volume is always present and gives a positive contribution to the virial coefficient, which is larger for the expanded D than for the more compact N. If this was the dominant contribution to $B_{ij}$, $K_{\text{D-N}}^\text{ref} < K_{\text{D-N}}$ in Eq. 5 and the equilibrium would be shifted toward N: This stabilization of the species of smallest volume is often referred to as the crowding effect (11–13). In addition to the repulsive excluded-volume effect, there are also attractive terms in the intermolecular potentials, giving a negative contribution to the virial coefficient. The dominant, but not the only, attractive contributions stem from local interactions between ion groups of opposite charge and patchy hydrophobic contacts. For SOD$^\text{I35A}$ with a small net charge and closely spaced anionic and cationic groups, the compact N species is expected to show relatively weak local electrostatic interactions with the other cell components. In the more expanded D state, on the other hand, where the charges are spread out and spatially flexible, there are larger possibilities to find such attractive interactions, tending to make $[B_{Dj}] > [B_{Nj}]$ in Eq. 5. The analogous argument holds for weak hydrophobic interactions where, again, the denatured ensemble will be stabilized due to its higher exposure of spatially amenable hydrophobic patches to the intracellular environment. An illustration of how the expanded D conformation shifts $K_{\text{D-N}}^\text{cell}$ (Eq. 5) by providing more opportunities for interactions with cellular components is given by the coupled equilibrium (see Fig. 4).

### Clues from in Vitro Crowders

To experimentally delineate the contributions to the in-cell destabilization of SOD$^\text{I35A}$, we mapped out the impact of a series of chemically distinct cosolutes in vitro (SI Data and Fig. S5 B–G). Consistent with predictions from excluded-volume effects (12) (Eqs. 3–5), the “hard-sphere mimic” ficoll$^\text{I35A}$ yields a progressive increase of SOD$^\text{I35A}$ stability (Fig. 3).

![Fig. 2. In-cell quantification of protein stability.](https://example.com/f2.png)
The data show, however, that the origin of this stabilization is molecularly more complex than excluded volume alone, because it is predominantly enthalpic in nature and without notable impact on $\Delta C_p$ (Table S2). This is not surprising as osmolytes in general not only occupy volume but also alter the osmotic pressure, yielding multiple components to the effect on protein stability (compare Eq. 3). Because the ficoll$^{40}$ effect contrasts with the in-cell data, the stabilizing excluded-volume/osmotic pressure contributions seem outweighed by opposing attractive interactions in live cells (Eq. 5). Next, we benchmarked PEG$^{400}$ that is reported to be an intermediate between a stabilizing osmolyte and a chemical denaturant (41). Similar to ficoll$^{35}$, PEG$^{40}$ yields an overall stabilization of SOD1$^{I35A}$ (Fig. 3), but with an accompanying increase of $\Delta C_p$ (Table S2). The latter indicates expansion of the denatured state of SOD1$^{I35A}$ consistent with the previously observed PEG$^{400}$ binding (41) and the present in-cell data (Table 1 and Table S2). To better isolate the attractive solute contributions we finally crowded SOD1$^{I35A}$ with a series of different globular proteins. The assumption is that these structurally fixed proteins represent hard spheres with variable surface properties determined by their respective amino acid composition. As a putative “strong” interacting partner we used folded lysozyme with a net positive charge (+8.5 e), allowing multiple electrostatic coordination possibilities with the negatively charged SOD1$^{I35A}$ species (−5.0 e) (Table S3). To minimize any opposing effects of excluded volume, we ran the experiments in the low-concentration regime of [lysozyme] $= 0 \text{mg/mL}$, 30 mg/mL, and 50 mg/mL. In contrast to the inert osmolytes, lysozyme promotes a marked destabilization of SOD1$^{I35A}$ (Fig. 3, Table 1, and Table S2). The net negative bovine serum albumin (BSA) (−8.5 e) and the bacterial putative heavy metal binding protein TTHA$^{\text{prom}}$ (−1.5 e), on the other hand, show no or little effect on SOD1$^{I35A}$ stability, whereas the cysteine-depleted SOD1 dimer, holosOD1$^{\text{Dimer}}$ (−5 e), yields a slight stabilization (Fig. 3, Table 1, and Table S2). Taken together, these results show that the effect of surrounding proteins is variable and depends on their detailed surface features. The observation not only complies with the rule that protein–protein interactions are sequence specific, but also emphasizes that the in-cell effect depends on the sequence of the target protein itself: The attraction potential relies on all partners in play (Eq. 3).

**Discussion**

**In-Cell Modulation of Protein Stability and Conformational Equilibria.** The destabilization and unfolding of SOD1$^{I35A}$ in mammalian cells illustrate well how classical in vitro analysis can easily overlook key physiological details (Fig. 2). With the caveat that cultured A2780 cells are not neuronal tissue, the in-cell destabilization observed here would suggest that the aggregation precursor in ALS, i.e., the reduced apoSOD1 monomer with a stability similar to that of SOD1$^{I35A}$, is largely unfolded in the neurons and not partly structured as envisaged in vitro (29, 42) (SI Data and Fig. S5 J–L). Such in vivo induced unfolding also explains why soluble apoSOD1 material in spinal cord of ALS mice is fully recognized by antibodies targeting disordered peptide epitopes (43). So, what causes this stability loss? Generally, the steric crowding experienced in the cellular compartment is predicted to stabilize proteins (11, 13). However, proteins engage also in various attractive interactions as they constantly search their environment for functional partners (19, 44–46). If these interactions are on average stronger for the folded state (N), they act stabilizing, and if they are stronger for the unfolded state (D), they act destabilizing (Eqs. 3–5 and Fig. 4). A key distinction here is that, unlike steric crowding, the protein’s interactions with the cellular environment depend on sequence identity (45, 46), governed by the same rules as protein folding itself (47). For SOD1$^{I35A}$, our results suggest that this sequence-specific crosstalk dominates the in-cell experience.

**Protein Identity and Cell Environment: Case-Specific Effects.** From the perspective of sequence-specific crosstalk (attractive interactions) it is not surprising that experiments targeting different proteins in different cell types and cell lysates yield different results. For example, intracellular stabilization has been observed for the lambda repressor in E. coli, using MS hydrogen/deuterium (HD)-exchange analysis (14); for GBI in E. coli cytols (19) and quenched E. coli lysozyme, using NMR HD-exchange analysis (15); and for FRET-labeled Tau (16) and phosphoglycerate kinase (PGK) (17, 18) in mammalian cell lines. The effect on PGK was also seen to vary with cell type, stage of cell cycle, and intracellular localization, underlining the importance of the detailed chemical context surrounding the target protein (17, 25). At the other end of the spectrum, FLASH-labeled CRABP (20) was found to become markedly destabilized in E. coli (21), and ubiquitin shows increased HD exchange rates in mammalian cells, suggested to arise from transient interactions with endogenous proteins (22). Similar stability losses are revealed upon titrating of the chymotrypsin inhibitor 2 (CI2) with bacterial lysozyme (23), by intracellular expression of a mammalian surface antigen (24), and by the mammalian- and bacterial-cell data presented here (Fig. 3, Table 1, and Table S2). Taken together, these observations underline the universal principle of structure–function relationships: The in vivo modulation of protein stability and structural behavior is by no means uniform, but case specific, determined by the interplay between an individual protein and its cellular “encounter interactome.”

**Nature of the Protein–Cell Crosstalk.** A ubiquitous source of in-cell interactions is the innate proteostasis system, which “buffers” structural stability and viable protein levels by a complex network of chaperones, transporters, and degradation pathways (8, 48). Somewhat surprisingly, the homogenous and temporally stable two-state equilibrium of SOD1$^{I35A}$ (SI Controls) shows that this proteostasis interference is either small or short-lived on the NMR timescale or sequesters strongly a minor, constant, fraction of the protein molecules that blinds out in the analysis and does not take part in the folding equilibrium. There is also no skewing of the $\Delta G_{D,N}(T)$ profiles (Fig. 2), indicating that the reversible interference from the proteostasis/chaperone system changes with temperature; i.e., there is no apparent heat- or cold-shock response (48). In terms of thermodynamics, this simplistic behavior allows us to assign the SOD1$^{I35A}$ destabilization to transient interactions alone (23) (Eqs. 3–5 and Fig. 4). The interpretation is also in full agreement with the similar effect of homogenous protein solutions (Fig. 3). At present it is not possible to deduce whether our inability to distinguish specific in-cell interactions relates to the specific chaperone-binding affinities of SOD1 itself (49) or reflects a general feature of soluble two-state proteins. Nevertheless, it can be safely assumed that, at a molecular level, one of the primary modulators of weak protein interactions is the side-chain charges, which not only steer macromolecular association and encounter complexes (19, 45) but also maintain solubility by negative design (50–52). The effect...



cold-unfolding temperature \( (T_c) \), which increases to just above zero in mammalian cells and to \(+8.6^\circ\text{C}\) in \( E.\ coli \) (Table 1). This inherent, yet rarely considered, phenomenon stems from the parabolic temperature dependence of protein stability \( (56) \) and moves both the cold-unfolding and melting temperatures of \( \text{SOD1}^{135A} \) into the physiological regime (Fig. 3 and Table 1). As analogous behavior is expected for any conformational transition involving sufficient exchange of coordinated water, it is surprising that physiological links to cold unfolding seem missing in the literature, except for an example in Antarctic fish \( (57) \). After all, some of the many proteomes of organisms adapted to low temperatures should contain members with structural properties that resemble, or partly overlap with, those of \( \text{SOD1}^{135A} \). Because natural two-state proteins in general are most stable around room temperature \( (58) \), the thermal behavior of \( \text{SOD1}^{135A} \) is also expected to be representative of marginally stable proteins rather than an odd exception. Regardless of what the physiological occurrence of cold unfolding turns out to be, settling this issue will help delineate the yet poorly understood biological constraints on protein stability.

Concluding Remarks. The answer to how protein behavior in vitro translates to in vivo conditions \( (13, 44, 59) \) seems now to be gradually unfolding. Although general confinement and excluded-volume effects must contribute, the rule of the game is in the molecular details: In-cell stability depends not only on the protein sequence itself, but also on how it interacts with its specific intracellular environment. From a sequence perspective alone, different proteins are thus expected to show different in vivo behavior, as is indeed observed in a series of independent in-cell studies, using a broad range of experimental techniques \( (14–24) \). Although some of these differences likely stem from experimental and molecular variation other than protein sequence, e.g., intracellular composition, stress response, and physical-chemical variation, they bring attention to the role of organism divergence. Because protein surfaces undergo much more rapid evolution than 3D structures \( (60) \), the surfaces exposed by the proteome of bacteria and mammalian cells are not the same. This divergence is found not only in functional interfaces, but also in “background” surfaces outside specific binding epitopes \( (61) \), leading to a new balance with the molecules in the cellular medium. The question is then, How will a protein behave in a foreign cellular environment? Our observations show that indeed there is a difference: Above room temperature \( \text{SOD1}^{135A} \) is more stable in \( E.\ coli \), whereas at lower temperatures it is better off in the mammalian cells (Fig. 2, Table 1, and Table S2). Along the same line Gruebele and coworkers have found that, even within mammalian cell lines, protein stability depends both on the phase of the cell cycle and on organelle localization \( (16, 18) \). Even though the details of these protein–environment relationships are yet to be pinned down, it is clear that the field of physical chemistry has finally moved in vivo \( (14–24) \): Molecular phenomena that were previously limited to speculation and inference from in vitro data can now be addressed directly in the environment where proteins are evolved to function.

Materials and Methods

Protein Engineering. Mutagenesis, expression, and purification were as in refs. 26 and 28.

Protein Internalization and in-Cell Analysis. For each in-cell NMR sample, 75–100 \times 10^6\) cells containing 1 mM \(^{15}\text{N}\)-isotope-labeled \( \text{SOD1}^{135A} \) were electroporated by a Super Electroporator NEPA21 (NEPa Gene). Measurements were performed on a Bruker Avance 700 MHz spectrometer. \(^{1}H\)(\(^{15}\text{N}\))-sofast-heteronuclear multiple quantum coherence \((62)\) spectra were used for all in vivo and in vitro experiments. The folded and unfolded populations were determined from the volumes of the C-terminal Q153 cross peaks and \( \Delta G_{D,N} \) was calculated from Eq. 1.

Structure Determination. Crystals of \( \text{SOD1}^{135A} \) were grown at 293 K by the sitting-drop vapor-diffusion method. Data were collected at 100 K on station I911-3 of the MAX IV Laboratory synchrotron, Lund, Sweden. Results have been deposited in the PDB, ID code 4XCR.

Physiological Occurrence of Cold Unfolding. Intriguingly, the biologically most striking effect of cell internalization is on the interaction sites \( (13, 54, 55) \) (Fig. 4). A similar picture is captured by Elcock’s full-scale simulation of the bacterial cytoplasm \( (44) \) where the stability of individual proteins either decreases or increases, depending on how the unfolded and folded material preferentially interacts with the surrounding. This intrinsic trade-off between steric crowding and weak encounter interactions explains why “passive” osmolytes like ficol\( l\) \( (2) \) and PEG \( (40) \) poorly mimic the physiological setting, why different proteins yield different results, and how crowding with chemically distinct proteins can induce the full spectrum of effects (Fig. 3, Table 1, and Table S2). From a theoretical perspective this is reassuring: Protein behavior in vivo seems after all defined by the mixing and environmental tweaking of individual folding funnels \( (47) \).
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