Mapping Temperature-induced Conformational Changes in the Escherichia coli Heat Shock Transcription Factor α32 by Amide Hydrogen Exchange*

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Stress conditions such as heat shock alter the transcriptional profile in all organisms. In Escherichia coli the heat shock transcription factor, α32, outcompetes upon temperature up-shift the housekeeping σ-factor, α70, for binding to core RNA polymerase and initiates heat shock gene transcription. To investigate possible heat-induced conformational changes in α32 we performed amide hydrogen (H/D) exchange experiments under optimal growth and heat shock conditions combined with mass spectrometry. We found a rapid exchange of around 220 of the 294 amide hydrogens at 37 °C, indicating that α32 adopts a highly flexible structure. At 42 °C we observed a slow correlated exchange of 30 additional amide hydrogens and localized it to a helix-loop-helix motif within domain α2 that is responsible for the recognition of the −10 region in heat shock promoters. The correlated exchange is shown to constitute a reversible unfolding with a half-life of about 30 min due to a temperature-dependent decrease in stabilization energy. We propose that this gradual decrease in stabilization energy of domain α2 with increasing temperatures facilitates the unfolding of α32 by the AAA+ protease FtsH thereby decreasing its half-life. Taken together our data show that the α2 domain of α32 can act as a thermosensor, which might be important for the heat shock regulation.

The heat shock response is a ubiquitously conserved protective mechanism of cells to cope with stress-induced damage in proteins. In Escherichia coli heat shock to 42 °C induces a 10–20-fold transient increase in the expression of about 20 heat shock genes through the stress-dependent increase in the level and activity of the heat shock transcription factor σ32 (1). In vivo α32 is very unstable with a half-life of approximately 1 min at 30 °C (2). Degradation of α32 in vivo requires the AAA+ protease FtsH and the DnaK chaperone system (3, 4). Immediately after temperature up-shift the synthesis as well as the half-life of α32 transiently increase by 10- and 8-fold, respectively. The transient stabilization of α32 is believed to be caused by the competition of unfolded proteins with α32 for binding to DnaK and FtsH (1, 5, 6). Under steady state conditions at 42 °C the half-life of α32 decreases to 10–15 s, probably due to an increase in the levels of available DnaK and FtsH.

However, it remains an important open question whether α32 itself can act as a thermosensor, similar to the heat shock transcription factor HSFI of Drosophila (7), by undergoing conformational alterations in response to heat shock. To investigate this question we determined the folding status of α32 under temperatures of optimal growth (37 °C) and under heat-stress conditions (42 °C).

In recent years amide hydrogen exchange combined with mass spectrometry has become an important method for studying the conformational properties of proteins (8–11). In combination with peptic digestion this method can resolve conformational changes down to the peptide level (8). We therefore built a high performance liquid chromatography mass spectrometry (HPLC-MS)1 setup that allowed us to monitor the incorporation of deuterons into full-length α32. Using an in-line column packed with immobilized pepticase for rapid reproducible digestion we were able to localize slow and fast exchanging regions within the entire sequence of α32. The results indicate that α32 has an unusually high degree of flexibility at 37 °C, and that heat treatment to 42 °C leads to unfolding of a stable subdomain. Our data support a role for α32 as a thermosensor.

EXPERIMENTAL PROCEDURES

Materials—α32 was prepared as described previously (12). D2O (99.9%) was from Cambridge Isotope Laboratories (Andover, MA), porcine immobilized pepticase and Poros R1 from Applied Biosystems (Darmstadt, Germany), and other chemicals from Sigma.

Amide Hydrogen Exchange Measurements—Amide hydrogen exchange was initiated by a 50-fold dilution of 100 pmol α32 into D2O containing 25 mM HEPES, pH 7.6, 50 mM KCl, 5 mM MgCl2, and 5% glycerol at 37 or 42 °C. After various times (0.2–90 min), the exchange reaction was quenched by decreasing the temperature to 0 °C and pH to 2.2 with 500 mM K2HPO4.

In-line Pepsin Digestion/Rapid Desalting HPLC Setup—The setup consisted of two HPLC pumps (Agilent 1100 series; Waldbronn, Germany), a Rhodyne injection valve (Model 7725i; Rhodyne, Rohnert Park, CA) with a 200-μl stainless steel sample loop, and a 2-position/10-port valve with microelectrode actuator (Valco C2–1000EP6; Schenkon, Switzerland). A schematic drawing of the setup is shown in Fig. 1. Pump A delivered the solvent for desalting (200 μl/min, 0.05% trifluoroacetic acid) and pump B for elution (20 μl/min, 70% acetonitrile, 30% water).

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Mass spectra were acquired on a quadrupole time-of-flight instrument to minimize back-exchange. For kinetic measurements of the full-length protein and peptide fragments, we needed to efficiently measure the masses of the full-length protein and of peptide fragments generated after digestion and desalting. Therefore, we needed to efficiently measure the masses of the full-length protein and of peptide fragments generated after the exchange reaction is quenched. To achieve this and to minimize back-exchange during digestion and desalting, we used an on-line “rapid desalting”-HPLC setup consisting of two HPLC pumps, a 2-position/10-port valve, and a 1 µl reversed-phase trap column. This system was coupled on-line to an electrospray ionization quadrupole time-of-flight mass spectrometer (Fig. 1). For the full-length protein, amide hydrogen exchange was initiated by diluting $\sigma^{32}$ into D$_2$O-buffer. The exchange reaction was stopped by pH shift to 2.2 and temperature shift to 0 °C. Under these conditions, amide hydrogens exchange on average with half-lives of 60–90 min (14), whereas deuterons incorporated into the side chains and amino/carboxyl termini rapidly exchange back to protium. Thus, the mass increase observed after deuteration and desalting reflects primarily deuterium incorporation at the backbone amide groups. For the localization of the fast and slow exchanging regions, we used a self-packed reversed-phase column (0.8 mm, Poros R1) for desalting. The resulting peptic fragments were immediately trapped on a trap column and analyzed by MS. The identity of the peptides was determined with the MagTran software (13). Protein mass spectra were deconvoluted with the MaxEnt software (Micromass). Peptic peptides of $\sigma^{32}$ were identified on the basis of their MS/MS spectra. The deuterium content of the peptides was calculated against the time; the curve is a fit of a first order rate equation to the data (see Table I for parameters).

RESULTS

Measurement of the Kinetics of Amide Hydrogen Exchange—To determine the solvent-accessible sites, temperature stability, and conformational changes of $\sigma^{32}$, we wanted to probe the overall kinetics of amide hydrogen exchange and then, under identical conditions, localize the fast and slow exchanging regions. Therefore, we needed to efficiently measure the masses of the full-length protein and of peptide fragments generated after the exchange reaction is quenched. To achieve this and to minimize back-exchange during digestion and desalting, we built up an on-line “rapid desalting”-HPLC setup consisting of two HPLC pumps, a 2-position/10-port valve, and a 1 µl reversed-phase trap column. This system was coupled on-line to an electrospray ionization quadrupole time-of-flight mass spectrometer (Fig. 1). For the full-length protein, amide hydrogen exchange was initiated by diluting $\sigma^{32}$ into D$_2$O-buffer. The exchange reaction was stopped by pH shift to 2.2 and temperature shift to 0 °C. Under these conditions, amide hydrogens exchange on average with half-lives of 60–90 min (14), whereas deuterons incorporated into the side chains and amino/carboxyl termini rapidly exchange back to protium. Thus, the mass increase observed after deuteration and desalting reflects primarily deuterium incorporation at the backbone amide groups. For the localization of the fast and slow exchanging regions, we used a self-packed reversed-phase column (0.8 mm, Poros R1) for desalting. The resulting peptic fragments were immediately trapped on a trap column and analyzed by MS. The identity of the peptides was determined with the MagTran software (13). Protein mass spectra were deconvoluted with the MaxEnt software (Micromass). Peptic peptides of $\sigma^{32}$ were identified on the basis of their MS/MS spectra. The deuterium content of the peptides was calculated against the time; the curve is a fit of a first order rate equation to the data (see Table I for parameters). The overall kinetics could be fitted to a biexponential equation or a charge state of $\sigma^{32}$ at 37 °C (open squares) and 42 °C (closed inverted triangle, lower mass peak; closed inverted triangle, higher mass peak). The curves are fits of a biexponential equation to the data (see Table I for parameters). c, correlated deuterium incorporation; the deconvoluted peaks at 42 °C are quantified using MagTran software, and percent of the higher mass peak is plotted against the time; the curve is a fit of a first order rate equation to the data. b and c, representative data of at least 4 independent experiments are shown.

Full-length $\sigma^{32}$ Protein Amide Hydrogen Exchange Kinetics—At 37 °C $\sigma^{32}$ exchanged about 190 of its 286 amide protons for deuterons within 1 min (Fig. 2a). The degree of deuterium incorporation increased over time leaving maximally 60–70 amide hydrogens (≤25% not counting back-exchange) that did not exchange under the experimental conditions within 60 min. The overall kinetics could be fitted to a biexponential equation (Fig. 2b and Table I), indicating that there are two kinetic categories of amide hydrogens in $\sigma^{32}$, 203 hydrogens exchanged with a time constant $\tau$ of ~8 s, whereas 30 exchanged with $\tau$ = 26 min. The remaining about 50 (17% of total) very slowly
exchanging hydrogens participate in a stable structure of specific hydrogen bonds, which is a surprisingly small number. For well folded globular proteins about 50% of the amide hydrogens are found to be protected against exchange after 60 min (examples in Ref. 15). These data can be interpreted in two ways. $\sigma^{32}$ either is a loosely folded protein with a low secondary structure content, or its structure has a high degree of flexibility.

At 42 °C the initial exchange kinetics was similar albeit slightly faster (205 hydrogens in the order of $\tau = 7 \pm 5$ min). However, after about 10 min the appearance of a second species was observed that had incorporated an additional 30 deuterons. The amount of this second species increased at the cost of the first species with a half-life of about 30 min (Fig. 2). The second species less than 30 hydrogens (10% not counting back-exchange) were protected against exchange.

This behavior could indicate a slow loss of secondary structure due to thermal denaturation. To test this possibility we pre-incubated $\sigma^{32}$ at 42 °C for one hour before diluting the protein into D$_2$O. The exchange kinetics under these conditions was, however, identical to the kinetics of the protein measured immediately after temperature shift (data not shown). These data indicate that the second species is not due to denaturation but to a reversible unfolding of a small structural motif. Because no intermediates between the first and the second species were observed, the exchange kinetics for the second species occurred in a correlated fashion, i.e. the refolding rate is much slower than the exchange rate (see “Discussion” for explanation).

**Localisation of the Slow and Fast Exchanging Regions by Peptit Digestion**—After deuteration, the $\sigma^{32}$ protein was digested under quenched conditions using the in-line pepsin column. Fig. 3 shows representative segments of the mass spectra of a fully protiated sample (bottom) and samples deuterated for various periods at 37 and 42 °C. In the fully protiated state each peptide is represented by several peaks on the mass-to-charge (m/z) scale due to the isotopic natural abundance. After incubation in D$_2$O the isotope envelope of most peptide peaks was shifted to higher m/z values due to incorporation of deuterons. For example, the peptides B, C, and D shown in Fig. 3 (m/z 867.69$^{+}$, a 121–155; m/z 877.46$^{+}$, a 257–294; m/z 880.45$^{+}$, a 2–18) exchanged all amide hydrogens within 12 s under the conditions used. In contrast, the peptides A and E (m/z 866.16$^{+}$, a 79–101; m/z 885.94$^{+}$, a 19–49) exchanged hydrogens much slower, leaving after 90 min 11 and 9 hydrogens unexchanged (Fig. 3 and data not shown). At 42 °C the exchange kinetics for peptide E was similar to that observed at 37 °C. In contrast, for peptide A the exchange kinetics is very different at 42 °C as compared with 37 °C. After 10 min deuteration at 42 °C a bimodal isotope distribution appears. As exchange proceeds, the abundance of the lower mass isotope distribution decreases while a corresponding increase is observed for the higher mass isotope distribution. Such a bimodal isotope distribution is the result of a local unfolding with a lifetime sufficiently long to allow complete exchange of several amide hydrogens within a single opening event. The lower mass isotope distribution represents a population of $\sigma^{32}$ molecules that has not yet unfolded whereas the higher mass population has unfolded and undergone the correlated exchange. Close inspection of the data revealed that such a correlated exchange in the part of the protein represented by peptide A also occurs at 37 °C albeit with much slower kinetics (see 60 min trace, Fig. 2, left panel: shoulder to the higher mass range; 45 and 60 min traces, Fig. 3: peaks between peptide A and B).

Using our HPLC-MS setup we were able to map the kinetic exchange profile for peptic peptides of $\sigma^{32}$ covering 99% of the sequence of the entire protein (Fig. 4a). Overlapping peptides that differed pairwise only by extensions at the C terminus were used to improve the resolution of the deuteron incorporation map (16, 17). For example, peptides 79–89 did not incorporate any deuterons at 37 °C within 10 s, whereas peptides 79–101 incorporated three deuterons under the same conditions. These deuterons can then be localized to the segment spanning residues 90–101. For all pairs of peptides the intrinsic chemical exchange rate for the C-terminal amide hydrogen in the short peptide at quenched conditions was found to be comparable with that of the corresponding residue in the extended peptide. In this way it was ensured that the deuterium occupancy for the non-overlapping C-terminal sequence extension was correctly assigned. Fig. 4b shows a plot of the relative deuteron incorporation per amide after 10 s, 2 min, and 90 min at 37 °C and after 90 min at 42 °C against the primary sequence of $\sigma^{32}$. The N terminus (residues 2–18) and the C-terminal half of the protein (residues 121–293) incorporate deuterons rapidly. The small decrease in relative exchange at the C terminus is due to the more rapid back-exchange of the amide hydrogens within the His$_6$-tag as calculated according to Englander’s algorithm (14). In contrast to the exchange behavior of the N terminus and the C-terminal half of the $\sigma^{32}$ protein, two regions, residues 79–89 and 104–115, were relatively well protected at 37 °C and showed correlated exchange at 42 °C (Fig. 4b, arrowheads).

**Homology Modeling of the $\sigma^{32}$ Structure**—To visualize fast and slow exchanging regions of $\sigma^{32}$ we wanted to obtain a suitable model of the $\sigma^{32}$ structure. For this purpose we extracted the coordinates of the atomic structure of *Thermus thermophilus* $\sigma^{70}$ out of the Protein Data Bank file of the RNA polymerase holoenzyme (Protein Data Bank code 1W7; see Ref. 18) and used it as a template for homology modeling using SWISS-MODEL (19–21). This approach resulted in a struc-

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**Table I**

| Overall amide hydrogen exchange kinetics |
|-----------------------------------------|
| $y = A_{n} - B_{n} \times e^{-k_{1}t} - C_{n} \times e^{-k_{2}t}$ |
| $A_{n}$ | $B_{n}$ | $k_{1} \times 10^{-3} s^{-1}$ | $C_{n}$ | $k_{2} \times 10^{-3} s^{-1}$ |
|---|---|---|---|---|
| 37 °C | 33777 ± 3 | 203.7 ± 3.1 | 118 ± 8 | 30.6 ± 2.9 | 0.63 ± 0.17 |
| 42 °C species 1 | 33774 ± 1 | 205.1 ± 2.0 | 147 ± 9 | 22.7 ± 1.3 | 1.75 ± 0.51 |
| 42 °C species 2 | 33806 ± 4 | 203.7 ± 2.0 | 146 ± 2.9 | 1.43 ± 0.31 |
| Conversion % | | | 0.36 ± 0.01 |

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* The centroids of the lower mass peak (see Fig. 2a) were fitted to the biexponential equation.

† The centroids of the higher mass peak were fitted to the biexponential equation. Because the higher mass peak was not visible before ten minutes no value for the fast rate is given.

The amounts of higher and lower mass peaks at 42 °C were determined by integration of the peak areas, and the change of the percentage of the higher mass peak over time was fitted to a first order rate equation.

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8 W. Rist and M. P. Mayer, unpublished data.
In recent years amide hydrogen exchange is being increasingly used for probing protein structure and stability (8–10) because it does not depend on the presence of proteolytic cleavage sites or fluorescent markers. Several methods are used to detect amide hydrogen exchange including NMR, matrix-assisted laser desorption/ionization time-of-flight-mass spectrometry, and electrospray ionization mass spectrometry. Our rapid desalting HPLC-MS setup has several advantages over other methods. First, low protein concentrations in the μL range can be used as compared with the mM range used in NMR. This was essential in the case of σ32 because the protein tended to aggregate at higher concentrations. Second, several coexisting conformations can be observed at the same time, which allowed the detection of the correlated exchange at 42 °C. In NMR an average exchange rate is determined. Third, sample handling

Fig. 3. Exchange kinetics of peptic fragments of σ32 at 37 and 42 °C. Representative region of the mass spectra showing five peptic peptides (A–E) of σ32 after exposure to D2O for different times. The isotopic natural abundance of the peptides is shown in the lowest trace (0 min, before amide hydrogen exchange). The traces above show the extent of deuterium incorporation of each peptide (0.2 to 60 min) at 37 °C (left panel) and 42 °C (right panel). For the triply charged peptide A the isotope envelopes are indicated as well as the bimodal distribution (open and closed triangles). A, m/z 866.16+; aa 79–101; B, m/z 887.89+; aa 121–155; C, m/z 877.45+; aa 257–294; D, m/z 880.45+; aa 5–18; E, m/z 885.94+; aa 19–49.

The precision with which this model fits our amide hydrogen exchange data is demonstrated by a helix-loop-helix element in domain σ2 where deuterium incorporation into the loop region (Fig. 4, b and c, open arrow head) was significantly faster than into the α-helices (Fig. 4, b and c, closed arrow heads). The domain σ2, which recognizes the heat shock promoter at the −35 region, forms a three helix bundle in our model but does not seem to be very tightly packed. Our exchange data show that the helix stabilizing H-bonds in this domain break and reform rapidly allowing deuterium incorporation. After 90 min at 42 °C even in domain σ2 significant deuteron incorporation is observed, demonstrating an increased flexibility and loosening-up of this domain.

**DISCUSSION**

**Mass Spectrometry and Amide Hydrogen Exchange**—In this study we developed an on-line rapid desalting-HPLC-electrospray ionization mass spectrometry setup that allows the rapid and efficient analysis of the kinetics of amide hydrogen exchange of full-length proteins and the on-line generation of peptic fragments for the localization of fast and slow exchanging regions.

Fig. 4c shows the structural model colored according to the degree of amide hydrogen exchange at different time points and temperatures with blue indicating 0–20%, green 20–45%, yellow 45–70%, and red 70–95% deuterium incorporation. 95% was the maximal amount of deuterium incorporation measured due to proton back-exchange during the desalting step. The N-terminal σ2 domain is well structured and shows little deuterium incorporation at 37 °C, whereas the C-terminal half of the model contains very little secondary structure and rapid deuterium incorporation is observed. Although this model of σ32 represents the RNA polymerase-bound form, our data are fully consistent with the model showing fast exchange in unstructured regions and slow exchange in the tightly folded α-helices of domain σ2 responsible for promoter recognition at the −10 region.
under quenched conditions was fully automated, which guaran-
teed an exact timing of the digestion and desalting and ther-
therefore easily reproducible results. In the case of matrix-
assisted laser desorption/ionization-MS pipetting must be very
accurate to obtain comparable results. Finally, the information
covering close to 100% of the entire protein sequence can be
acquired with just one spectrum. Our setup generated highly
reproducible peptic digests of the \( \text{H9268} \)
protein as clearly visible
in Fig. 3 and minimized back-exchange to generally less than
10%. Parallel to our work, Smith and co-workers (22) developed
a similar setup. In our study, no adjustment was made for deu-
terium loss during quenched conditions as described previously
(17), because we are mainly interested in the difference between
the exchange kinetics of \( \text{H9268} \) at 37 and 42
°
C and not an exact
determination of exchange rate constants of individual peptides.

Overall Deuteron Incorporation into \( \sigma^{32} \)—We analyzed the
exchange kinetics of full-length \( \sigma^{32} \) and the propensity for
deuterium incorporation within 99% of its primary sequence.
Our data indicate that \( \sigma^{32} \) is a relatively loosely folded or
highly flexible protein already at the regular growth tempera-
ture of \( E. \) coli (37 °C). The rapid incorporation of deuterons into
the full-length protein as well as the localization of slow and
fast exchanging regions showed that only domain \( \sigma_2 \) is pro-
tected against exchange indicating a stable domain. Except for
the part that exchanged in a correlated fashion, the stability of
\( \sigma^{32} \) at 42 °C was similar to its stability at 37 °C as indicated by
similar overall exchange kinetics. Heat shock conditions
(42 °C) caused a correlated exchange of several amide hydro-
gen in domain \( \sigma_2 \) with a mean half-life of about 30 min that is
indicative for a local, reversible unfolding event.

These data fit well to our model of the \( \sigma^{32} \) structure despite
the fact that this model was derived by homology modeling to
the RNA polymerase-bound form of \( \sigma^{70} \) (18) and not to the
uncomplexed structure of this protein. Due to the high se-
quence coverage and to the precision with which deuteron
incorporation could be determined (see helix-loop-helix ele-
ment), our setup can be used to verify structural models of
proteins where no crystal structure is available and to analyze
the dynamics of protein structures. A surprising result was
that domain \( \sigma_4 \), which is responsible for recognition of the −35
region of heat shock promoters, is not better protected against
deuteron incorporation and therefore not tightly folded. Maybe
the structure of this domain is stabilized upon binding to RNA
polymerase and/or DNA.

Using circular dichroism and fluorescence spectroscopy on
\( \sigma^{32} \), Chattopadhyay and Roy (23) recently observed that the
molar ellipticity between 200 and 230 nm decreases between 35
and 42 °C, whereas the intrinsic tryptophan fluorescence
stayed constant. They speculated that the region between res-
idges 181 and 208 unfolds upon temperature up-shift. In con-
trast, we could now map precisely the unfolding region at the
peptide level to residues 79–105 and demonstrate that the

**Fig. 4.** Time- and temperature-dependent exchange of \( \sigma^{32} \). a, amino acid sequence of \( \sigma^{32} \) and peptic peptides used for the analysis (underlined). Sequence coverage was close to 100%. b, the propensity for amide hydrogen exchange at different times and temperatures is plotted against the primary sequence of \( \sigma^{32} \). Overlapping peptides were used to increase the resolution of the deuteron incorporation map. Correlated exchange at 42 °C occurred mainly in two regions indicated with closed arrowheads. The intervening loop region is indicated with an open arrowhead. c, secondary structure representation of the model of the \( \sigma^{32} \) structure colored according to their exchange behavior at 37 °C after 10 s, 2 min, and 90 min as well as at 42 °C after 90 min (left to right). In the third structure from the left (90 min, 37 °C), the two helices with their connecting loop are marked by closed and open arrowheads, respectively, to demonstrate the precision with which the deuteron incorporation data fit this structural model.
unfolding event occurs in a cooperative and reversible manner. This clearly shows the power of amide hydrogen exchange as compared with global structural methods as circular dichroism spectroscopy.

In uncomplexed $\sigma^{232}$ the domains $\sigma_2$ and $\sigma_4$ may pack against each other as suggested by the recent structure of $\sigma^{232}$ in complex with a fragment of its anti-\(\sigma\)-factor RseA (24). However, such a packing does not stabilize the domains $\sigma_3$ and $\sigma_4$, because our data clearly demonstrate that $\sigma^{232}$ adopts loosely folded and highly flexible conformations allowing rapid exchange of the majority of its amide hydrogens. These data and our model show that only the N-terminal $\sigma_2$ domain is tightly folded and the C-terminal half resembles an unfolded protein. This is consistent with the short in vivo half-life of $\sigma^{232}$ at all temperatures and with the fact that $\sigma^{232}$ is prone to aggregation at higher concentration. Furthermore, our data explain why $\sigma^{232}$ constitutes a substrate for Hsp70 chaperones, which otherwise recognize unfolded proteins.

**Correlated Exchange (EX1)—**To understand the physiological implications of the observed correlated exchange in the helix-loop-helix element of domain $\sigma_2$, the exchange mechanisms must be considered. For amide hydrogen exchange to occur a structural-special H-bond between an amide hydrogen and an H-bond acceptor has to open, thereby allowing the catalyst (hydroxide ion) to attack. Such an opening can be a global or local unfolding of a secondary structural element within the native structure of a protein according to Equation 1,

$$k_{\text{F}}(\text{H}) \rightarrow k_{\text{F}}(\text{D}) \rightarrow k_{\text{D}}(\text{D}) \rightarrow k_{\text{U}}(\text{D}) \rightarrow k_{\text{U}}(\text{H}) \rightarrow k_{\text{U}}$$

(Eq. 1)

with F and U representing the folded and unfolded conformation of the structural element and $k_1$, $k_{-1}$, and $k_2$ being the unfolding, refolding, and intrinsic chemical exchange rate constants. There are two limiting cases for the observed exchange rates, called EX1 and EX2. In the EX1 case, the refolding is rate-limiting for the overall exchange kinetics ($k_{-1} \ll k_2$), and the amide hydrogens exchange in a correlated manner in each opening event. The observed exchange rate ($k_{\text{obs}}$) is then equal to the unfolding rate ($k_{\text{obs}} = k_2$). The signature for EX1 kinetics is a bimodal isotope distribution in peptide mass spectra and the occurrence of two separate, interconverting mass peaks in protein mass spectra. Under native state conditions, however, EX2 is most commonly observed because the rates for unfolding/refolding are generally much greater than the intrinsic chemical exchange rate ($k_{-1} \gg k_2, k_{\text{obs}} = k_2 \times k_{-1}$).

The occurrence of two distinct interconverting mass peaks observed in the $\sigma^{232}$ protein mass spectra (Fig. 2, 42 °C, 30 min) as well as the bimodal isotope distributions in the mass spectra of some of its peptic peptides (peptide A in Fig. 3 and data not shown) clearly indicate a correlated exchange according to the EX1 mechanism. This correlated exchange observed here in $\sigma^{232}$ is interesting from the following structural and physiological points of view.

Usually EX1 kinetics are observed in the presence of chemical denaturants because they decrease the refolding rate $k_{-1}$ without affecting the intrinsic chemical exchange rate $k_2$. Refolding of a protein implies the formation of H-bonds and a hydrophobic core, which usually occurs very fast in the absence of denaturants (ns to \(\mu\)s; see Ref. 26). Because the intrinsic chemical exchange rates are in the ms time scale, EX1 is rarely observed under native state conditions (27, 28). Therefore, some property of the domain $\sigma_2$ must impose a slow rate-limiting step on the refolding reaction. Despite extensive analysis of the existing structures of $\sigma$-factors and our structural model of $\sigma^{232}$, it remained unclear which property of domain $\sigma_2$ and in particular the highly conserved helix-loop-helix element is responsible for the correlated exchange behavior. The stability of this structural element, however, could be estimated. Because $k_{-1}$ is at least five-times smaller than the intrinsic chemical exchange rate $k_2$ (for 95% exchange to happen before refolding occurs), $k_2/(k_{-1} \leq k_2/5F)$. The resulting $\Delta\text{G} = |R \times T \times \ln(k_2/(k_{-1}F))|$ with R being the universal gas constant and T the absolute temperature. With the values for $k_2 (3.6 \times 10^{-4}$ s$^{-1}$), as determined from the kinetics of the correlated exchange, and $k_{-1}$ (50 s$^{-1}$), as calculated according to Englisher’s algorithm using the HXPep-program (Ref. 14, courtesy Z. Zhang), $\Delta\text{G}$ at 42 °C can be calculated to $|\Delta\text{G}| \leq 27 \text{ kJ/mol}|$. At 37 °C the unfolding rate $k_1$ was much smaller than at 42 °C, and the helix-loop-helix element was therefore more stable. At intermediate temperatures the correlated exchange occurred with intermediate rates, and the stability of the helix-loop-helix element was between the stability at 37 °C and 42 °C. This structural element seems to be able to “measure” temperature by a gradual increase in unfolding rate, which is equivalent to a gradual decrease in activation energy necessary for its unfolding. Because refolding is comparatively slow, the helix-loop-helix element could act like a metastable switch that becomes more labile with increasing temperatures.

What are the physiological implications of these observations? $\sigma^{232}$ is a very unstable protein with a half-life of 1 min at 30 °C and 10–15 s at 42 °C in vivo. Recently, it was shown that the membrane AAA+ protease FtsH, which is mainly responsible for the degradation of $\sigma^{232}$, does not have a robust unfolding activity and that the thermodynamic stability of the substrate protein is rate-limiting for degradation by FtsH (29). In vitro, FtsH degrades $\sigma^{232}$ with a half-life of $>$60 min at 35 °C and 40 min at 44 °C (30). These data are reflected very closely by our exchange kinetics, suggesting that in vitro the unfolding kinetics of the helix-loop-helix element may be rate-limiting for the degradation of $\sigma^{232}$ by FtsH. Consistent with this view is the fact that FtsH degrades proteins processively from one terminus, whereby it needs 5–20 aa for an initial interaction (31–33). For $\sigma^{232}$ it has been shown that the C terminus is not responsible for degradation by FtsH suggesting that degradation proceeds from the N terminus (12). According to our data and our model of $\sigma^{232}$, the N terminus contains a short unfolded stretch, which could be sufficient for recognition by FtsH, followed by the only stable domain $\sigma_2$.

In contrast to in vitro degradation data, in vivo the half-life of $\sigma^{232}$ is 10 to 15 s at 42 °C suggesting additional factors may aid degradation of $\sigma^{232}$ by promoting the unfolding of the N-terminal domain. In fact, the lack of significant deuteron incorporation at 37 °C within 60 min and the slow in vitro unfolding half-life of 30 min at 42 °C indicate a relatively high stability of this structural element. Furthermore, high stability is very often interrelated to a high refolding rate. The fact that the helix-loop-helix element combines a low unfolding rate with a low refolding rate may facilitate regulation of the heat shock response because once unfolded, degradation of $\sigma^{232}$ by FtsH is not hampered by rapid refolding.

It has been shown that $\sigma^{232}$ production after temperature up-shift is increased due to the melting of the secondary structure of its encoding mRNA and consequently increased translation rates (34, 36). The $\sigma^{232}$ encoding mRNA therefore has a built-in thermosensor. Our data now suggest that the $\sigma^{232}$ protein also has a built-in thermosensor that counteracts the $\sigma^{232}$ encoding mRNA. This mechanism of temperature-dependent synthesis and degradation rates may contribute to the stabilization of the $\sigma^{232}$ steady-state levels at all temperatures.

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