Amide proton exchange measurements as a probe of the stability and dynamics of the N-terminal domain of the ribosomal protein L9: Comparison with the intact protein

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Abstract

Amide H/D exchange rates have been measured for the N-terminal domain of the ribosomal protein L9, residues 1–56. The rates were measured at pD 3.91, 5.03, and 5.37. At pD 5.37, 18 amides exchange slowly enough to give reliable rate measurements. At pD 3.91, seven additional residues could be followed. The exchange is shown to occur by the EX2 mechanism for all conditions studied. The rates for the N-terminal domain are very similar to those previously measured for the corresponding region in the full-length protein (Lillemoen J et al., 1997, *J Mol Biol* 268:482–493). In particular, the rates for the residues that we have shown to exchange via global unfolding in the N-terminal domain agree within the experimental error with the rates measured by Hoffman and coworkers, suggesting that the structure of the domain is preserved in isolation and that the stability of the isolated domain is comparable to the stability of this domain in intact L9.

Keywords: H/D exchange; L9; protein folding; protein stability

The ribosomal protein L9 forms an unusual bilobal structure with a compact N-terminal domain connected by a long-solvent exposed α -helix to a compact C-terminal domain (Hoffman et al., 1994, 1996) (Fig. 1). The structure of the protein has been shown to be preserved in solution (Lillemoen et al., 1997). Studies of a peptide consisting of the long central helix show that it is monomeric and essentially fully structured in isolation, thus providing additional evidence that the unusual structure of L9 is preserved in solution (Kuhlman et al., 1997).

We have been using the N-terminal domain, residues 1–56, referred to as NTL9, as a model system to study protein folding (Kuhlman et al., 1998). The structure of the domain is one of the simplest examples of an important class of layered sheet-helix structures that are found in several other ribosomal proteins (Orengo & Thornton, 1993; Efimov, 1995). NMR experiments performed on the N-terminal domain show that its structure is preserved in isolation. The intact L9 protein also offers an interesting system to

study the assembly and folding of multidomain proteins. The folding of the two domains of L9 may not be independent because they are connected via a structured element of secondary structure, which in turn extensively interacts with each domain. In this communication we use H/D exchange measurements to probe the stability of NTL9 and compare it to the apparent stability of the domain in the intact protein.

Results

A general mechanism of the exchange process for the protonated form of a protein in D₂O has the following form (Linderstrøm-Lang, 1958):

where N(H) is the protonated form of the native protein, OP(H) is the exchange-competent form, OP(D) is the deuterated exchange competent form, N(D) is the deuterated native form, k_1 and k_{-1} are

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Fig. 1. Ribbon diagram of the ribosomal protein L9 from *Bacillus stearothermophilus* (PDB access code, 1div. Drawing made using the program MOLSCRIPT) (Kraulis, 1991). The N-terminal domain, residues 1–56, is shaded.

unfolding and refolding rate constants, respectively, and k_{ch} is the rate constant for amide proton exchange in the exchange-competent form (the dependence on the catalyst concentration is included in k_{ch}).

The kinetic expression for the rate of exchange is

$$k_{ex} = k_1 k_{ch} / (k_{-1} + k_{ch}). \quad (2)$$

Two limiting cases of the general kinetic expression are distinguished (Hvidt & Nielsen, 1966). The EX2 regime corresponds to $k_{-1} \gg k_{ch}$. In this case, the refolding rate, either global or local, is much faster than the intrinsic exchange rate and the overall rate of exchange is given by

$$k_{ex} = K_{op} k_{ch} \quad (3)$$

where K_{op} is the apparent equilibrium constant for the unfolding to the exchange competent form for the amide proton of interest, $K_{op} = k_1/k_{-1}$. The inverse of K_{op} , k_{ch}/k_{ex} is referred to as a protection factor (P) (Englander & Kallenbach, 1984). For the EX2 case, the standard apparent free energies for the opening reactions are defined by the equation:

$$\Delta G_{ex}^\circ = -RT \ln(k_{ex}/k_{ch}) = RT \ln P. \quad (4)$$

In the EX1 regime the intrinsic rate of exchange is much faster than the refolding step ($k_{-1} \gg k_{ch}$). Exchange via the EX1 limit is relatively rare (Roder et al., 1985; Englander & Mayne, 1992).

Demonstration that exchange occurs via the EX2 limit

To interpret exchange rates in terms of equilibrium constants for local or global unfolding, it is necessary to prove that the exchange is occurring in the EX2 limit. There are many lines of evidence that indicate exchange is occurring via the EX2 limit in NTL9 under the conditions studied:

1. The rate constant for the folding of NTL9 in D₂O at pD 5.4 and 303 K (k_{-1} in Equation 1) has been directly measured via stopped-flow fluorescence experiments and is 1,400 s⁻¹ (B. Kuhlman, P. Evans, & D.P. Raleigh, unpubl. obs.). The highest intrinsic exchange rate constant (k_{ch}) of 0.3 s⁻¹ at pH 5.37 and 303 K is considerably less than the refolding rate. Thus, even those amides that exchange via global unfolding satisfy the EX2 condition.
2. Residues with very different intrinsic rates give the same protection factors.
3. The measured protection factors are the same at pD 5.03 and 5.37, where the stability of the protein is the same (as estimated by urea titrations).
4. The protection factors for the most protected residues scale with the change in protein stability.

Measurement of exchange rates and determination of protection factors

Out of 54 residues that contain backbone amides, 18 exchanged slowly enough at pD 5.37 and 30°C to accurately measure rates. The rates were also measured at pD 5.03 and pD 3.91. At the lower pH several additional amides could be followed because of slower intrinsic exchange rates.

The protection factors and apparent free energies were calculated according to Equation 4. Figure 2A shows a histogram of the apparent free energies of the opening reactions for NTL9 versus residue number at pD 5.37. The apparent free energies of the opening reactions for the intact protein, calculated using the rates measured by Hoffman and coworkers (Lillemoen et al., 1997), are included for comparison. Urea denaturation experiments were performed in order to estimate the free energy of global unfolding. ΔG° of global unfolding is estimated to lie between 3.9 and 4.9 kcal/mol (95% confidence limit). The linear extrapolation method was used to analyze the urea denaturations. The excellent agreement between the free energy of folding calculated from the hydrogen deuterium exchange measurements and the value determined by the urea denaturation experiments, indicates that the linear extrapolation method is valid for this system. In Figure 2B we show the apparent free energies for the opening reactions at pD 3.9. The protein is less stable at this pH (ΔG° of global unfolding lies in the range of 2.1 to 3.3 kcal/mol, 95% confidence limit) and the protection factors are smaller. However, we were able to obtain protection factors for an additional seven residues because the intrinsic rates are lower at this pH. The additional residues include four amino acids in the C-terminal helix, 44–47, and residues 33, 34,

Fig. 2. Histogram of the apparent free energy change for the opening reaction, ΔG° , versus residue number for the N-terminal domain of L9 (slanted rule columns) measured at 303 K and (A) pD 5.37 and (B) pD 3.91. The apparent free energy change for the opening reaction for the corresponding region in the full-length L9 (open columns) is also shown in part (A). The apparent free energies for the full-length L9 were calculated from the exchange rates measured by Hoffman and coworkers at pD 5.37 and 303 K (Lillemoen et al., 1997). Error bars were calculated using the standard deviations for the exchange rates. Horizontal lines represent the upper and the lower limit of the free energy for the global unfolding of the isolated N-terminal domain as estimated by urea denaturation experiments conducted under the same conditions.

and 38. Amide resonances for seven additional residues were observed in the initial two-dimensional spectrum.

Discussion

The apparent free energies for the opening reactions for the residues that we observe in NTL9 agree very well with the free energies calculated using the rates measured by Hoffman and coworkers (Lillemoen et al., 1997). The comparison is represented in Figure 2A. There are only two apparent discrepancies. The rate for Leu30 is 2.5 times higher than the rate in L9, and the rate for Gly16 is 3.2 times lower than in L9.

In their studies of the intact protein, Hoffman and coworkers were able to measure rates for more residues than we could measure in the isolated domain. This does not necessarily mean that exchange from the isolated domain is more rapid. Our experiments involved homonuclear two-dimensional measurements that require a longer acquisition time than the ^{15}N -proton correlation experiments used by Hoffman and coworkers. Hence, it is possible that

we would not be able to detect certain residues in the N-terminal domain even if they exchange with the same rate as was measured for the intact L9. Out of 37 amides that we cannot measure, only four residues, D8, K15, N20, and E38, might have yielded reliable rates based on the reported rates for the intact protein.

The vast majority of amide resonances that yield reliable rate measurements at pD 5.37 appear to be exchanging via global unfolding. Hoffman and coworkers observed that these residues were the most protected in the N-terminal domain of the full-length protein. If these slow exchanging residues in the intact protein undergo the exchange process via the global unfolding pathway, then our results indicate that the stability of the isolated N-terminal domain is similar to the stability of the domain in the full-length protein.

The residues that exchange too rapidly to be measured presumably do so via local unfolding. As can be seen from Figure 2A, there are 3 residues out of the 18 observed that are likely to undergo exchange via local unfolding at pD 5.37: Val 9, which is in a turn-like structure at the end of the first β -strand in the crystal structure of L9 and is strongly hydrogen bonded; Gly13 is in the loop that connects the first and second strands and is hydrogen bonded; and, finally, Leu30, which is part of the first helix. At pD 3.91 residues 9, 13, and 30 undergo exchange via global unfolding instead of by local unfolding. It is reasonable to expect that more residues will exchange by global unfolding at pD 3.91 because NTL9 is less stable at pD 3.91 than at pD 5.37.

There is clear evidence that some of the residues are exchanging via local unfolding at pD 3.91. A set of residues corresponding to the N-terminus of the second helix (44 through 47) all have similar apparent free energies with values clearly less than for global unfolding, suggesting that the first turn of the helix unfolds as a cooperative unit. Residues 33 and 34 are on the inside of the first helix and may exchange via local unfolding at pD 3.91. Gln33 is strongly H-bonded in the crystal structure of L9 and Gly34 is weakly H-bonded. Glu38 is another residue for which the apparent free energy of opening is less than the free energy of global unfolding. Glu38 has also been shown to exchange at a rate significantly faster than its neighbors (Ile37 and Ala39) in the full-length protein.

In conclusion, the H/D exchange studies of the isolated NTL9 have demonstrated that the pattern of exchange in this domain is very similar to the pattern observed for the corresponding region in full-length L9, suggesting that the structural and dynamic features that contribute to the exchange process are similar. If the slow exchanging residues in the intact protein exchange via global unfolding, then our studies suggest that the stability of the isolated N-terminal domain is similar to the stability of the domain in the intact protein. This result implies that there is little cooperativity in the folding of the intact protein. At first glance, this seems surprising because we have previously demonstrated that a peptide corresponding to the central helix of L9 is folded but less stable in isolation. It is, however, important to realize that the N-terminal portion of this helix is part of the N-terminal domain and that the first 16 residues of the helix are included in the domain studied here.

Materials and methods

NTL9 was synthesized and purified as described in Kuhlman et al. (1998).

Sample preparation and data collection

The protonated form of about 3 mM NTL9 was adjusted to the required pH, frozen, and lyophilized, dissolved in a buffer (20 mM deuterated acetic acid in D_2O at the required pD), and immediately transferred to an NMR tube and inserted into the spectrometer.

NMR spectra for the H/D exchange studies were collected on a Varian Instrument Inova 600 MHz spectrometer. Experiments were conducted at 303 K. The pD of the sample was measured immediately before and immediately after the NMR spectra were taken, the values agreed within one-tenth of a pH unit. Reported pD values are corrected for glass-electrode pH artifacts. Total correlated spectroscopy (TOCSY) was used to monitor the rates of exchange. Eight scans per t_1 value were collected and each TOCSY spectrum took 52 min. Weak presaturation for 1.25 s was used to suppress the residual solvent signal.

Data processing

The data were processed on a Silicon Graphics workstation using Felix 95.0 BIOSYS/MSI software. A sinebell window function in both dimensions and zero filling to a final matrix of $512 \times 2,048$ were used. Peak volumes (vol) were calculated in an automated fashion and normalized to a nonexchangeable cross-peak using the equation:

$$\text{vol} = (v_i - v_o)/(v_{st} - v_o), \quad (5)$$

where v_i is the observed volume, v_{st} is the volume of the nonexchangeable reference cross-peak (the γ -methyl to γ -methyl cross-peak of Val 3 was used), and v_o is the average of 8 vol taken in the fingerprint region of the spectra where no peaks are present.

Amide proton exchange rate constants were calculated by fitting the normalized peak volumes to a first-order exponential decay using Kaleidagraph™ (Albeck Software, Reading, Pennsylvania). For peaks with visible systematic error in the baseline, the following equation was used:

$$I(t) = I_0 \exp(-k_{ex}t) + b, \quad (6)$$

where I is peak intensity, k_{ex} is the exchange rate constant, I_0 is the peak intensity at time zero, and b is the baseline correction term.

Random coil hydrogen exchange rate constants were obtained according to the method of Bai et al. (Molday et al., 1972; Bai et al., 1993; Connelly et al., 1993). For most residues this was accomplished using the SPHERE program provided by the Roder laboratory (internet address: <http://www.fccc.edu/research/labs/roder/>). For acidic residues (D8, E17, and E38) and residues adjacent to those residues the intrinsic rates were calculated taking into account the fraction ionized at each pH. This was determined using pK_a values explicitly measured for NTL9 (B. Kuhlman, D. Luisi, P. Young, & D.P. Raleigh, unpubl. obs.).

Estimates of the limits of error in the measured H/D exchange rates were obtained using the standard error values for the rates given by Kaleidagraph™ and appropriate Student t -values. The

limits of error in the free energy obtained from the exchange studies using Equation 5 were calculated by standard propagation of the errors in the rates.

Estimates of NTL9 stability

Urea titrations were performed as described in Kuhlman et al. (1998). The uncertainty in the free energy values was estimated by the statistical procedure described in Shoemaker et al. (1989).

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