Characterization of the free energy spectrum of peptostreptococcal protein L

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Background: Native state hydrogen/deuterium exchange studies on cytochrome *c* and RNase H revealed the presence of excited states with partially formed native structure. We set out to determine whether such excited states are populated for a very small and simple protein, the IgG-binding domain of peptostreptococcal protein L.

Results: Hydrogen/deuterium exchange data on protein L in 0–1.2 M guanidine fit well to a simple model in which the only contributions to exchange are denaturant-independent local fluctuations and global unfolding. A substantial discrepancy emerged between unfolding free energy estimates from hydrogen/deuterium exchange and linear extrapolation of earlier guanidine denaturation experiments. A better determined estimate of the free energy of unfolding obtained by global analysis of a series of thermal denaturation experiments in the presence of 0–3 M guanidine was in good agreement with the estimate from hydrogen/deuterium exchange.

Conclusions: For protein L under native conditions, there do not appear to be partially folded states with free energies intermediate between that of the folded and unfolded states. The linear extrapolation method significantly underestimates the free energy of folding of protein L due to deviations from linearity in the dependence of the free energy on the denaturant concentration.

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Introduction

Characterization of the denaturant dependence of hydrogen/deuterium (HD) exchange from the native states of cytochrome *c* [1] and RNase H [2] revealed the presence of excited states with partially formed native structure. Independent kinetic experiments have confirmed that conformations resembling these excited states are populated during refolding [3]. These elegant studies show that native state HD exchange can be a powerful method for obtaining information about partially unfolded states of a protein which may shed light on possible folding mechanisms.

A number of very small (50–80 residues) single-domain proteins, lacking cofactors and disulfide bonds, have been found to fold in a highly cooperative two-state reaction lacking any observable intermediates. These proteins are smaller than RNase H (155 residues) and cytochrome *c* (104 residues), and it is unclear whether for such simple proteins there are states analogous to the excited states observed for RNase H and cytochrome *c* that are intermediate in free energy between the unfolded and folded forms of the protein under native conditions.

One such protein is the IgG-binding domain of peptostreptococcal protein L, which apparently folds in a highly cooperative two-state reaction [4]. Using a combined mass spectrometry and NMR analysis of HD experiments carried out under EX1 conditions, we previously determined that all of the HD exchange in protein L could be accounted for by global unfolding events in combination with very rapid local fluctuations around the native state [5]. Partial opening reactions analogous to the excited states observed for cytochrome *c* and RNase H were conspicuously absent. However, to ensure that exchange was in the EX1 regime, the earlier protein L experiments were carried out at high temperature and pH, while the partially folded states observed for cytochrome *c* and RNase H were populated only under mild conditions.

Our initial goal was to determine whether partially folded states exist in equilibrium with the native state for very small (<80 amino acid) proteins under mild conditions. In the course of these experiments, a substantial discrepancy emerged between free energy estimates from the HD exchange experiments and earlier guanidine denaturation experiments. To resolve the discrepancy, we carried out a more thorough study of the denaturant and temperature dependence of global unfolding of protein L. The results suggest, firstly, that there are no states intermediate in free energy between the native and unfolded states of protein L under native conditions and, secondly, that the denaturant dependence of the free energy of folding of protein L deviates from linearity.

Results

The HD exchange rates of the amide protons in protein L were measured at pH 7.0 for a series of guanidine concentrations ranging from 0 to 1.2 M. Of the 63 backbone amides in protein L, 31 exchange slowly enough to be measured by two-dimensional N^{15}–H^1 HSQC NMR experiments. The exchange rate constants (k_{ex}) were obtained from simple exponential fits of the volumes of the N^{15}–H^1 crosspeaks as a function of the exchange time (Figure 1).

A simple model for HD exchange in proteins is that structural 'opening' reactions expose otherwise protected protons to exchange with solvent [6]. The rate of exchange in the open states is assumed to be similar to that of exchange in unstructured peptides (k_{rc}):

$$\text{closed} \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} \text{open} \xrightarrow{k_{rc}} \text{exchange} \qquad (1)$$

Under EX2 conditions, where $k_{cl} >> k_{rc}$:

$$k_{ex} \approx (k_{op} / k_{cl}) * k_{rc} = K_{op} * k_{rc} \qquad (2)$$

Since k_{rc} is known from model studies, the measurement of k_{ex} leads to the determination of K_{op} and hence the opening free energy ΔG_{HD}:

$$\Delta G_{HD} = -RT * \ln(K_{op}) = -RT * \ln(k_{ex} / k_{rc}) \qquad (3)$$

Extraction of opening free energies from observed exchange rates in this manner requires that exchange be in the EX2 regime. The folding rate decreases with increasing denaturant concentration, and thus HD exchange may leave the EX2 regime at higher denaturant concentrations. To investigate this, we examined the pH dependence of the exchange rates at the higher guanidine concentrations. As the random coil exchange rate is mainly base-catalyzed above pH 4 [7], k_{ex} under EX2 conditions should be proportional to $[OH^-]$:

$$\log k_{ex}^{pH1} = \log k_{ex}^{pH2} + pH_1 - pH_2 \qquad (4)$$

Thus, a plot of the logarithm of the rate constants for exchange at one pH against those for another pH should yield a line with a slope of 1.0. The pH dependence of the observed exchange rates for different guanidine concentrations is shown in Figure 2. At 0.7 M guanidine, pH 7.0 (Figure 2a), and, by inference, at lower concentrations of guanidine, exchange for all observed protons in protein L is in the EX2 regime. At 1.2 M guanidine and pH 7.0, exchange is no longer in the EX2 limit (Figure 2b). Thus, ΔG_{HD} cannot be obtained from exchange rate measurements at 1.2 M guanidine, pH 7.0. Similar results were obtained with barnase [8] and RNase A [9] in moderate concentrations of denaturant.

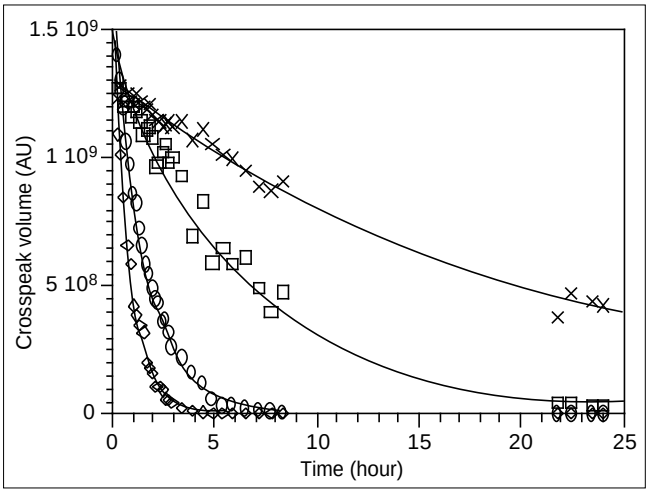
Comparison of the exchange rates at pH 4.3 and pH 5.0 (Figure 2c) indicates that exchange is under EX2 control at 1.2 M guanidine pH 5.0, and thus ΔG_{HD} can be computed for each proton under these conditions. However, these results can be combined with the results obtained at lower guanidine concentrations at pH 7.0 only if the opening and closing rates in equation 1 are independent of pH over this range. To investigate this, folding and unfolding rates were measured for different guanidine concentrations at pH 5.0 and compared to the data obtained previously at pH 7.0 [4]. As is clear from Figure 3, the rates of the global opening and closing reactions are virtually identical at pH 5.0 and pH 7.0, so for the remainder of the paper we treat the apparent free energy obtained from the 1.2 M guanidine pH 5.0 data together with the lower guanidine pH 7.0 data. Measurement of the exchange rates at lower denaturant concentrations at pH 7.0 rather than pH 5.0 has the advantage that the NMR data can be collected under identical instrument conditions within 24 hours (exchange at pH 7.0 is 100 times faster than exchange at pH 5.0).

The denaturant dependence of the apparent ΔG_{HD} for individual residues has been analyzed using a simple model [6,10] in which the only contributions to exchange are from local and global fluctuations:

$$\Delta G_{HD} = -RT * \ln(K_{op}^{local} + K_{op}^{global}) \qquad (5)$$

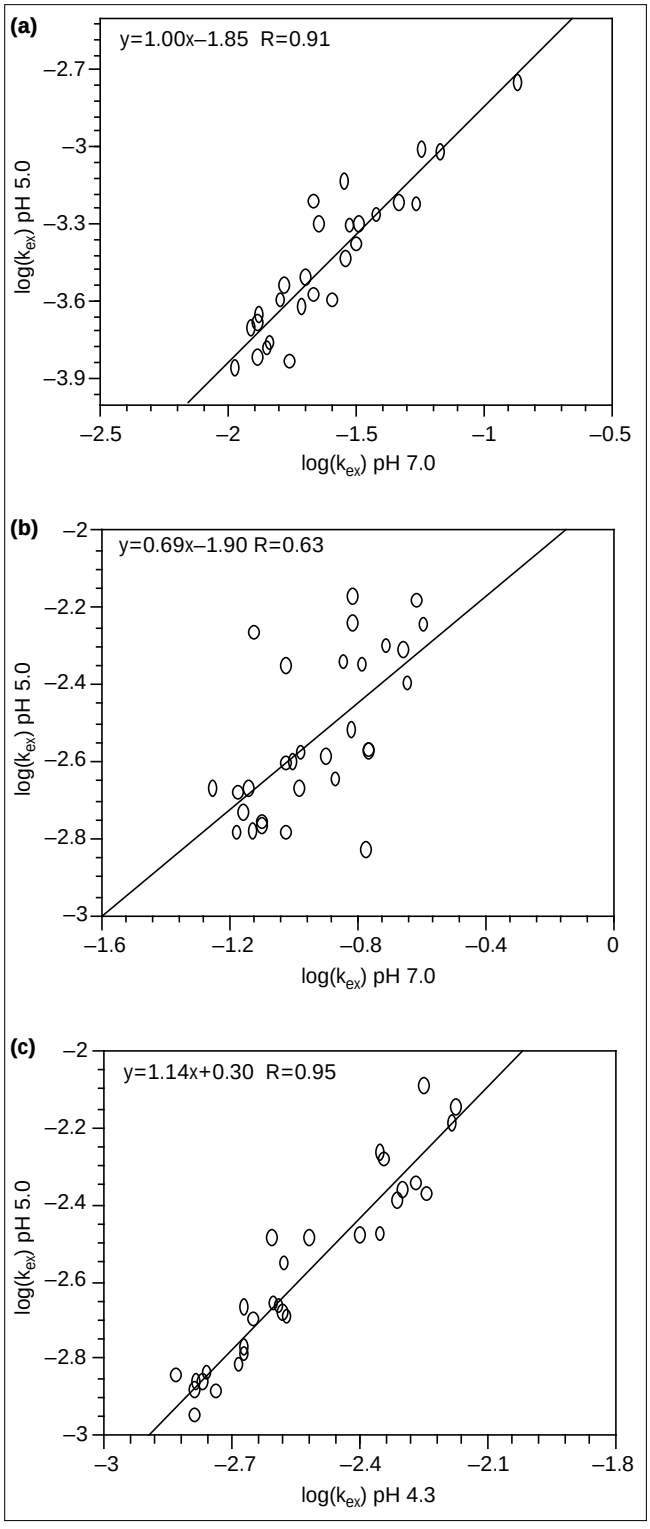
A similar model was used in the earlier mass spectrometry study of protein L HD exchange at high pH and temperature [5]. The equilibrium constant for local fluctuations,

Figure 1



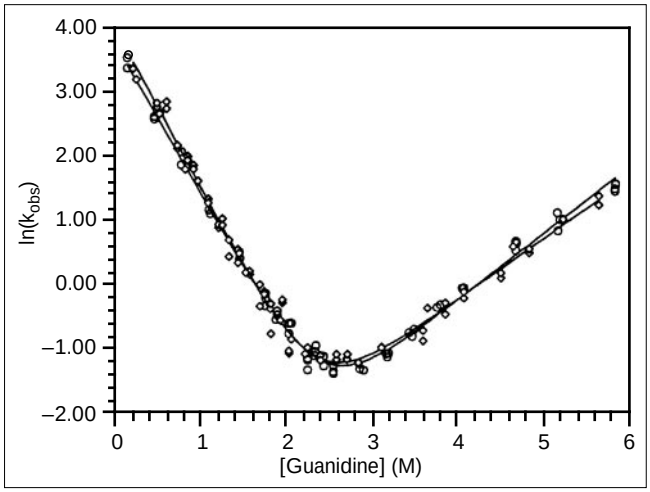
Representative examples of exchange kinetics for four amides at 0 M guanidine, 100 mM potassium phosphate, pH 7.0, 100 nM KCl in D_2O. Exchange rate constants were obtained from simple exponential fits (solid lines) to the data; I4 (crosses), L56 (squares), T37 (circles) and W45 (diamonds).

Figure 2



pH dependence of amide exchange rates at different guanidine concentrations. Each symbol corresponds to an amide proton; (**a**) pH 5.0 versus pH 7.0 at 0.7 M guanidine; (**b**) pH 5.0 versus pH 7.0 at 1.2 M guanidine; (**c**) pH 5.0 versus pH 4.3 at 1.2 M guanidine. Lines are linear fits to the data.

Figure 3



Denaturant dependence of folding and unfolding at pH 5.0 (diamonds) and pH 7.0 (circles).

K_{op}^{local}, is different for each residue but is assumed to be independent of guanidine concentration, while the equilibrium constant for global unfolding, K_{op}^{global}, is the same for all residues and has the usual dependence on the denaturant concentration:

$$K_{op}^{global} = \exp(-(\Delta G_{HD}^{0} - m*[\text{guanidine}]) / RT) \qquad (6)$$

Equations 5 and 6 were fit to all of the HD exchange data simultaneously (see Figure 4 legend); the free parameters are the two global variables ΔG_{HD}^{0} (free energy in the absence of denaturant) and m, and the 31 residue-specific variables K_{op}^{local}. The estimates of the global parameters obtained in the fitting procedure were ΔG_{HD}^{0} = 7.0 ± 0.1 kcal mol^{-1} and m = 3.5 ± 0.1 kcal mol^{-1} M^{-1}. Figure 4 shows the fit of the HD exchange data for residues throughout the protein.

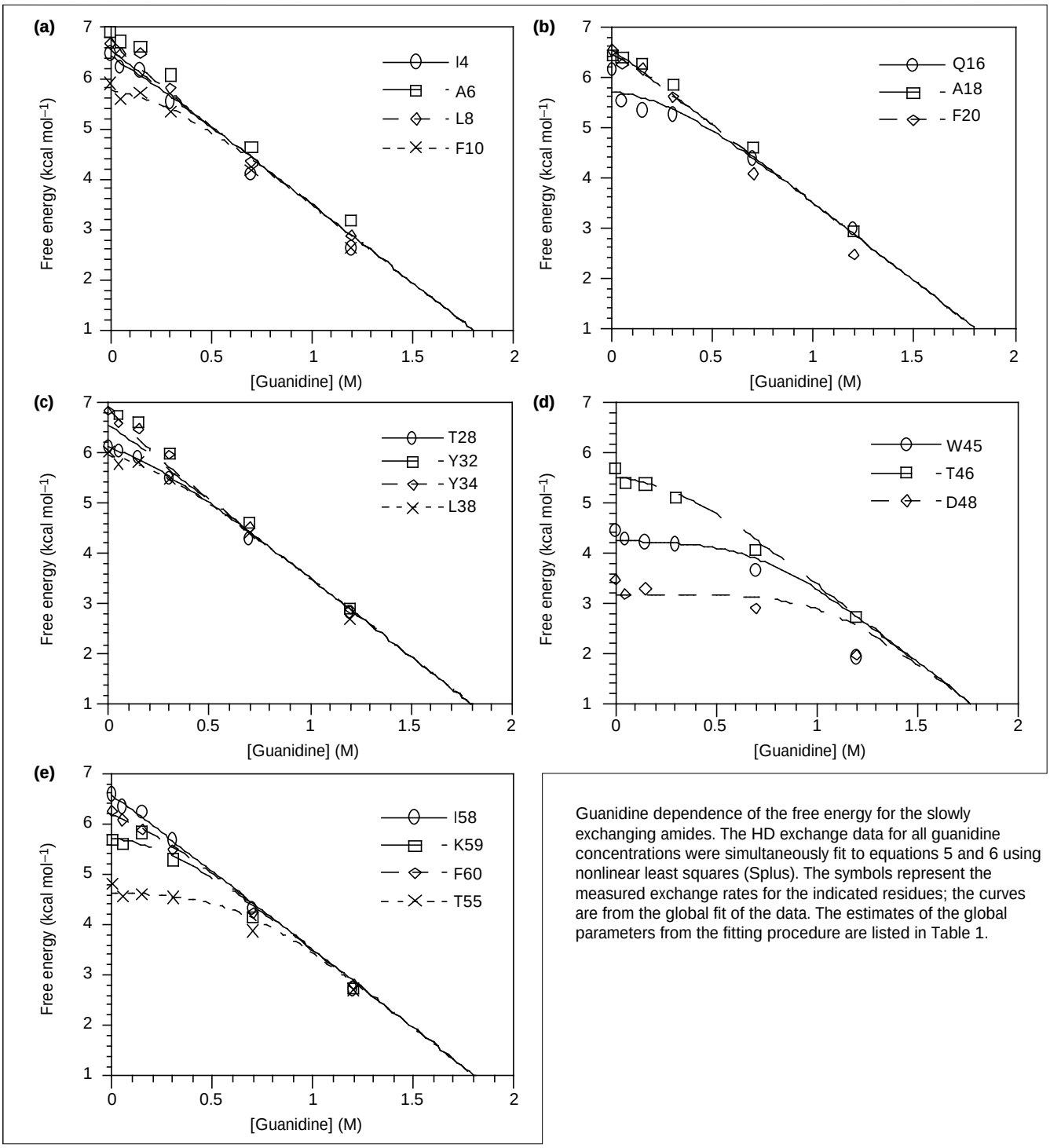
A signature of the partially folded states in RNase H and cytochrome *c* was the clustering of subsets of amides into isotherms with different apparent *m* values. The fit (Figure 4) of the simple model (equations 5 and 6) to the data in which the only denaturant-dependent transition is global unfolding suggests that partially folded species do not contribute significantly to HD exchange of protein L under native conditions. Similar results were obtained with RNase A [9,10].

The K_{op}^{local} obtained for each of the 31 residues for which the exchange rate could be measured are summarized in Figure 5. The equilibrium constant is particularly large for local fluctuations in the third β-strand and the beginning of the fourth β-strand. Interestingly, the global fit of the exchange data assigned negative values of K_{op}^{local} to several

residues in the middle of the first β-strand and the helix (Figure 5, arrows). These residues may be partially protected from exchange in the unfolded state. In the structurally related protein G, the overall pattern of protection is reversed: protons in the second hairpin exchange more slowly than those in the first hairpin [11].

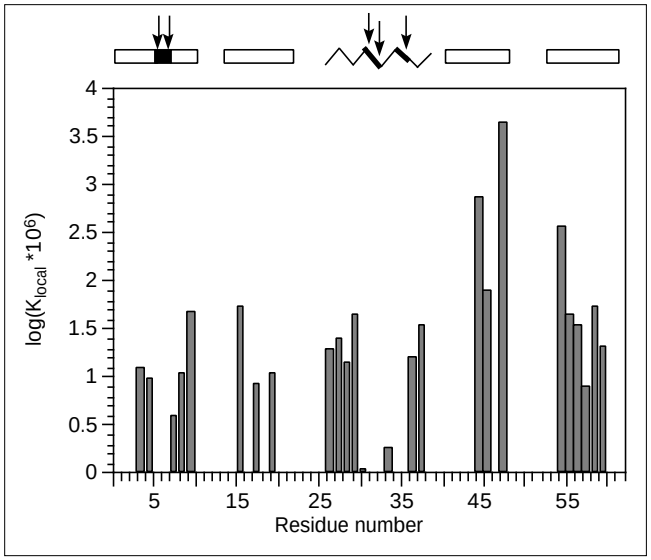
While the fitting of the HD exchange data was internally consistent, the ΔG^0_{HD} estimate of 7.0 kcal mol^{-1} is considerably larger than the value of 4.6 kcal mol^{-1} obtained previously [4] from linear extrapolation of global unfolding data. The earlier measurements were made in H_2O; Figure 6 compares the HD exchange data to circular

Figure 4



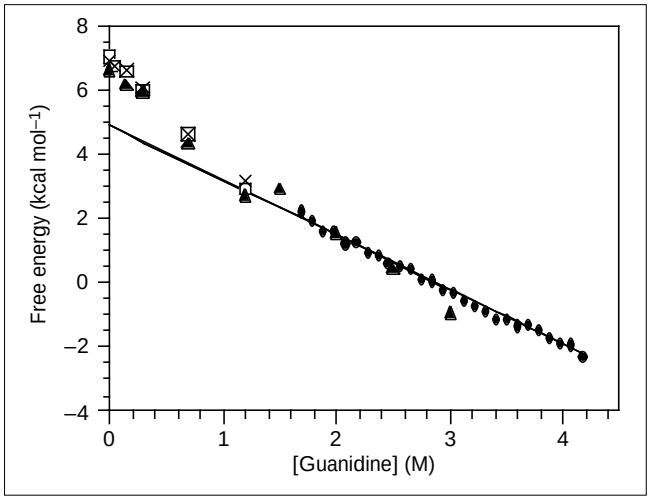
Guanidine dependence of the free energy for the slowly exchanging amides. The HD exchange data for all guanidine concentrations were simultaneously fit to equations 5 and 6 using nonlinear least squares (Splus). The symbols represent the measured exchange rates for the indicated residues; the curves are from the global fit of the data. The estimates of the global parameters from the fitting procedure are listed in Table 1.

Figure 5



Equilibrium constants for local fluctuations. The K_{op}^{local} were obtained by globally fitting the HD exchange data with equations 5 and 6 as described in the text. The fitting procedure assigns negative values of K_{op}^{local} to the residues indicated by arrows on the secondary structure diagram at the top of the figure (A6, N7, Y32, A33 and A35); these residues exchange more slowly than expected given the free energy of unfolding. The overall HD exchange pattern is similar to that observed in previous studies of protein L at pH 6.0 [29] and pH 11.0 [5]. The analysis depends on the assumptions that the K_{op}^{local} are guanidine independent and that the intrinsic rate constants for exchange upon local fluctations are well modeled by k_{rc} values obtained in model peptide studies; neither assumption is likely to be completely accurate.

Figure 6



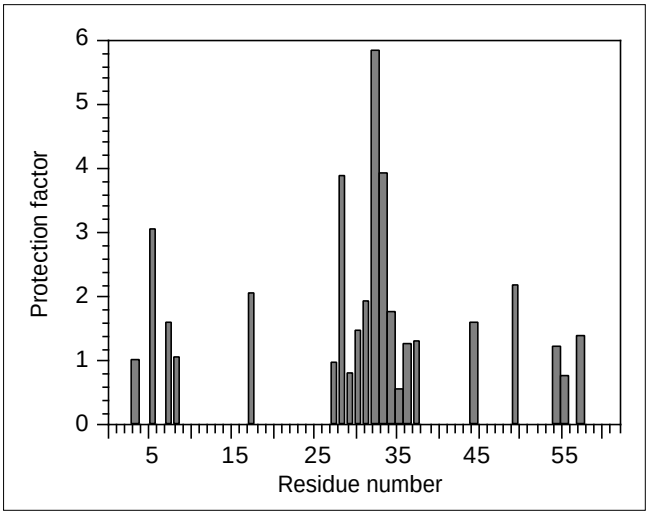
Comparison of free energy estimates obtained from HD exchange and guanidine and temperature melts monitored by CD. The symbols represent free energy estimates from HD exchange of residues A6 (crosses), Y32 (open squares), thermal melts (closed triangles), and guanidine denaturation (closed circles). The line is the best fit of the transition region data used in the LEM (ΔG([guanidine]) = 4.9–1.7*[guanidine]). The thermal melts at varying guanidine concentrations were fit to equation 8 with fixed ΔC_p and baseplanes obtained from global fitting of the thermal and chemical denaturation melts. The free energy estimates obtained from the individual fits are in good agreement with those calculated from the global fit.

dichroism (CD) data monitoring global unfolding in the same buffer used for the HD exchange experiments (500 mM KCl, 100 mM potassium phosphate, pH 7.0 in D_2O). The protein is slightly more stable in D_2O (~0.3 kcal mol^{-1}) than in H_2O, but there remains a significant discrepancy between ΔG^0_{LEM} (free energy derived from the linear extrapolation method [LEM]) and ΔG^0_{HD} (4.9 versus 7.0 kcal mol^{-1}). This discrepancy has been previously observed for a number of proteins, including cytochrome *c* and RNase A [6]. It was suggested that proline isomerization could account for at least part of the discrepancy in these two proteins, but there are no prolines or other obvious complicating structural features in protein L. Therefore, the discrepancy must stem from other factors.

The first possibility is electrostatic effects or residual structure in the denatured protein that partially blocks HD exchange. In this case, the k_{rc} values in equation 1, which are based on unstructured peptides, would be too large and this would result in an overestimate of the ΔG^0_{HD}. This possibility was tested directly by measuring the protection factors in the unfolded protein immediately after dilution of denatured protein into refolding buffer. Deuterated denatured protein was rapidly diluted into H_2O buffer lacking denaturant with pH 8.5, 9.0 or 10.0. After 3.5 ms, proton exchange was quenched by addition of low pH buffer. Protection factors were estimated from the fractional proton occupancy at each pH [12]. As shown in Figure 7, the protection factors for almost all of the slowly exchanging protons were considerably less than the ~20 (exp($\Delta\Delta G/RT$)) needed to account for the discrepancy (~2 kcal mol^{-1}) between the global unfolding and the native state HD exchange estimate of the free energy of folding. These results are consistent with mass spectrometric analysis of pulsed HD exchange labeling in the first 20 ms after the initiation of refolding of protein L: the mass distribution of the unfolded population was very close to that of the fully exchanged protein, indicating very little protection from exchange in the unfolded state [4]. Taken together, these results suggest that the discrepancy in the free energy estimates is unlikely to result from deviations in the k_{rc}s from the model peptide values.

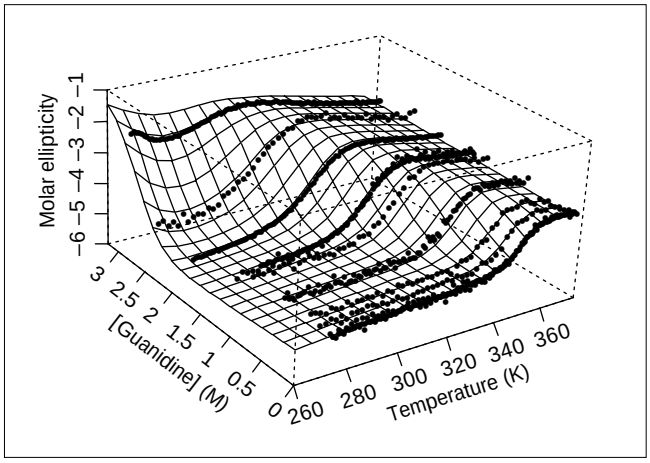
A second possibility is that the estimate of the free energy of global unfolding obtained by linear extrapolation of the CD data is incorrect. The linear dependence of the free energy on the guanidine concentration may break down in the low concentration region making the long extrapolation from the data in 2–4 M guanidine no longer valid. To

Figure 7



Amide proton exchange protection factors in the unfolded state of protein L. Protection factors were estimated from fractional proton occupancies after a 3.5 ms pulse at pH 8.5 using the model described in [12]. Qualitatively similar results were obtained with a pH 9.0 pulse. Estimates could not be made for residues 5, 7, 16, 20, 57 and 59 due to substantial peak overlaps.

Figure 8



Thermal and chemical denaturation surface of protein L. The circles represent experimental data and the surface depicts the best global fit of the data. The folded baseline is described by the equation $\theta = (-6.5 \pm 0.1) + (0.0062 \pm 0.0003)T - (0.14 \pm 0.01)$*[guanidine], and the unfolded baseline by $\theta = (2.9 \pm 0.2) - (0.016 \pm 0.001)T + (0.058 \pm 0.007)$*[guanidine]. The units for molar ellipticity are deg cm^2 dmol^{-1} 10^{-3}.

investigate this possibility, temperature denaturation experiments were carried out in the presence of increasing concentrations of guanidine. The thermodynamic parameters are better determined by such experiments because the transition regions span a broader range of guanidine concentrations. Attempts to determine the thermodynamic parameters by differential scanning calorimetry were unsuccessful due to protein aggregation at the concentrations required for the experiments.

The dependence of protein stability on temperature and denaturant concentration can be expressed in several different ways. One possibility is to expand each of the thermodynamic parameters (ΔH^0, ΔS^0 and ΔC_p) as linear functions of the denaturant concentration [13]. However, in denaturant melts carried out at different temperatures, we found the apparent m value for unfolding to be temperature independent within the error of the measurements. Because of this, a model was fit to the global denaturation data in which the denaturant dependence is described by a single overall m value:

$$\Delta G(T,[\text{guanidine}]) = \Delta H^0 - T\Delta S^0 - \Delta C_p\left(T_0 - T + T\ln\frac{T}{T_0}\right) - m[\text{guanidine}] \quad (7)$$

As found in studies of HPr [14], introduction of additional parameters describing the denaturant dependence of ΔC_p and ΔS^0 did not improve the quality of the fit (decrease Chi square), and thus equation 7 is probably a sufficient model. A further justification for this is that the dependence of ΔC_p [13,15] on guanidine concentration is usually very small, and the correlation between the estimates of ΔH^0 and ΔS^0 obtained from the fit is very high (0.99).

Figure 8 displays the CD signal as a function of temperature and guanidine along with a three-dimensional surface representing the best fit of the data. The thermodynamic parameters calculated from the global fit as well as those from HD exchange and the linear extrapolation of the guanidine denaturation melt (Figure 6) are listed in Table 1. The ΔC_p derived from the fit (0.75 kcal mol^{-1} K^{-1}) is very close to the estimate obtained from the difference in solvent-accessible surface area in the folded and unfolded states using the method of Gomez *et al.* [16] (0.76 kcal mol^{-1} K^{-1}).

The free energy estimate from the global fitting is close to that from the HD exchange experiments but considerably higher than that obtained by linear extrapolation from CD measurements in the transition region (Table 1). Also, these experiments yield different guanidine dependencies of the free energy; the m values obtained from HD exchange, global fitting and LEM are 3.5, 2.4, and 1.7 kcal mol^{-1} M^{-1}, respectively. Such a discrepancy could arise if the dependence of the free energy on the guanidine concentration is nonlinear: the HD exchange data were obtained at low (0–1.2 M) guanidine concentrations, the LEM data at relatively high concentrations of guanidine (2–4 M), and the global denaturation data at 0–3 M guanidine. The global fitting of the data was repeated using extensions of equation 7 with additional terms

Table 1

Thermodynamic parameters for protein L.

	Method of determination		
Parameter	Thermal melts	HD exchange	LEM
ΔG^0 (kcal mol^{-1})	6.4 ± 0.1	7.0 ± 0.1	4.9 ± 0.1
m (kcal mol^{-1} M^{-1})	2.4 ± 0.1	3.5 ± 0.1	1.7 ± 0.1
ΔC_p (kcal mol^{-1} K^{-1})	0.77 ± 0.02		
ΔS^0 (kcal mol^{-1} K^{-1})	0.047 ± 0.001		
ΔH^0 (kcal mol^{-1})	20.1 ± 0.4		

The parameters in the first column were determined from the global fit of equations 7 and 8 to temperature melts carried out at different guanidine concentrations (reference temperature 295K). The parameters in the second column are from the fit of equations 5 and 6 to the HD exchange data. Perfect agreement between the thermal melt and HD exchange estimates is not expected since protein L is slightly more stable (~0.3 kcal mol^{-1}) in the D_2O buffer used in the HD exchange experiments. Standard deviations reported for linear extrapolation and thermal melt parameters are from least squares fit to the data and are probably underestimates of the true errors.

allowing for a nonlinear dependence of the free energy on denaturant concentration, but better fits were not obtained, possibly because there is a relatively small amount of curvature in the 0–3 M guanidine range.

An alternative method to determine the denaturant dependence of the free energy from the data shown in Figure 8 is to estimate the free energy of unfolding from the temperature melts at different guanidine concentrations. To reduce the number of parameters in such fits, ΔC_p and the baseplanes describing the dependence of the signals of the native and unfolded states on the guanidine concentration and temperature were estimated using global fits of the CD data to equation 7. Equation 8 (see Materials and methods) was fit to each of the temperature melts keeping these parameters fixed. The free energies from all the experiments are in good accordance with one another (Figure 6): the free energy estimates from the thermal melts match the free energies obtained from the guanidine melt at higher guanidine concentrations and, importantly, the free energy estimates from the HD exchange experiment at low concentrations of guanidine. Thus, the resolution of the discrepancy between the original guanidine unfolding data and the HD exchange data appears to be a breakdown of the linear extrapolation model used to obtain the free energy of unfolding in the absence of denaturant.

Discussion

The protein L HD exchange data are fit well using a simple model in which there are only two contributions to exchange: denaturant-independent local fluctuations and global unfolding transitions. Local fluctuations contribute to the exchange of the more rapidly exchanging amide protons at low denaturant concentrations; as the denaturant concentration increases, exchange is increasingly dominated by global unfolding events. Unlike RNase H [2] and cytochrome *c* [1], there do not appear to be partially folded states of protein L that are intermediate in free energy between the folded and fully denatured states. This conclusion is consistent with the previous study of HD exchange in protein L under EX1 conditions [5] and with the two-state unfolding at neutral pH for protein L detected by CD, fluorescence and mass spectrometry [4].

At equilibrium under conditions in which the native state is stable, a partially folded state with free energy between the folded and unfolded states would be more populated than the unfolded state, and provided that its HD exchange properties are different from those of the folded and unfolded states, the presence of such an intermediate should be readily detectable. For protein L, CI2 [17] and perhaps most very small proteins, there may be no conformations with free energies intermediate between that of the folded and unfolded states because the loss of configuration entropy during refolding is compensated only by formation of stabilizing hydrophobic and other interactions relatively late in folding. For larger proteins, there may be partially folded conformations with stabilizing interactions sufficient to reduce their free energy below that of the unfolded state.

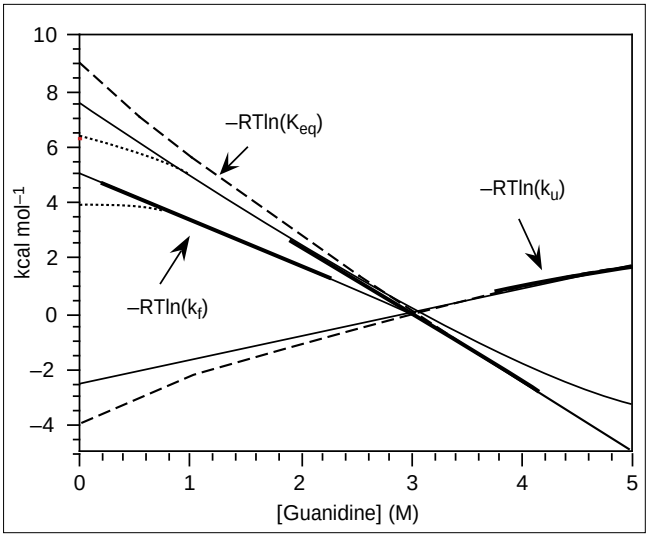
The discrepancy between the free energy estimate from the HD exchange measurements and earlier estimates based on linear extrapolation from CD and fluorescence data in the transition region is largely resolved by global fitting of temperature denaturation data taken at a series of different guanidine concentrations. The free energy estimates from LEM, global fitting, and HD exchange are 4.9, 6.4 and 7.0 kcal mol^{-1}, respectively. Because of the larger amount of data at low guanidine concentrations contributing to the estimates from global fitting and HD exchange relative to LEM, and the consistency in the HD exchange and global fitting estimates, it seems likely that the true free energy is close to 6.5 kcal mol^{-1}. Linear extrapolation from data in the transition region (2–4 M guanidine) may underestimate the free energy for unfolding in the absence of denaturant due to a nonlinear dependence of the free energy on the denaturant concentration. Consistent with an upward curvature ($d^2\Delta G/d[\text{guanidine}]^2 < 0$) in the denaturant dependence of the free energy of folding, the m values of destabilized mutants of protein L, which derive from data collected at lower guanidine concentrations, are larger on average than that of the wild-type protein (data not shown). This has also been noted for barnase [18].

The origin of the deviation from linearity can be better defined by examining the guanidine dependence of the folding and unfolding rates. Free energy estimates from kinetic studies ($-RT^*\ln(k_u/k_f)$) were close to that obtained

from guanidine melts using linear extrapolation, suggesting that similar errors are involved in both calculations. Since k_f at low guanidine concentrations is measured directly and the logarithm of the folding rate was found to be linear with denaturant (Figure 3 and [4]), the deviation in linearity is probably due to errors in the extrapolation of k_u from data collected at high guanidine concentrations. As illustrated in Figure 9 (dashed line) for a two-state folding reaction, deviations from a linear dependence of the free energy on the denaturant concentration together with the observed linearity of ln k_f imply a nonlinear dependence of ln k_u on denaturant concentration. Such curvature in ln k_u was directly observed for barnase [19] and Arc repressor [20]. Rollover in ln k_f at low denaturant concentrations (Figure 9, dotted line) could produce a similar rollover in free energy, leading to an overestimation of the free energy of folding by linear extrapolation.

A nonlinear dependence of ln k_u on the denaturant concentration could result either from a change in the nature of the rate-limiting step for unfolding at low concentrations of denaturant [19,20] (e.g. movement of the position of the transition state as suggested by the Hammond postulate [21]), or from changes in the interaction of denaturant with the native and transition states. One possibility is that denaturant binding sites [15] are exposed upon opening of the native structure at the rate-limiting step in unfolding, and that these sites become saturated at 1–2 M guanidine. In such a picture, denaturants would have two different effects on proteins: first, a general effect on solvation by reduction of the hydrophobic effect, and second, specific and saturable interactions, perhaps with the peptide backbone. Another possible source of curvature is suggested by the nonlinear dependence of the transfer free energy of the amino acids to guanidine-containing solutions [22]. However, nonlinear effects of denaturant on the free energy of nonpolar solvation would be expected to primarily influence the folding rate, since the largest changes in exposed surface area appear to occur in this step as measured by the denaturant dependence of folding and unfolding (for protein L, m_f/m = 0.75 [4]).

Figure 9



Possible sources of LEM discrepancies. Bold lines, measurable data; thin lines, extrapolations; dotted lines, rollover in ln k_f at low guanidine concentrations; dashed lines, nonlinear dependence of ln k_u on guanidine.

The linear dependence of the free energy of folding on denaturant concentration appears to be protein dependent. For a number of proteins, such as barstar [23], HPr [14], lambda repressor [13,24] and RNase H [2], the estimate from linear extrapolation is fairly close to that obtained with either differential scanning calorimetry, combined temperature and chemical denaturation studies, or HD exchange. For protein L, barnase [18], protein G [11,25], RNase A [26] and cytochrome *c* [6,7], on the other hand, there are significant deviations from linearity. It is unclear what sequence/structural features control the extent of linearity of response to denaturant.

From the theoretical point of view, if exchange is dominated by global unfolding and the k_{rc} values are accurate, then the estimate $\Delta G = -RT^*\ln(k_{obs}/k_{rc})$ should be a good estimate of global stability under EX2 conditions. Unlike the other commonly used methods, there are no extrapolations from unfolding transitions measured under nonnative conditions and no assumptions are needed about potentially complex interactions of proteins with denaturant. Recent measurements ([12,27]; D Shortle, personal communication) of the protection factors of amides in denatured states suggest that the k_{rc} in equation 1 for exchange from the open state will in general be reasonably accurate; the proton exchange rates are usually within a factor of three of the random coil exchange rates. A threefold error in k_{rc} estimation limits the accuracy of free energy estimates from HD exchange to ± 0.5 kcal mol^{-1}; because there is likely to be more protection on average in unfolded large polypeptide chains than in the short peptides used for the k_{rc} determinations [7], the errors are most likely to produce slight overestimates in the free energy of folding. Empirically, for protein L, barnase [28] and protein G [11], the HD exchange estimate of ΔG^0 is much closer to the estimate obtained from differential scanning calorimetry or global fitting of denaturation data than is the LEM estimate. Previously, discrepancies between HD exchange and LEM estimates of the free energy of unfolding have been ascribed to complications with the interpretation of the HD exchange data (proline isomerization, partial blockage in the unfolded state, etc), but, given the results reported here, part of the discrepancy may instead be due to breakdown of the linear extrapolation method.

Interestingly, the global fit of the native state HD exchange data assigned negative values of K_{op}^{local} to several residues in the middle of the first β-strand and the helix (Figure 5, arrows). This suggests that these residues may be partially protected from exchange in the unfolded state. Furthermore, the small amount of protection from exchange observed in the dead-time labeling experiment (Figure 7) was primarily in the middle of the first strand and helix. The possibility of residual structure in the unfolded state of protein L involving this part of the protein will be investigated in future experiments.

Materials and methods

Sample preparation

Uniformly N^{15}-labeled Trp-protein L was prepared as described previously [4]. Throughout this paper, protein L refers to protein L with the Y45W mutation. The HD exchange buffer was 100 mM potassium phosphate, pH 7.0 in D_2O containing guanidine ranging from 0 to 1.2 M. For buffers with guanidine concentrations less than 0.5 M, KCl was added to a final concentration of 0.5 M in order to minimize salt effects [7]. pH values were not corrected for isotopic effects.

HD exchange and data analysis

HD exchange experiments were carried out by acquiring a series of N^{15}–H^1 HSQC spectra on a Bruker DMX500 immediately after dissolving lyophilized protein L in deuterated exchange buffer. Time points were taken to be the end of 9.5 min acquisition periods for each HSQC experiment. For each guanidine concentration, 33 HSQC experiments were collected over a 24 h period. The spectra were processed and the volumes of the N^{15}–H^1 crosspeaks were integrated using Felix 2.30 (Biosym, CA). The data of N^{15}–H^1 volume versus exchange time were fit to a single exponential using KaleidaGraph (Abelbeck Software).

Dead-time labeling

The dead-time proton labeling experiments were carried out using a Bio-logic quenched-flow instrument as previously described [12]. About 10 mg ml^{-1} N^{15}-labeled protein L in 4.5 M GuDCl/D_2O was incubated at 40°C for 3 h to allow all the amide protons to exchange with deuterons. The denatured protein solution in D_2O was initially diluted 11-fold into 200 mM borate buffer/H_2O at pH 8.5, 9.0, or 10.0 and the protein was allowed to refold and exchange for 3.5 ms. After the exchange period, the reaction mixture was quickly mixed with 0.5 M acetic acid/H_2O pH 2.0 quench solution in a ratio of 11:10 to a final pH of 4.0. The quenched reaction solution was finally concentrated and the buffer was exchanged to 50 mM potassium phosphate/D_2O pH 4.0 using an Amicon ultrafiltration unit (YM3 filters). The degree of labeling at each pH was determined by N^{15}–H^1 HSQC NMR experiment. The data analysis was as described previously [12].

Temperature denaturation

Temperature denaturation experiments were performed using an Aviv CD spectrometer Model 62A DS. The observation cell was thermostated within 0.2K using a Peltier device. The samples contained 25 ± 0.6 μM protein L, 100 mM sodium phosphate, pH 7.0, 0.5 M KCl, and varying amounts of guanidine HCl (0.0, 0.15, 0.3, 0.7, 1.2, 1.5, 2.0, 2.5 and 3.0 M). The temperature melts were carried out with 30 s equilibration times and a 30 s sampling period. All measurements were made in a 0.2 cm cuvette at 220 nm. Under these conditions, the temperature melts were completely reversible (data not shown).

Thermodynamic parameters for protein L folding were obtained by fitting equation 8:

$$\theta(T,[\text{guanidine}]) = \frac{\theta_{folded}(T,[\text{guanidine}]) + \theta_{unfolded}(T,[\text{guanidine}]) * e^{-\Delta G(T,[\text{guanidine}])/RT}}{1+e^{-\Delta G(T,[\text{guanidine}])/RT}} \quad (8)$$

to temperature melts monitored by CD at nine different concentrations of guanidine using the nonlinear least square function in Splus (MathSoft, Inc.) [13] (see Figure 8 legend). The temperature and denaturant dependence of ΔG was described by equation 7. The error in ΔG^0 was computed using equation 9:

$$\text{Variance}(\Delta G^0) = \text{Variance}(\Delta H^0 - T\Delta S^0) =$$
$$\text{Variance}(\Delta H^0) - 2T * \text{Covariance}(\Delta H^0, \Delta S^0) - T^2 * \text{Variance}(\Delta S^0) \quad (9)$$

The square roots of the variance (the standard deviation) of ΔG^0, ΔH^0 and ΔS^0 are listed in Table 1.

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