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Direct evidence for a two-state protein unfolding transition from hydrogen–deuterium exchange, mass spectrometry, and NMR

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Abstract

We use mass spectrometry in conjunction with hydrogen–deuterium exchange and NMR to characterize the conformational dynamics of the 62-residue IgG binding domain of protein L under conditions in which the native state is marginally stable. Mass spectra of protein L after short incubations in D_2O reveal the presence of two distinct populations containing different numbers of protected protons. NMR experiments indicate that protons in the hydrophobic core are protected in one population, whereas all protons are exchanged for deuterons in the other. As the exchange period is increased, molecules are transferred from the former population to the latter. The absence of molecules with a subset of the core protons protected suggests that exchange occurs in part via a highly concerted transition to an excited state in which all protons exchange rapidly with deuterons. A steady increase in the molecular weight of the population with protected protons, and variation in the exchange rates of the individual protected protons indicates the presence of an additional exchange mechanism. A simple model in which exchange results from rapid ($>10^5$/s) local fluctuations around the native state superimposed upon transitions to an unfolded excited state at ~0.06/s is supported by qualitative agreement between the observed mass spectra and the mass spectra simulated according to the model using NMR-derived estimates of the proton exchange rates.

Keywords: hydrogen–deuterium exchange; IgG binding domain; MS; NMR; protein folding; protein L; two-state model

The folding reactions of small globular proteins are often modeled as two-state transitions (Segawa & Sugihara, 1984; Chen et al., 1989; Kuwajima et al., 1989; Jackson & Fersht, 1991; Alexander et al., 1992a, 1992b; Schmid, 1992; Serrano et al., 1992; Chen & Matthews, 1994). Both thermodynamic and kinetic data on a number of these proteins are very well fit by models in which the signal $S(x)$ (detected using a variety of spectroscopic methods) is the sum of signals from folded and unfolded forms:

$$S(x) = S_u \cdot F_u(x) + S_n \cdot F_n(x),$$

where x is the experimental variable (time, denaturant concentration, temperature, etc.), S_u and S_n are the signals from unfolded and folded states, and $F_u(x)$ and $F_n(x)$ are the fraction of molecules in unfolded and folded states. F_u and F_n are not observed directly; only the ensemble average value $S(x)$ is measured. More direct evidence for a two-state transition requires methods that can measure F_u and F_n directly.

As illustrated by previous work on lysozyme (Miranker et al., 1993), mass spectrometry in conjunction with amide H-D exchange is potentially such a method. Different conformational states of proteins can be resolved provided that different numbers of protons are protected from exchange in the different states. Electrospray mass spectrometry can resolve mass differences as small as a single proton, and thus states with different numbers of deuterons can be distinguished readily. Here we use H-D exchange, mass spectrometry, and NMR to probe the conformational dynamics of the small IgG binding domain of the 62-residue peptostreptococcal protein L (Wikstrom et al., 1993, 1994) under conditions in which the native state is marginally stable.

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Abbreviations: H-D exchange, hydrogen–deuterium exchange. ESI-MS, electrospray ionization mass spectrometry; HSQC, heteronuclear single quantum coherence.

Results and discussion

H-D exchange

Amide H-D exchange in proteins is often described using the following simple mechanism (Roder et al., 1985; Bai et al., 1994):

$$\text{NH(closed)} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{NH(open)} \xrightarrow{k_{ch}} \text{ND}. \quad (1)$$

The closed to open transition exposes the proton to solvent; the opening reaction can range from a local perturbation of the structure in the vicinity of the proton to global unfolding of the molecule. The observed steady state exchange rate k_{ex} that follows from these mechanism is

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

If k_{-1} is much larger than k_1 and k_{ch}, $k_{ex} \approx k_{op}\, k_{ch}$, where k_{op} is the equilibrium constant for the opening reaction (k_1/k_{-1}). This is the EX2 limit, and most H-D exchange work in proteins has been conducted under such conditions (Roder et al., 1985; Bai et al., 1994, 1995). The exchange rate in this limit depends on the free energy difference ($-RT \ln k_{op}$) between the closed state and the relevant open state, and thus hydrogen exchange experiments can probe the energetics of localized changes in a manner analogous to that of conventional spectroscopic methods such as CD and fluorescence for global unfolding.

If k_{ch} is much larger than k_1 and k_{-1}, the overall exchange rate becomes a function of the opening rate rather than the opening equilibrium constant: $k_{ex} \approx k_1$. This is the EX1 limit, and experiments under this regime probe the kinetics rather than the thermodynamics of protein conformational changes (Bai et al., 1994, 1995).

MS analysis of H-D exchange in protein L

We began by investigating the H-D exchange of protein L using ESI-MS as a function of temperature and pH. As the pH (Fig. 1A)

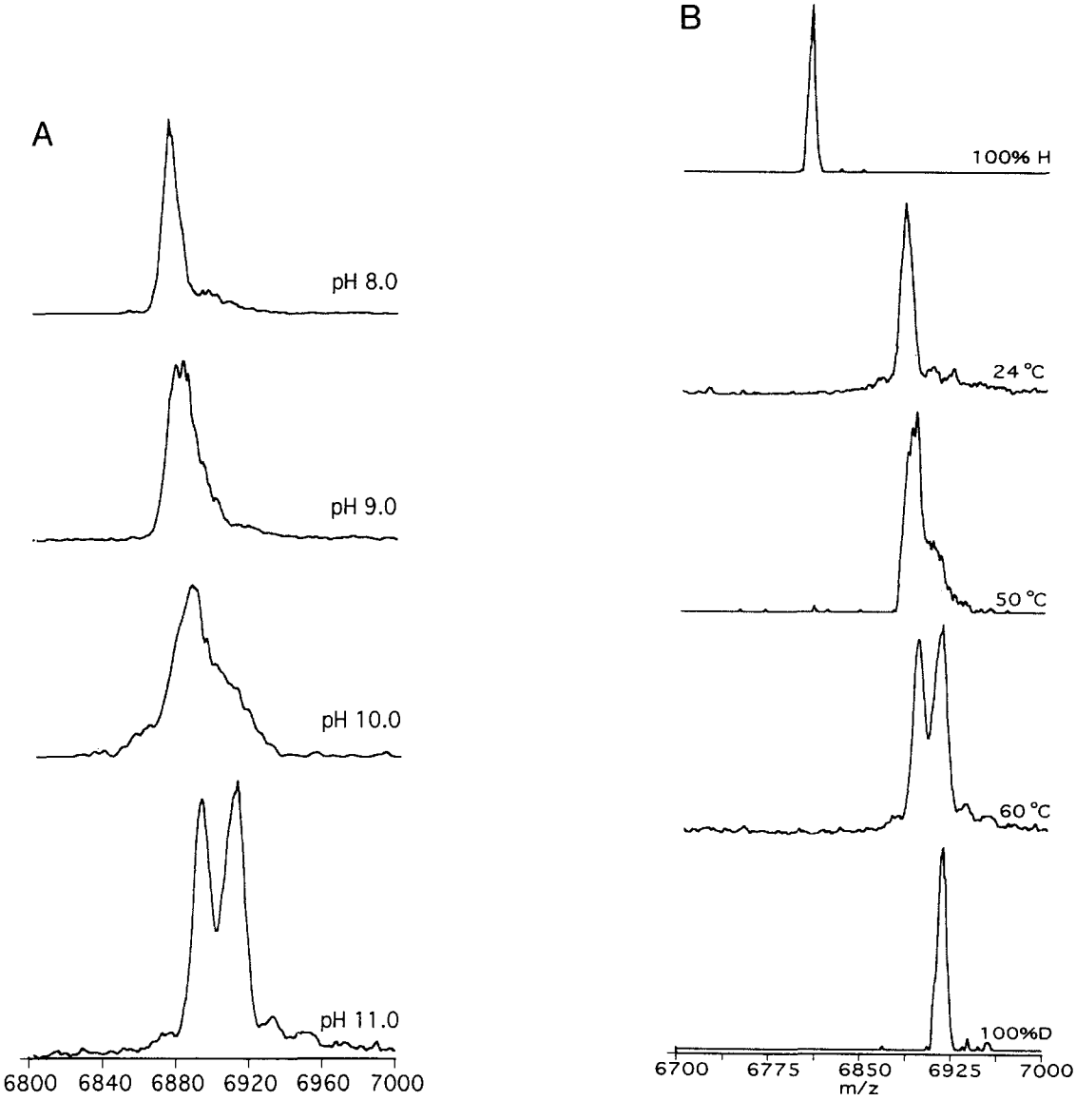

Fig. 1. pH and temperature dependence of exchange. **A:** MS spectra of protein L following H-D exchange at different pHs at 60 °C. **B:** MS spectra of protein L following H-D exchange at different temperatures at pH 11.0. The exchange time for these experiments is about 4 s.

and temperature (Fig. 1B) are increased, the mass spectra change considerably. The mass of the protein increases as more protons are replaced by deuterons due to the increase in intrinsic exchange rate (k_{ch} in Equation 1) with temperature and pH (Roder et al., 1985). Interestingly, the peak becomes increasingly broad with increasing temperature and pH, finally splitting into two distinct peaks at 60 °C and pH 11.0. The mass of the higher molecular weight peak at pH 11.0 and 60 °C is very close to that of a sample boiled in D_20 (Fig. 1B, 100% D), suggesting that all protons in the molecules contributing to the peak have undergone exchange.

Such broadening and eventual splitting is expected for a two-state transition as the exchange mechanism approaches the EX1 limit starting from EX2 conditions. Under EX1 conditions, where the rate of exchange is much faster than the rate of the closing reaction, a single opening reaction will lead to the exchange of nearly all the newly exposed protons. Thus, there will be two classes of molecules in solution after a brief exchange period: those molecules that have undergone an opening reaction and hence have all of the relevant protons exchanged for deuterons, and those that have never opened, and hence have all of the sites still protonated. Under EX2 conditions, in contrast, many opening and closing reactions will occur prior to the exchange of any particular proton, and thus there will be little correlation between the occupancies of protons/deuterons at different sites on a given molecule. Instead, each molecule will have, on average, the same number of protons exchanged, and the population will appear as a single peak on a mass spectrum. In between the EX1 and EX2 extremes at intermediate intrinsic exchange rates, there will be some correlation between the protons exchanged on individual molecules, and thus different molecules will have different masses and the peak will become increasingly broad.

The absence of peaks intermediate between the protected and fully exchanged peaks in the mass spectrum at pH 11.0 and 60 °C suggests that the exchange of the core protons is likely to involve a single cooperative unfolding reaction and clearly suggests a two-state model of unfolding. The concerted nature of the H-D exchange of protein L under these conditions may be in part due to the extreme nature of the conditions. The melting temperature of protein L at pH 11.0 was found to be ~68 °C using CD spectroscopy (data not shown; a fit to a two-state model suggests that 85–90% of molecules are folded at 60 °C). The interpretation of the temperature and denaturant dependence of H-D exchange data for proteins such as cytochrome *c* and RNAse (Mayo & Baldwin, 1993; Bai et al., 1995; Kiefhaber & Baldwin, 1995) has suggested that global unfolding becomes an increasingly important exchange mechanism at increasing temperatures and concentrations of denaturant; this conclusion is supported by the spectra in Figure 1.

Kinetics of exchange probed by mass spectrometry and NMR

To further characterize the conformational dynamics of protein L at pH 11 and 60 °C, the protein was incubated in D_2O for increasing lengths of time and then analyzed by mass spectrometry. To determine which amide protons are protected in the lower molecular weight peak in the mass spectra (Fig. 1), NMR ^1H-^{15}N HSQC experiments were performed on the same samples. The methods are complementary: MS reports on the population distribution, whereas NMR experiments determine the proton occupancy at individual sites averaged over all species in solution.

Figure 2 shows a side-by-side comparison of the MS and NMR results. As the exchange time increases, the fully exchanged population increases and the population protected from exchange decreases as expected for a two-state transition. The intensities of the protected ^1H-^{15}N crosspeaks show a corresponding decrease with time. The average mass of the lower molecular weight peak in the mass spectrum slowly increases, indicating some exchange in the absence of global unfolding (see below).

The exchange rates for all the protected protons were estimated from the NMR data and are summarized in Table 1. Of the 62 backbone amide protons in protein L, 29 exchange too rapidly at pH 11 and 60 °C to be detected in our experiments. These sites are colored yellow in Figure 3 and lie primarily in solvent-exposed portions of the protein. Fourteen of the remaining protons have exchange rates close to 0.06 s^{-1}. These are primarily in the hydrophobic core of the protein (Table 1) and

Table 1. *Correlation between the slow H-D exchanging residues and the structural factors*

Residue	Exchange rate (s^{-1})[a]	H-Bond partner	Secondary structure	Tertiary structure
Ile 4	0.069	Phe 20	1st β-Sheet	Hydrophobic core
Lys 5	0.178	Tyr 54	1st β-Sheet	Partially buried
Ala 6	0.053	Ala 18	1st β-Sheet	Hydrophobic core
Asn 7	0.135		1st β-Sheet	Partially buried
Leu 8	0.050	Gln 16	1st β-Sheet	Hydrophobic core
Ile 9	0.074	Ile 58	1st β-Sheet	Hydrophobic core
Phe 10	0.136		1st β-Sheet	Hydrophobic core
Ala 18	0.137	Ala 6	2nd β-Sheet	Partially buried
Phe 20	0.053	Ile 4	2nd β-Sheet	Hydrophobic core
Thr 28	0.230	Glu 25	α-Helix	Partially buried
Ser 29	0.250	Glu 25	α-Helix	Exposed
Glu 30	0.227	Lys 26	α-Helix	Partially exposed
Ala 31	0.055	Ala 27	α-Helix	Partially buried
Tyr 32	0.056	Thr 28	α-Helix	Hydrophobic core
Ala 33	0.074	Ser 29	α-Helix	Exposed
Tyr 34	0.067	Glu 30	α-Helix	Hydrophobic core
Ala 35	0.056	Ala 31	α-Helix	Partially buried
Asp 36	0.058	Ala 33	α-Helix	Partially buried
Thr 37	0.223	Tyr 34	α-Helix	Exposed
Leu 38	0.129		α-Helix	Partially buried
Lys 39	0.201		Turn	Partially exposed
Tyr 45	0.244		3rd β-Sheet	Hydrophobic core
Thr 46	0.250	Lys 59	3rd β-Sheet	Exposed
Asp 48	0.240	Asn 57	3rd β-Sheet	Partially exposed
Ala 50	0.243		3rd β-Sheet	Exposed
Tyr 54	0.219	Lys 52	4th β-Sheet	Hydrophobic core
Thr 55	0.234		4th β-Sheet	Partially buried
Leu 56	0.067	Lys 5	4th β-Sheet	Hydrophobic core
Asn 57	0.176		4th β-Sheet	Partially buried
Ile 58	0.045		4th β-Sheet	Hydrophobic core
Lys 59	0.066		4th β-Sheet	Partially buried
Phe 60	0.141	Ile 9	4th β-Sheet	Hydrophobic core
Ala 61	0.219	Glu 44	4th β-Sheet	Exposed

[a] The exchange rate was estimated by fitting the relative ^1H-^{15}N peak volume versus exchange time with a single first-order exponential.

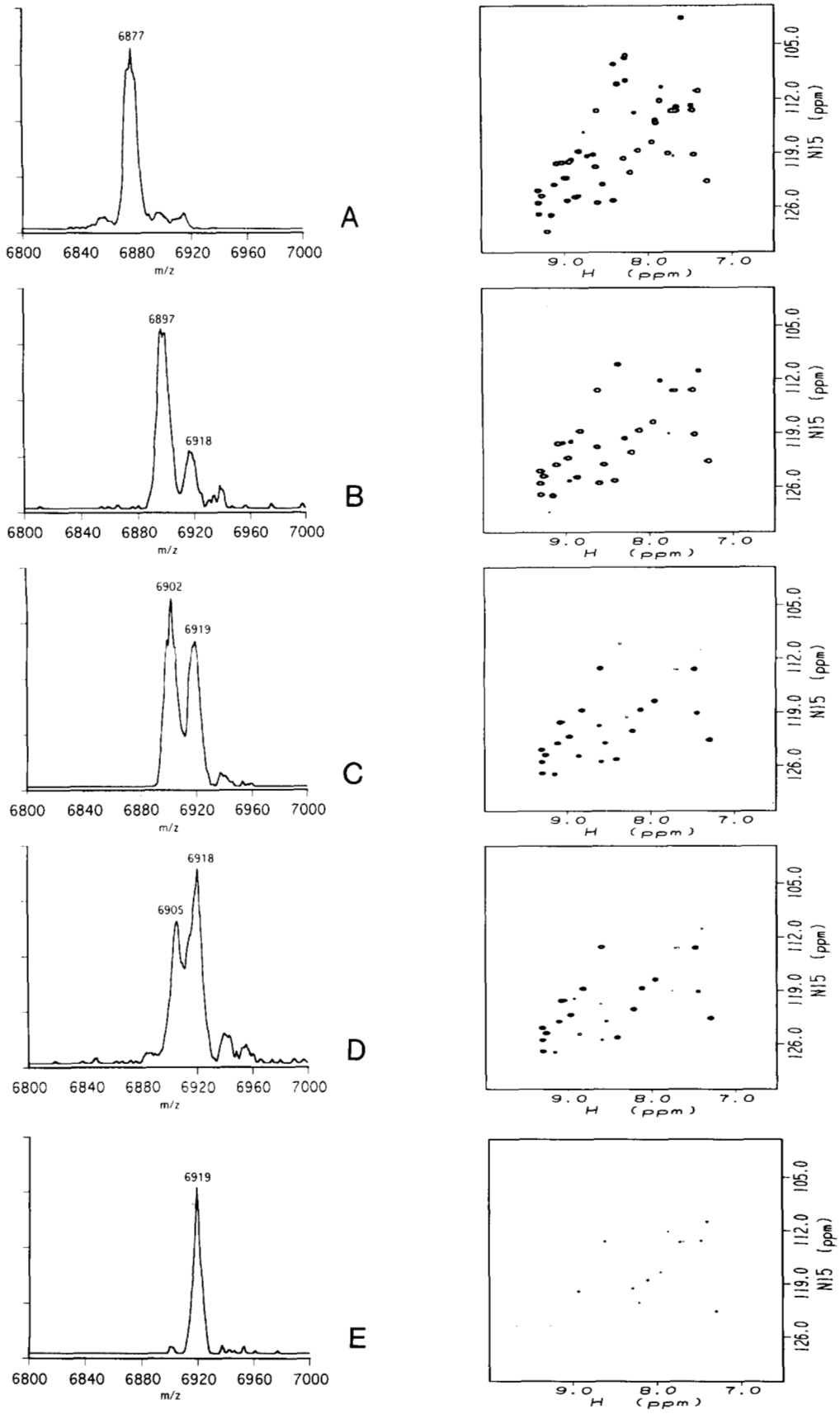

Fig. 2. Time course of exchange. MS and NMR HSQC spectra of protein L after exchange with D_2O at pH 11.0 and 60 °C for different lengths of time. The exchange time is **(A)** 0 s; **(B)** 4 s; **(C)** 7 s; **(D)** 10 s; and **(E)** 10 min, respectively.

are colored red in Figure 3. The 19 remaining protons have exchange rates ranging from 0.14 to 0.23 s^{-1} and are primarily associated with the ends of the secondary structural elements and with the outer β strands. Overall, the distribution of slow and fast exchanging sites is similar to that observed previously in protein L (Wikstrom, 1995), and the related IgG binding domain of protein G (Orban et al., 1994) at neutral pH.

Conformational dynamics of protein L at high pH and temperature

An important feature of our experiments is that the combination of NMR and MS data put strong constraints on possible H-D exchange mechanisms. In this section, we investigate different models for exchange by comparing the observed mass spectra with spectra simulated using NMR-derived estimates of the individual proton exchange rates.

The two separated peaks in the mass spectra suggest that the core protons exchange via a highly concerted global unfolding transition that exposes all protons in the protein to solvent. Exchange following global unfolding under these conditions is well into the EX1 limit. The unfolding rate can be estimated from both the NMR data (the exchange rate of the core protons) and the MS data (the rate of conversion of the lower molecular weight population into the higher molecular weight population); both estimates are close to 0.06/s. A rough estimate of 0.3/s for the rate of folding can be obtained from the rate of unfolding and the folding equilibrium constant. In contrast, estimates of the amide proton exchange rates in a random coil with the protein L sequence at 60 °C and pH 11 (Bai et al., 1993) range from 8×10^4 to 4×10^6/s, at least five orders of magnitude greater than the global folding and unfolding rates, and exchange following global unfolding is thus certainly in the EX1 limit (see Equation 2).

The increased rate of H-D exchange of protons outside of the central hydrophobic core (Table 1) could result either from local fluctuations around the native state, or from transitions to partially folded excited states. We use the intrinsic rate of exchange ($\sim 10^5$/s) to resolve the somewhat semantic issues involving the definition of the native state and the distinction between local fluctuations around the native state and transitions to intermediate or excited states: configurations of the chain that interconvert with the native configuration at rates greater than 10^5/s are considered to belong to the native state, whereas configurations that interconvert with the native configuration at lesser rates are considered to belong to distinct excited states.

Fig. 3. Ribbon diagram of protein L with the exchange rates represented with different colors. The backbone of protein L is depicted in yellow, the red regions represent residues with an exchange rate of ~0.06 s^{-1}, and the blue regions represent residues with exchange rates ranging from ~0.14 to ~0.23 s^{-1} at pH 11.0 and 60 °C.

To distinguish between the possibilities, mass spectra were simulated using the individual proton H-D exchange rates from the NMR data (Table 1) and two different models. In both models, the rate of global unfolding was taken to be the average of the H-D exchange rates of the slowly exchanging core protons (0.06/s). In the first model, the increase in the exchange rates at the more rapidly exchanging sites was assumed to result from rapid ($>10^5$/s) fluctuations around the native state. In the second model, the increase was assumed to result from transitions to discrete excited states at rates less than 10^5/s (Fig. 4, upper panel and legend). The distinguishing feature of the two models with regard to the mass spectra is that the additional exchange is in the EX2 regime in model I and in the EX1 regime in model 2.

Comparison of the simulated spectra to the observed mass spectra strongly supports the first model (Fig. 4). There is striking qualitative agreement between the amplitude, position, and breadth of the peaks in the simulated and observed mass spectra for model 1, but little resemblance for model 2. The success of model 1 in reproducing the steady increase in the mass and the breadth of the lower molecular weight population supports the assumption that the variations in the exchange rates among the different amide protons result from very rapid local fluctuations in the EX2 regime. We note that EX2 exchange behavior in proteins at high pH and temperature has been observed previously (Roder et al., 1985).

There has been a considerable amount of discussion in the literature about the nature of the opening reaction in the first step of Equation 1. The temperature and denaturant dependence of exchange rates, and the number and location of protons with similar exchange rates in the EX2 limit have been used to make inferences about the structural changes and energetics of the opening reactions (Mayo & Baldwin, 1993; Bai et al., 1994, 1995; Kiefhaber & Baldwin, 1995); a particularly elegant example is the probing of excited states of cytochrome *c* (Bai et al., 1995). The qualitative agreement between the MS and NMR results in our experiments provides direct support for the distinction between exchange via local fluctuations and exchange via global unfolding implicit in current treatments of H-D exchange in proteins. Although the MS experiments performed here do not provide residue-specific information, the comparisons in Figure 4 illustrate that MS data can provide strong constraints for distinguishing between alternate models for H-D exchange in proteins.

By working in the EX1 limit, our approach potentially allows access to the kinetics of transitions to excited states and hence $\Delta G^{\ddagger}$ rather than ΔG. For example, here we obtain the rate of global unfolding under conditions in which the native state is stable, a quantity not readily accessible through direct measurement and instead usually inferred from extrapolation. The experiments do not carry information on the equilibrium unfolding transition; the conclusion from the two distinct populations observed in the mass spectra is that the breaking of hydrogen bonds during kinetic unfolding is a two-state process. Other probes of structure could potentially indicate a more complex process; for example, NMR chemical shift (Kiefhaber et al., 1995) and H-D exchange data (Kiefhaber & Baldwin, 1995) on RNAse A un-

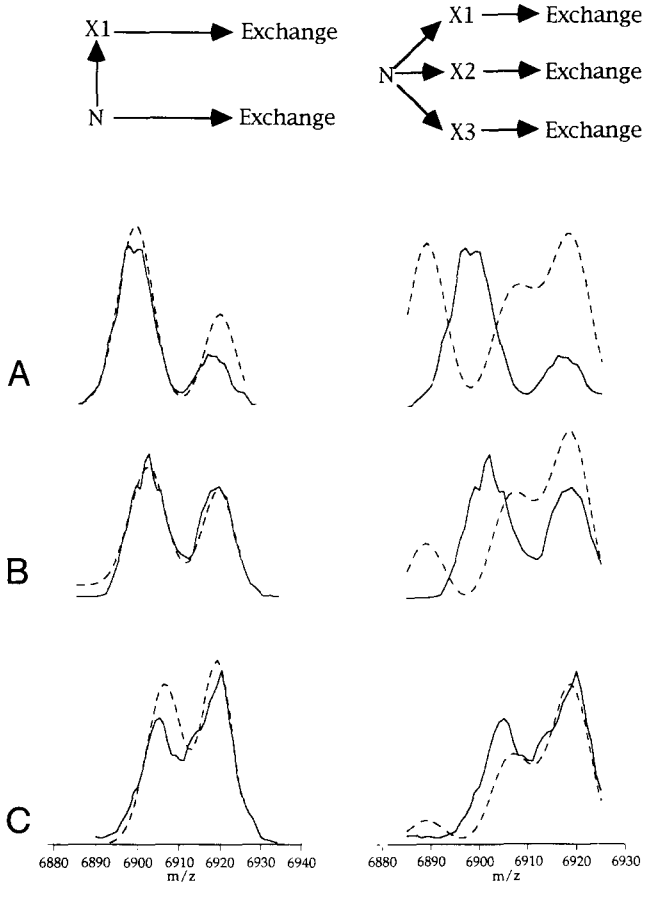

Fig. 4. Comparison of observed and simulated mass spectra. Mass spectra were reconstructed from the NMR data using the exchange rates for the individual protons given in Table 1 and one of two models. Top: Models for exchange. N indicates the native state, and X1, X2, and X3, higher energy excited states. In the model on the left, exchange can occur from either N or X1. Transitions from N to X1 occur at the average of the rates of the slowest exchanging protons (0.06/s) and cause exchange of all protons for deuterons (EX1 limit). Exchange from N is in the EX2 limit and occurs with a rate of $(k_{obs}(i) - 0.06)$, where $k_{obs}(i)$ is the observed rate constant for exchange of the ith proton estimated from the NMR data. In the model on the right, exchange occurs from any of the three excited states, but not from N. The exchange rates in Table 1 fall roughly into three groups with average values of 0.06, 0.15, and 0.23/s. Protons with exchange rates of ~0.06/s are assumed to only exchange in state X1, protons with exchange rates of ~0.15/s, in states X1 and X2, and protons with exchange rates of ~0.23/s, in states X1, X2, and X3. Lower three panels: Comparison of simulated and observed mass spectra. Simulated distributions were calculated from a total of 30,000 molecules using either model I (left) or model II (right). Differences between the simulated and observed spectra for model I are of the same order of magnitude as those resulting from changes of ~10% in the values of the input exchange rates. **A:** 4 s. **B:** 7 s. **C:** 10 s. Solid lines, observed spectra; dashed lines, simulated spectra.

folding suggest the accumulation of an intermediate state prior to the highly cooperative breaking of the hydrogen bonding network.

The observed mass spectra and the strong agreement between these spectra and the simulated spectra suggest strongly that H-D exchange in protein L at high pH and temperature occurs from either the native state or a largely unfolded excited state. However, the extent to which different states can be resolved depends on the resolution of the measurements. Thus, the unfolded state could include configurations with protection factors of up to 1,000, because, with an intrinsic exchange rate of 10^5/s, all protons would be exchanged from these configurations prior to the first time point. Similarly, rapid transitions to discrete excited configurations may be responsible for the H-D exchange from the native state, but such transitions would have to occur at rates greater than 10^5/s.

Materials and methods

Protein

Protein L was purified in a $(His)_6$-tag form from *Escherichia coli* strain BL21 (DE3/plys S) as described previously (Gu et al., 1995). ^{15}N-labeled protein was purified from cells grown in M9 minimal media (Sambrook et al., 1989) using $^{15}NH_4Cl$ as a sole source of nitrogen. The $(His)_6$-tag in the N-terminal was removed by CNBr digestion with 50-fold excess of CNBr relative to methionine in 0.1 N HCl at 22 °C for 16 h. The cleavage products were separated by SP-sepharose (Phamacia Biotech) ion exchange chromatography; roughly 50% of the input protein L was recovered after cleavage. The mass of the final purified protein was determined by MS and matched that expected for protein with complete ^{15}N isotope incorporation after cleavage at the methionine residue. The protein was greater than 99% pure.

H-D exchange

The H-D exchange reaction was conducted as follows: 4 mg/ml ^{15}N-labeled protein L in H_2O, and D_2O adjusted to the desired pH with saturated ammonia were preequilibrated at the desired temperature. H-D exchange was initiated by manually injecting protein L in H_2O into an Amicon concentrator containing 10 volumes of D_2O with constant stirring. Control experiments with a chymotrypsin and benzoyl tyrosine ethyl ester (Sigma) test reaction showed that the mixing dead time is less than 2 s under these conditions. The exchange reaction was allowed to proceed for a variable length of time (4 s, 7 s, 10 s, and 10 min). At the end of the exchange period, ice cold D_2O at pH 3.0 (adjusted by formic acid) was added directly into the exchange mixture at a 4:1 volume ratio to quench further exchange. The final pH of the mixture was 3.2. One portion of the sample was analyzed directly by ESI-MS; for NMR analysis, the remainder was washed and concentrated in 50 mM potassium phosphate in D_2O at pH 3.2 using an Amicon concentrator with a YM3 membrane. Samples were sometimes frozen and stored at −80 °C prior to MS or NMR analysis. In a control experiment, the same amount of protein L stock solution was added directly into pre-mixed and pre-cooled exchange and quench solutions in the same ratios as described above. Subsequent manipulations were the same as above; data from this sample was used as the zero time point of exchange. The pH measurements were not corrected for isotope effects.

ESI-MS

All the ESI-MS spectra were recorded using a Perkin Elmer Sciex API III instrument under standard ESI-MS conditions, except that 150 μL of D_2O was injected prior to each sample to reduce exchange of protons for deuterons within the instrument.

The mass of each sample was determined to within a standard deviation of ±1.1 amu. The observed masses were corrected for the residual water in the experiments; with this correction, the mass of a fully deuterated protein control was within a mass unit of that calculated from the sequence. This indicates that very few deuterons exchange with protons within the instrument.

NMR

The sensitivity enhanced HSQC experiment (Palmer et al., 1991; Kay et al., 1992) was used to monitor the exchange reactions. The NMR experiments were performed using a Bruker 500 MHz DMX instrument; 256 increments with 1,024 data points and eight scans for each increment were collected in each HSQC experiment. NMR data were processed using FELIX 2.30 (Biosym Technologies, San Diego, California). The intensities of ^{1}H-^{15}N cross peaks in the HSQC spectra were estimated from the volumes of the peaks and corrected for variations of protein concentration in each sample using the intensities of three nonexchangeable upfield methyl resonances in the proton 1D-NMR spectra.

Molecular graphics

Molecular images were generated using MidasPlus (UCSF). The coordinates of the protein L IgG binding domain were generously provided by Drs. Lars Bjorck and Mats Wikstrom.

Acknowledgments

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References

Alexander P, Fahnestock S, Lee T, Orban J, Bryan P. 1992a. Thermodynamic analysis of the folding of the streptococcal Protein G IgG-binding domains B1 and B2: Why small proteins tend to have high denaturation temperatures. *Biochemistry 31*:3597–3603.

Alexander P, Orban J, Bryan P. 1992b. Kinetic analysis of folding and unfolding the 56 amino acid IgG-binding domain of streptococcal protein G. *Biochemistry 31*:7243–7248.

Bai Y, Milne JS, Mayne L, Englander SW. 1993. Primary structure effects on peptide group hydrogen exchange. *Proteins Struct Funct Genet 17*: 75–86.

Bai Y, Milne JS, Mayne L, Englander SW. 1994. Protein stability parameters measured by hydrogen exchange. *Proteins Struct Funct Genet 20*:4–14.

Bai Y, Sosnick TR, Mayne L, Englander SW. 1995. Protein folding intermediates: Native-state hydrogen exchange. *Science 269*:192–197.

Chen BL, Basse WA, Schellman JA. 1989. Low temperature unfolding of a mutant of phage T4 lysozyme. 2. Kinetic investigation. *Biochemistry 28*:691–699.

Chen X, Mattews CR. 1994. Thermodynamic properties of the transition state for the rate-limiting step in the folding of the α-subunit of tryptophan synthase. *Biochemistry 33*:6356–6362.

Gu H, Yi Q, Bray ST, Riddle DS, Shiau AK, Baker D. 1995. A phage display system for studying the sequence determinants of protein folding. *Protein Sci 4*:1108–1117.

Jackson SE, Fersht AR. 1991. Folding of chymotrypsin inhibitor 2. 1. Evidence for a two-state transition. *Biochemistry 30*:10428–10435.

Kay LE, Keifer P, Saarinen T. 1992. Pulse absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity. *J Am Chem Soc 114*:10663–10665.

Kiefhaber T, Baldwin RL. 1995. Kinetics of hydrogen bond breakage in the process of unfolding of ribonuclease A measured by pulsed hydrogen exchange. *Proc Natl Acad Sci USA 92*:2657–2661.

Kiefhaber T, Labhardt AM, Baldwin RL. 1995. Direct NMR evidence for an intermediate preceding the rate-limiting step in the unfolding of ribonuclease A. *Nature 375*:513–515.

Kuwajima K, Mitani M, Sugai S. 1989. Characterization of the critical state in protein folding. *J Mol Biol 206*:547–561.

Mayo SL, Baldwin RL. 1993. Guanidinium chloride induction of partial unfolding in amide proton exchange in RNase A. *Science 262*:873–876.

Miranker A, Robinson CV, Radford SE, Aplin RT, Dobson CM. 1993. Detection of transient protein folding populations by mass spectrometry. *Nature 262*:896–900.

Orban J, Alexander P, Bryan P. 1994. Hydrogen–deuterium exchange in the free and immunoglobulin G-bound protein G B-domain. *Biochemistry 33*:5702–5710.

Palmer AG III, Cavanagh J, Wright PE, Rance M. 1991. Sensitivity improvement in proton-detected two-dimensional heteronuclear correlation NMR spectroscopy. *J Magn Reson 93*:151–170.

Roder H, Wagner G, Wuthrich K. 1985. Amide proton exchange in proteins by EX1 kinetics: Studies of the basic pancreatic tyrosine inhibitor at variable $p^{2}H$ and temperature. *Biochemistry 24*:7396–7407.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning 3*. Coldspring Harbor Press: New York. p A3.

Schmid FX. 1992. In: Creighton TE, ed. *Protein folding*. New York: Freeman. pp 197–239.

Segawa S, Sugihara M. 1984. Characterization of the transition state of lysozyme unfolding. I. Effect of protein-solvent interaction on the transition state. *Biopolymers 23*:2437–2488.

Serrano L, Matouschek A, Fersht AR. 1992. The folding of an enzyme. III. Structure of the transition state for unfolding of barnase analyzed by a protein engineering procedure. *J Mol Biol 224*:805–818.

Wikstrom M. 1996. Backbone dynamics of an immunoglobulin light chain-binding domain of protein L. *Eur J Biochem* (in press).

Wikstrom M, Drabenberg T, Forsen S, Sjobring U, Kaster W, Bjorck L. 1994. Three dimensional solution structure of an immunoglobulin light chain-binding domain of protein L. Comparison with the IgG-binding domains of protein G. *Biochemistry 33*:14011–14017.

Wikstrom M, Sjobring U, Kastern W, Bjorck L, Drabenberg T, Forsen S. 1993. Proton nuclear magnetic resonance sequential assignments and secondary structure of an immunoglobin light chain-binding domain of protein L. *Biochemistry 32*:3381–3386.