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Hydrogen Exchange in Native and Denatured States of Hen Egg-White Lysozyme

Sheena E. Radford,[1] Matthias Buck,[1] Karen D. Topping,[1] Christopher M. Dobson,[1] and Philip A. Evans[2]
[1]*Inorganic Chemistry Laboratory and Oxford Centre for Molecular Sciences, University of Oxford, Oxford, OX1 3QR, England; and* [2]*Department of Biochemistry and Cambridge Centre for Molecular Recognition, University of Cambridge, Cambridge CB2 1QW, England*

ABSTRACT **The hydrogen exchange kinetics of 68 individual amide protons in the native state of hen lysozyme have been measured at pH 7.5 and 30°C by 2D NMR methods. These constitute the most protected subset of amides, with exchange half lives some 10^5–10^7 times longer than anticipated from studies of small model peptides. The observed distribution of rates under these conditions can be rationalized to a large extent in terms of the hydrogen bonding of individual amides and their burial from bulk solvent. Exchange rates have also been measured in a reversibly denatured state of lysozyme; this was made possible under very mild conditions, pH 2.0 35°C, by lowering the stability of the native state through selective cleavage of the Cys-6–Cys-127 disulfide cross-link (CM^{6-127} lysozyme). In this state the exchange rates for the majority of amides approach, within a factor of 5, the values anticipated from small model peptides. For a few amides, however, there is evidence for significant retardation (up to nearly 20-fold) relative to the predicted rates. The pattern of protection observed under these conditions does not reflect the behavior of the protein under strongly native conditions, suggesting that regions of native-like structure do not persist significantly in the denatured state of CM^{6-127} lysozyme. The pattern of exchange rates from the native protein at high temperature, pH 3.8 69°C, resembles that of the acid-denatured state, suggesting that under these conditions the exchange kinetics are dominated by transient global unfolding. The rates of folding and unfolding under these conditions were determined independently by magnetization transfer NMR methods, enabling the intrinsic exchange rates from the denatured state to be deduced on the basis of this model, under conditions where the predominant equilibrium species is the native state. Again, in the case of most amides these rates showed only limited deviation from those predicted by a simple random coil model. This reinforces the view that these denatured states of lysozyme have little persistent residual order and contrasts with the behavior found for compact partially folded states of proteins, including an intermediate detected transiently during the refolding of hen lysozyme.**

Key words: protein folding, denatured states, hydrogen exchange, NMR

INTRODUCTION

The kinetics of hydrogen exchange have become increasingly important as a means of characterizing the structure and dynamics of proteins. In native globular proteins there has long been an interest in the dynamic processes by which labile hydrogens, ordinarily buried in the structural core, become accessible to solvent water permitting hydrogen exchange to occur.[1–3] More recently, the utility of hydrogen exchange as a probe for residual specifically stabilized structure in nonnative states, including transient folding intermediates, has become established.[4–6]

The mechanisms of exchange in native proteins are still not well understood. In the case of a number of proteins, including hen egg-white lysozyme, it has been observed that the dominant exchange mechanism for many of the more highly protected hydrogens changes with temperature.[7,8] Under conditions remote from those of denaturation, exchange is often mediated by low activation energy processes and it is supposed that these reflect localized fluctuations of the structure. Closer to the denaturation transition, however, exchange may become dominated by larger scale fluctuations of much higher activation energy which are of considerable interest in relation to protein folding.[7–10]

In the case of nonnative states of proteins, exchange kinetics have in some instances been observed to resemble rather closely those anticipated

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Address reprint requests to Dr. Christopher M. Dobson, Inorganic Chemistry Laboratory, Oxford Centre for Molecular Sciences, University of Oxford, South Parks Road, Oxford OX1 3QR, England.

for a random coil polypeptide, as might be expected on the basis of a simple all-or-none folding model.[11,12] In other cases, however, it has been possible to detect specific protection from exchange of individual amides both in compact denatured states at equilibrium,[13–15] and in transient refolding intermediates.[16–19] The existence of elements of structure in compact denatured proteins which is thus revealed is of great interest in relation to the protein folding problem, having implications both for the organization of the native structure and for the early events in protein folding pathways. In the case of lysozyme, differential rates of protection of hydrogens have been observed during the refolding of the protein, indicative of a transient population of partially structured folding intermediates.[19] In the case of the related protein α-lactalbumin, protection from exchange of groups of amide hydrogens has revealed the persistence of specific elements of secondary structure in the acid-denatured state.[13,20]

In the present paper we compare the patterns of amide exchange kinetics observed in lysozyme under a variety of conditions. Under strongly native conditions, similar to those under which the pattern of protection during the refolding process was previously investigated,[19] the rates of exchange of over half of the 126 amide hydrogens were determined. This extends considerably our previous qualitative examination of the exchange properties of hen lysozyme.[21] Although under a single set of conditions a full interpretation of the hydrogen exchange kinetics cannot be deduced, the data are of particular importance since the pattern of exchange kinetics can be interpreted in terms of structural protection afforded by the native conformation. This pattern of exchange kinetics is compared with that observed for a denatured state under comparably mild conditions; this was made possible by selective cleavage of the 6–127 disulfide bridge in lysozyme which considerably diminishes the stability of the native conformation.[22] The pattern of exchange kinetics from the native state when it is only marginally stable relative to thermal denaturation is then considered. These exchange rates are interpreted in terms of the kinetics of transient global unfolding, which are independently measurable by NMR methods, and used to determine the intrinsic exchange rates from the unfolded state. In this way we have been able to use hydrogen exchange to probe the unfolded state under conditions where it is interconverting with a predominant population of the native state.

MATERIALS AND METHODS

Hen egg-white lysozyme was obtained from Sigma and further purified by extensive dialysis against distilled water at pH 3.0 before use. CM^{6-127} lysozyme was prepared as described previously.[22]

Amide NH Exchange in the Native State at pH 7.5, 30°C

Exchange was carried out at 30°C in a D_2O incubation mixture containing 0.1 M NaCl, 3 mM sodium azide, and 20 mM sodium phosphate buffer, pH 7.5. pH meter readings, calibrated against standard buffers (Aldrich), were not corrected for isotope effects and remained constant to within ±0.1 pH units throughout the experiment. Hydrogen exchange was initiated by dissolution of lyophilized protein in the exchange buffer to give a protein concentration of 2.5 mg/ml (0.17 mM). This protein concentration was chosen so that aggregation of lysozyme was negligible (<5%) under these conditions.[23,24] This was confirmed by the observation that exchange rates were identical when selected time points were repeated at a lower protein concentration of 0.25 mg/ml. Sixteen samples were withdrawn from the incubation mixture after time intervals ranging from 40 sec to 70 days and the exchange quenched by addition of 2.3 ml of 240 mM deuterated sodium acetate buffer, pH 3.6, giving a final pH of 3.8. Samples were concentrated 30-fold by ultrafiltration at 4°C to give a final protein concentration of 5 mM. Samples were stored at either 4°C (for short intervals) or −20°C (for longer intervals) before acquisition of the NMR spectrum.

Amide NH Exchange in Acid-Denatured CM^{6-127} Lysozyme

A solution of CM^{6-127} lysozyme (0.25 mM) was prepared in D_2O at 35°C and the pH immediately lowered to 2.0. Under these conditions the protein is reversibly denatured with negligible aggregation.[22] After various times samples were taken and hydrogen exchange quenched by rapid refolding of the protein on raising the pH to 3.8. The samples were then concentrated by lyophilization followed by redissolution in 0.5 ml D_2O, immediately before acquisition of a COSY spectrum.

Equilibrium and Kinetics of Reversible Thermal Denaturation

A solution of lysozyme (6 mM), freshly dissolved in 200 mM deuterated sodium acetate buffer in D_2O at pH 3.8, was prepared in bulk. The thermal denaturation transition was followed by taking an aliquot and measuring intensity changes of resolved native state ^1H NMR resonances, as described previously.[8,25] The rate of unfolding was determined by analysis of the time dependence of magnetization transfer between corresponding resonances of the native and denatured states in presaturation-inversion NMR experiments[25]; the resonances of H15 $H^{\epsilon 1}$ and C64 H^{α} were used for this purpose. On the basis of these experiments a temperature (69°C) was selected at which the unfolding rate could be determined reliably but where the majority of the protein

(>90%) was in the folded state. These conditions were then employed for the hydrogen exchange study. In order to ensure the compatibility of the thermodynamics and the hydrogen exchange data the temperature controller of the NMR spectrometer was checked carefully by undertaking melting point determinations of *p*-toluenesulfonyl chloride (68°C) and naphthalene (80°C) inside and outside the probe. In this way we were able to achieve a confidence of ±0.5°C in the actual probe temperature.

Amide NH Exchange at pH 3.8, 69°C

Fresh samples (0.5 ml) of the lysozyme solution used for the thermodynamic and kinetic measurements described above were taken and incubated in a water bath at 69°C. After varying lengths of time, hydrogen exchange was quenched by immersion of the sample in an ice-water bath. The samples were then stored at −20°C until the NMR spectra were recorded. Additional experiments were performed at a lower protein concentration (~0.5 mM) to confirm that the exchange rates were not significantly perturbed by intermolecular interactions under these conditions.

NMR Spectroscopy

NMR experiments were performed using the 500 MHz GE/Nicolet and Bruker spectrometers belonging to the Oxford Centre for Molecular Sciences. Phase sensitivite COSY spectra were acquired on the former, using the States–Haberkorn-Ruben method[26] and on the latter using TPPI.[27] The spectra were taken at 30°C in the case of the lysozyme sample which had been subjected to high temperature hydrogen exchange and at 35°C in the case of the native enzyme and CM^{6-127} lysozyme which had been subjected to acid denaturation. Data sets comprised 256 × 2,048 data points in the frequency domain. The intensity of each NH–C_αH cross peak was taken to be the average of the heights (neglecting sign) of the four components of the cross peak, and were normalized by comparison with the intensities of correlations between nonexchangeable protons, either W63 C7H-C6H and W108 C4H-C5H or Y23 C3,5H–C2,6H and Y53 C3,5H–C2,6H. These intensities were then normalized with respect to those measured for a control sample in which exchange of protected amide protons was negligible. This sample was obtained by dissolving lysozyme in D_2O at pH 3.8 and immediately acquiring a COSY spectrum.

RESULTS

Hydrogen Exchange at 30°C, pH 7.5

The rates of exchange of 68 amides were measured at pH 7.5, 30°C. The data are listed in Table I and the half-lives for exchange are shown versus the protein sequence in Figure 1. Rates of exchange for all of the amides measured here are extremely slow; values for the half-life vary from 1 to 10^5 min, in comparison with a theoretical half-life of about 10^{-4} min, calculated for small model peptides under these conditions.[28,29] Indeed, for two amides (Met-12 and Val-29) only upper limits for the exchange rates could be estimated since exchange of the amides was negligible, even after 70 days at pH 7.5 and 30°C. The remaining amides exchanged too quickly to be measurable in this experiment.

In order to compare the exchange properties of individual amides in the native protein (and, indeed, of the same amide in different folded states) protection factors were calculated. These are defined as $P = k_p/k_{ex}$, where k_{ex} is the measured exchange rate and k_p is the predicted exchange rate for each amide hydrogen in small unstructured peptides under the same conditions, based on data for the pH, temperature, and sequence dependence of amide exchange.[28,29] The protection factors measured for the native enzyme are shown as a function of the protein sequence in Figure 2.

Amide hydrogens in lysozyme have previously been classified into four categories (I–IV) based upon their exchange behaviour in solution at pH 4.2 and 7.5.[21] Amides in class I and two members of class II (Arg-14 and Ser-85) correspond to those which exchanged too fast to be measured under the conditions of the present study. Protection factors for the majority of amides for which rates were obtained ranged from 10^4 to 10^8, consistent with their previous classification into the exchange categories II–IV.

Hydrogen Exchange Kinetics in Acid-Denatured CM^{6-127} Lysozyme

Exchange rates were measured for 41 amides at pH 2.0 and 35°C; these are listed in Table I and the half-lives shown versus the protein sequence in Figure 1. The protection factors given by the ratio of these experimental and predicted rates are shown versus the protein sequence in Figure 2. It is apparent that many amides exchange at rates very similar to those predicted by the random coil model; the majority have protection factors close to 1. However, for nine amides (Asp-52, Tyr-53, Leu-56, Ile-58, Trp-63, Ile-78, Trp-108, Trp-111, Ile-124), the rate of exchange is significantly (more than 5-fold) slower than predicted.

Reversible Thermal Denaturation

The midpoint of the denaturation transition of lysozyme at pH 3.8 was determined to be 74.8 ± 0.5°C and the enthalpy of denaturation (effectively constant over the narrow observable temperature range) was determined from a Van't Hoff plot to be 392 ± 30 kJ mol^{-1}. At 69°C the equilibrium constant, $K_{eq} = [U]/[N]$, is equal to 0.10 ± 0.03, indicating that about 90% of the protein is in the native state under these conditions.

TABLE I. Exchange Kinetics of Amide Hydrogens in Lysozyme

	30°C, pH 7.5 k_{ex} (s^{-1})	69°C, pH 3.8 $10^2 \cdot k_{ex}$ (s^{-1})	69°C, pH 3.8 k_3 (s^{-1})	CM^{6-127} lysozyme 35°C, pH 2.0 $10^3 \cdot k_{ex}$ (s^{-1})
Phe-3	1.28×10^{-2}	1.76	0.25 (0.18,0.40)	
Leu-8	5.38×10^{-5}	0.96	0.12 (0.09,0.17)	3.59
Ala-9	7.58×10^{-6}	0.85	0.10 (0.08,0.15)	5.45
Ala-10	2.65×10^{-6}	1.07	0.13 (0.10,0.19)	8.74*
Ala-11	4.90×10^{-5}	0.90	0.11 (0.08,0.16)	
Met-12	8.33×10^{-8}	1.10	0.13 (0.10,0.19)	8.74*
Lys-13	2.25×10^{-5}	1.55	0.21 (0.15,0.33)	2.93*
His-15	1.39×10^{-3}			
Leu-17	2.87×10^{-3}	1.70	0.24 (0.17,0.37)	
Tyr-23	3.09×10^{-3}	1.72	0.24 (0.17,0.37)	7.85
Asn-27	1.28×10^{-4}	2.73	0.44 (0.29,0.74)	
Trp-28	4.39×10^{-7}	0.90	0.11 (0.08,0.16)	1.65
Val-29	8.33×10^{-7}	0.23	0.02 (0.02,0.03)	1.23
Cys-30	8.33×10^{-8}			
Ala-31	4.07×10^{-7}			3.64*
Ala-32	1.39×10^{-6}			2.97
Lys-33	1.39×10^{-6}			
Phe-34	2.87×10^{-5}	0.98	0.12 (0.09,0.17)	3.21
Glu-35	1.39×10^{-3}	1.59	0.21 (0.15,0.33)	
Ser-36	2.92×10^{-3}	2.27	0.35 (0.23,0.57)	
Asn-37	1.87×10^{-3}			
Phe-38	3.14×10^{-4}	0.91	0.11 (0.08,0.16)	
Asn-39	5.50×10^{-5}	1.75	0.24 (0.17,0.37)	2.55
Thr-40	2.06×10^{-3}	1.73	0.24 (0.17,0.37)	
Gln-41	1.28×10^{-2}	1.57	0.21 (0.15,0.33)	2.69*
Ala-42	2.56×10^{-4}	1.35	0.18 (0.13,0.27)	
Asn-44	6.67×10^{-4}	1.97	0.29 (0.20,0.48)	1.69
Asn-46	5.56×10^{-3}	3.22	0.56 (0.37,0.93)	7.43
Ser-50	6.67×10^{-3}	1.70	0.23 (0.17,0.37)	2.62
Thr-51	9.80×10^{-3}	1.82	0.26 (0.18,0.41)	3.34
Asp-52	2.49×10^{-6}	3.50	0.65 (0.42,1.10)	0.61
Tyr-53	1.28×10^{-6}			0.77
Gly-54	1.04×10^{-6}			
Ile-55	4.07×10^{-3}			
Leu-56	3.97×10^{-4}	0.38	0.04 (0.03,0.06)	1.11
Gln-57	1.79×10^{-6}	0.65	0.08 (0.06,0.12)	1.79
Ile-58	7.58×10^{-7}	0.19	0.02 (0.02,0.03)	1.39
Asn-59	1.28×10^{-3}			
Ser-60	2.08×10^{-6}			
Arg-61	6.94×10^{-5}	1.42	0.18 (0.13,0.27)	1.59
Trp-63	1.11×10^{-4}	0.21	0.02 (0.02,0.03)	0.63
Cys-64	1.75×10^{-6}	0.65	0.07 (0.05,0.10)	1.35
Asn-65	1.28×10^{-4}	1.62	0.22 (0.16,0.35)	1.08
Leu-75	5.95×10^{-4}			
Cys-76	1.70×10^{-4}	1.53	0.20 (0.15,0.30)	2.05
Ile-78	3.62×10^{-4}	0.22	0.02 (0.02,0.03)	0.55
Cys-80	6.17×10^{-4}			
Ala-82	1.51×10^{-2}	1.39	0.18 (0.13,0.27)	3.59
Leu-83	9.26×10^{-6}	0.90	0.11 (0.08,0.16)	2.09
Leu-84	4.39×10^{-3}	0.38	0.04 (0.03,0.06)	2.35
Ser-85		0.96	0.12 (0.09,0.17)	
Val-92	8.77×10^{-5}	0.55	0.06 (0.05,0.08)	0.99
Asn-93	3.03×10^{-3}			
Cys-94	3.33×10^{-3}	1.90	0.28 (0.19,0.45)	4.69*
Ala-95	5.75×10^{-8}			
Lys-96	8.33×10^{-8}			2.74
Lys-97	6.17×10^{-5}	1.47	0.20 (0.15,0.30)	1.74
Ile-98	6.94×10^{-7}			

(*continued*)

TABLE I. Exchange Kinetics of Amide Hydrogens in Lysozyme (Continued)

	30°C, pH 7.5 k_{ex} (s^{-1})	69°C, pH 3.8 $10^2 \cdot k_{ex}$ (s^{-1})	69°C, pH 3.8 k_3 (s^{-1})	CM^{6-127} lysozyme 35°C, pH 2.0 $10^3 \cdot k_{ex}$ (s^{-1})
Val-99	2.73×10^{-6}	0.30	0.00 (0.03,0.05)	1.76
Ser-100	4.76×10^{-3}			
Trp-108	1.11×10^{-3}			0.78
Trp-111	4.07×10^{-4}	0.66	0.08 (0.06,0.12)	0.63
Arg-112	2.11×10^{-3}			1.83*
Arg-114	1.28×10^{-2}			
Cys-115	2.49×10^{-3}	1.53	0.20 (0.15,0.30)	1.66
Trp-123	1.19×10^{-3}	0.70	0.08 (0.06,0.12)	
Ile-124	5.05×10^{-4}			0.74
Arg-125	4.50×10^{-3}	0.85	0.10 (0.08,0.15)	

*Estimated value; exchange was too fast for rates to be accurately measured (less than four time points defined the curve). Numbers in parentheses denote the minimum and maximum values for k_3 estimated from the errors in determining k_1 and k_2.

The rate of unfolding, k_1, for both C64 H^{α} and H15 $H^{\epsilon 1}$ at 69°C was determined to be 0.08 ± 0.04 s^{-1} from magnetization transfer measurements. These residues lie in different lobes of the lysozyme structure and confirm that the unfolding process is a highly cooperative event. The folding rate, k_2, was then determined by comparison with the equilibrium constant ($K_{eq} = k_1/k_2$) to be 0.8 ± 0.5 s^{-1}.

Hydrogen Exchange Kinetics at 69°C

Exchange rates were measurable for 46 amides (Table I). These rates vary very much less widely than was the case at lower temperature and in this way resemble much more closely the pattern observed for the acid denatured state. This suggests that under these conditions exchange is probably associated with reversible global unfolding. If we make this assumption, exchange can be described by the following scheme:

$$H_n \underset{k_2}{\overset{k_1}{\rightleftharpoons}} H_d \xrightarrow{k_3} D.$$

Here, H denotes an unexchanged amide proton and D an amide where the proton has been replaced by a deuteron through exchange with solvent. The subscripts n and d refer to the native and denatured states, respectively. Since the reaction occurs in an overwhelming excess of solvent deuterons (>99.8% D_2O) exchange is effectively irreversible. The rate constants k_1 and k_2 are the unfolding and refolding rates while k_3 is, according to this model, the exchange rate from the denatured protein. The dependence of the overall hydrogen exchange rate, k_{ex}, on these three rate constants can be expressed generally by[1]

$$k_{ex} = \frac{k_1+k_2+k_3-[(k_1+k_2+k_3)^2-4k_1k_3]^{1/2}}{2}.$$

Since we have determined k_{ex}, k_1 and k_2 experimentally, as described above, it is possible to use this expression to calculate apparent exchange rates (k_3) from the denatured state; the results are listed in Table I and the half-lives for exchange shown, as a function of the protein sequence, in Figure 1.

Protection factors in the denatured state under these conditions were calculated and compared with those observed in the acid denatured CM^{6-127} lysozyme species (Fig. 2). Again, it is clear that the protection factors are relatively small for all but two amides the experimental rates are within an order of magnitude of the predicted values for a random coil. The protection factors in the acid-denatured state are, on average, somewhat smaller than those obtained for the thermally denatured enzyme but there is some similarity between the two profiles. In both cases the majority of amides with significant protection factors ($k_p/k_{ex} > 5$) occur in a central part of the sequence—approximately between residues 50 and 85, while in the N-terminal region of the sequence (residues 1–20), there is a marked absence of significant protection factors. At an individual residue level the agreement between the distributions is, however, limited. Thus, the amide of Cys-64 exchanges significantly more slowly in the thermally denatured state, whereas that of Asp-52 is much more slowly exchanging in CM^{6-127} lysozyme. The one marked exception to this is the amide of Ile-78, which has the largest individual protection factor in both denatured states.

DISCUSSION

Pattern of Exchange Kinetics Under Strongly Native Conditions

The protection factors of between 10^4 and 10^8, shown in Figure 2, emphasize both that the exchange rates of protected amide hydrogens in lysozyme at pH 7.5, 30°C are very slow and that they

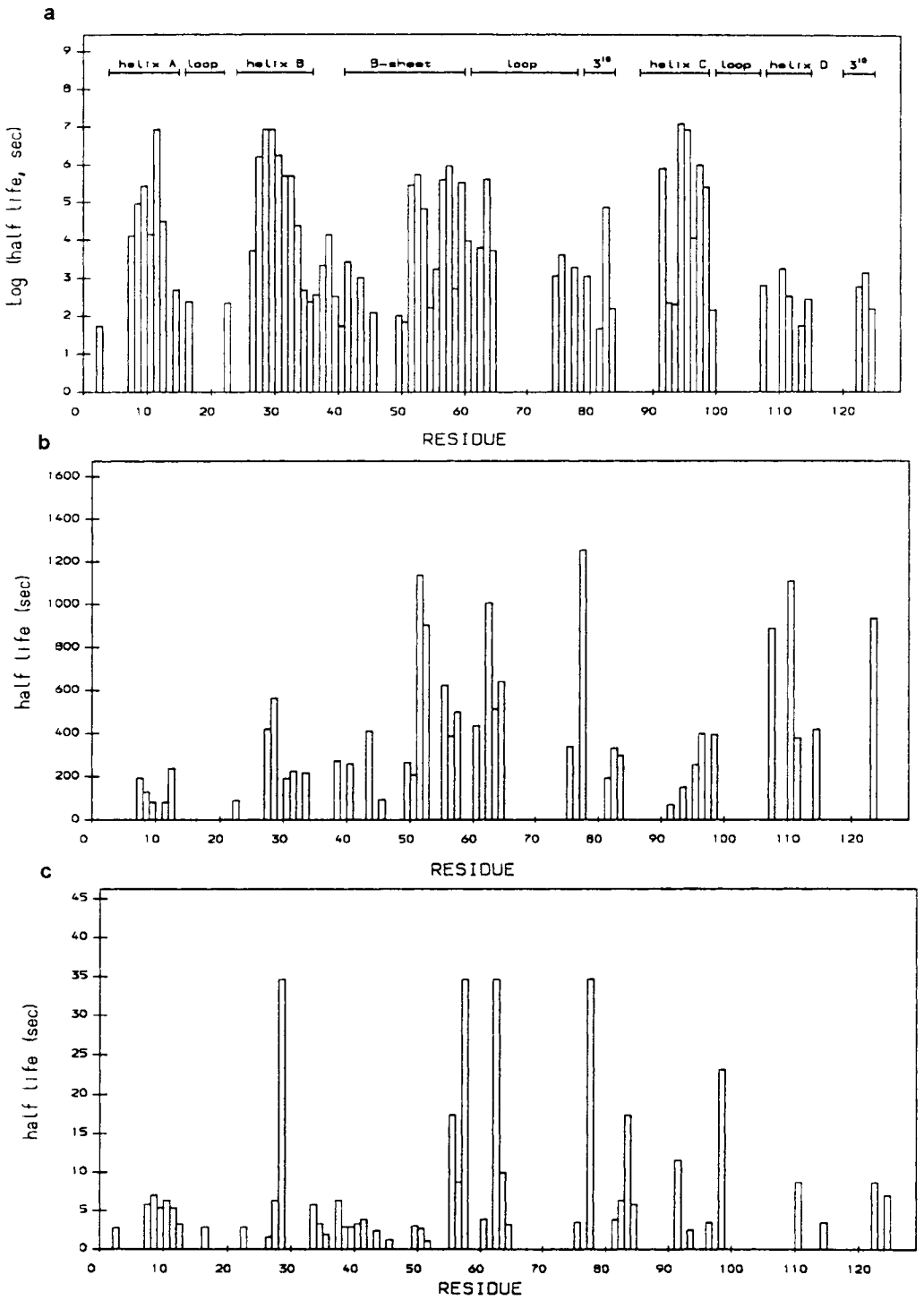

Fig. 1. Profile of the half-life for hydrogen exchange versus the protein sequence. (**a**) pH 7.5, 30°C, (**b**) pH 2, 35°C (CM^{6-127} lysozyme), and (**c**) pH 3.8, 69°C. The location of elements of structure in the native protein are shown in **a**. Note the scale in **a** is logarithmic.

vary widely. This implies that the mechanism by which these amides become transiently exposed to solvent, permitting exchange to occur, is not a cooperative unfolding of the structure under these conditions but rather that localized fluctuations, of widely differing frequency, are involved. Only for

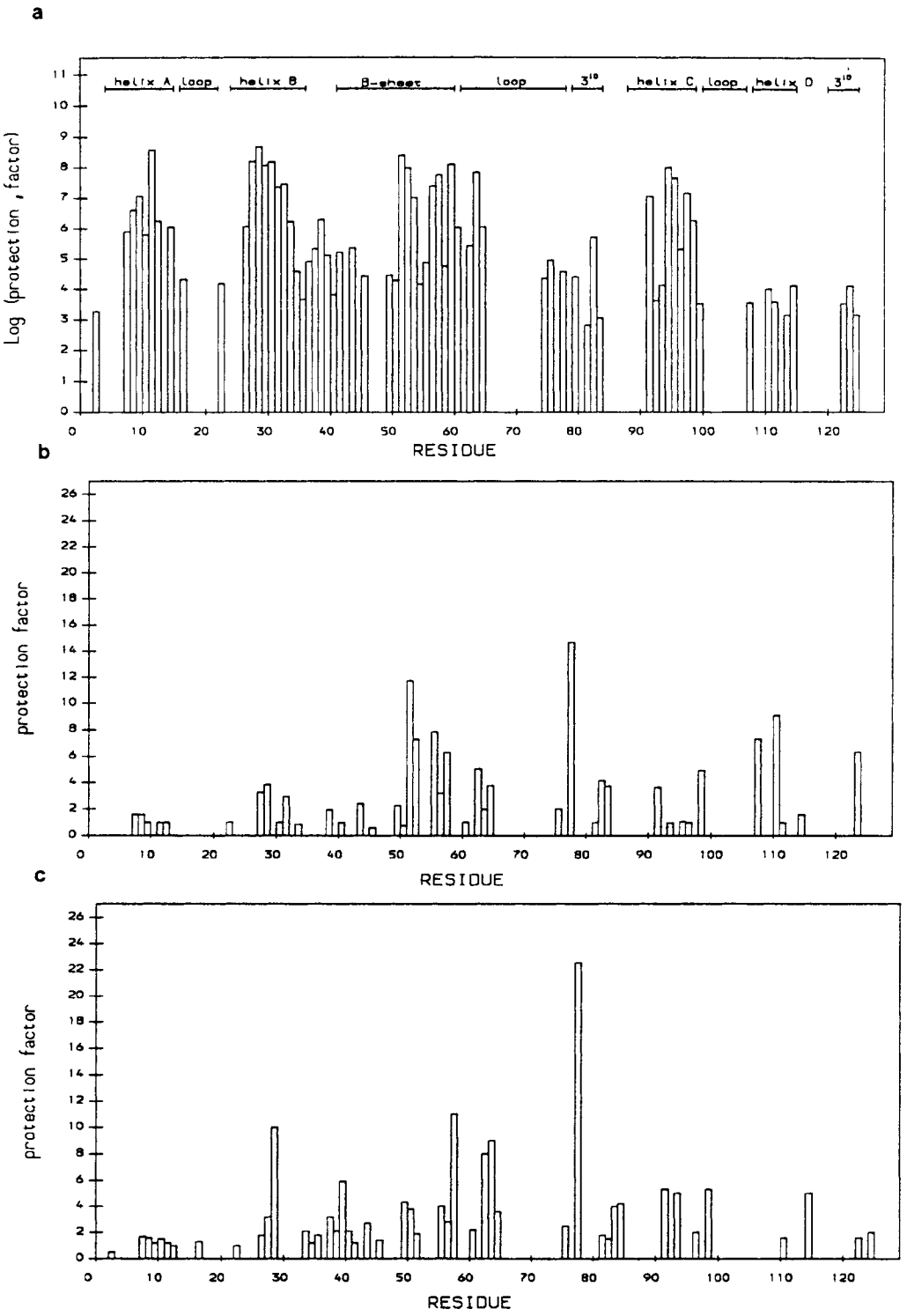

Fig. 2. Profile of protection factors versus the protein sequence. (**a**) pH 7.5, 30°C, (**b**) pH 2, 35°C (CM^{6-127} lysozyme), and (**c**) pH 3.8, 69°C. The location of elements of structure in the native protein are shown in **a**. Note the scale in **a** is logarithmic.

the slowest exchanging amides of all, for which rates were too slow to measure under the present conditions, does the possibility remain that exchange occurs by a global unfolding mechanism. It is thus of considerable interest to look for relationships between the distribution of exchange rates and features of the folded structure of the protein. Lysozyme is an $\alpha+\beta$ protein, comprising two structural lobes.[30] One is constructed largely from helical backbone segments enclosing a large hydrophobic core; the other includes a rather distorted three stranded β-sheet and an extended loop of irregular structure.

It is clear from Figure 2 that there is a close correlation, under these conditions, between slow amide exchange and participation of the particular amide in a region of secondary structure. Thus, the majority of amides in helical or β-sheet structures have protection factors exceeding 10^5. In general, exchange of amides in short, irregular helical regions is faster than that for amides which occur in more regular and extensive segments of secondary structure; for example, the short D helix (encompassing residues 108–115) is much more weakly protected than the three longer α-helices (residues 4–15, 24–36, and 88–99, respectively); protection factors in helix D were some 100-fold smaller than those seen for residues in helices A, B, and C. In addition, the two short segments of 3^{10} helix which are located in opposite lobes of the lysozyme structure have similar protection factors to each other and to the D-helix.

It is interesting to note that there are pronounced differences in exchange rate within individual helices; protection at the extremities of the helices is, in general, smaller than that at the center. Thus, for example, the four slowest exchanging amides of all (Met-12, Val-29, Ala-95, and Lys-96) lie in the center of their respective helices. In addition, amides which line the exposed side of amphipathic helices exchange much more rapidly than those which abut the hydrophobic core of the enzyme. Thus residues Val-92, Ala-95, and Ile-98 in helix C exchange much more slowly than those along the opposite face (Asn-93, Cys-94, and Lys-97). Such a pattern is not observed for helix B, which is the most deeply buried and hydrophobic helix of all; in this case all amides exchange relatively slowly. It is apparent, therefore, that individual helices do not unfold as cooperative elements to permit exchange under these conditions; rather the dominant exchange mechanism seems to involve much smaller scale structural fluctuations, the rates of which vary widely depending on the details of the structural context of the individual hydrogen bonded amide. The nature of the fluctuations, however, is not known.[1–3]

Protection factors for amides in the triple stranded β-sheet (residues 41–60) are similar to those in the four α-helical segments. This is interesting, since these lie in opposite lobes of the lysozyme molecule and have been shown to be stabilized independently on the folding pathway.[19] As discussed above, the widely disparate rates observed for different amides indicate that their exchange is mediated by independent, small scale fluctuations. The implication is, therefore, that the energy required to bring about these structural distortions is similar in the most stable regions of the α and β domains. A second, much shorter double stranded β-sheet is formed between residues 1–3 and 38–40 in native lysozyme. Though this is stabilized by only two hydrogen bonds, the amides of Phe-38, Asn-39, and Thr-40 have protection factors exceeding 10^5, while that of Phe-3 is about 10^3. Clearly participation in hydrogen bonding is of fundamental importance in determining the rate of hydrogen exchange. The majority of amides not involved in elements of secondary structure exchange rapidly under these conditions and an upper limit for their protection must be about 10^2. Significant exceptions to this relationship are, however, found to exist. Four amides (Trp-63, Leu-75, Cys-76, and Ile-78) are involved in a region of the protein which is irregular in structure yet are also highly protected from exchange (by factors greater than 10^5). Two of these amides (Leu-75 and Cys-76) form main chain hydrogen bonds in the native enzyme. The exchange properties of the remaining two amides cannot, however, be rationalized in this manner, Ile-78 forms only a long and weak hydrogen bond to the side chain of Asn-74 and Cys-64 is not identified in the X-ray structure as forming a hydrogen bond at all.

Proximity of the amide nitrogen to surface water has also been used to rationalize the exchange properties of amides in the native states of several proteins.[31] The overall relationship between the rate of exchange and surface proximity, estimated as the distance to the nearest accessible water molecule,[21] is shown in Figure 3. The six amides (Trp-28–Ala-32, inclusive and Cys-64) most deeply buried in the core of the enzyme are among those with the slowest rates of exchange. On the other hand several amides (e.g., Ala-11 and Lys-97) lie in regions of secondary structure quite close to the surface of the enzyme and yet exchange quite slowly, while a number of others (Thr-40, Gln-41, Ser-50, Thr-51, and Arg-112) lie at intermediate distance from the nearest accessible water molecule yet have some of the fastest rates of exchange. The hydrophobic core of lysozyme is formed essentially from the side chains of Leu-17, Tyr-20, Tyr-23, Trp-28, Ile-98, Met-105, Trp-108, and Trp-111. Interestingly, the amides of only two of these residues (Trp-28 and Ile-98) show protection factors exceeding 10^5 and both of these lie in helical elements of structure and are quite deeply buried from the solvent. Two of the remaining six amides (Tyr-20 and Met-105) exchange at a rate too fast to measure under the con-

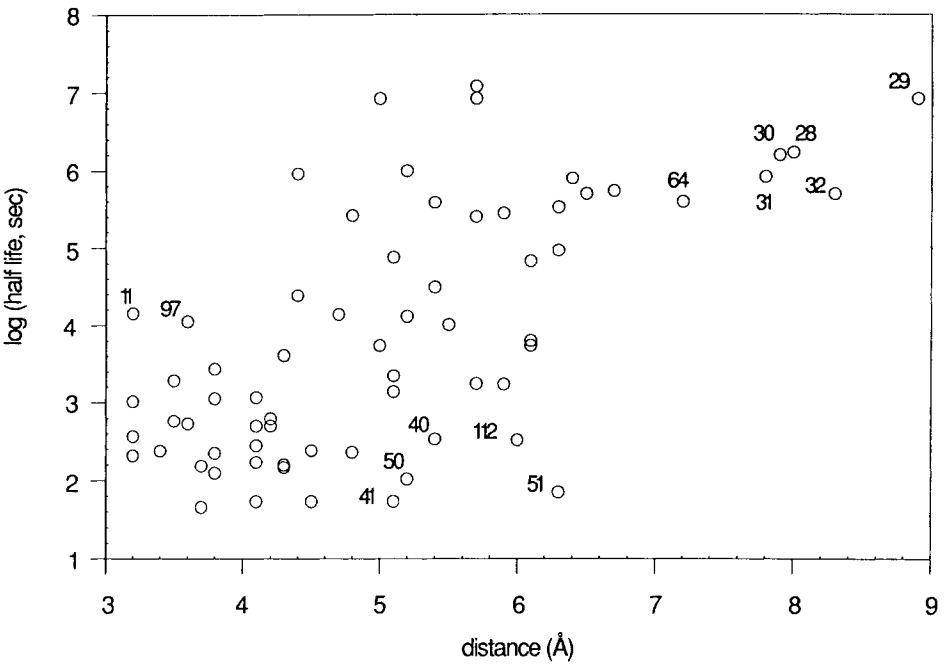

Fig. 3. Relationship between the half-life for amide exchange in the native state of lysozyme (pH 7.5, 30°C) and surface proximity, estimated as the distance of the individual amide nitrogen to the nearest accessible water molecule.[21] Amides of particular interest are labeled.

ditions employed. Involvement of the side chain in a hydrophobic environment, apparently, is not itself sufficient to retard significantly the rate of hydrogen exchange of the backbone. The pattern of amide protection also shows no obvious correlation with the distribution of hydrogen bond energies, the *B* factors estimated from the crystal structure or the temperature dependence of the amide hydrogen chemical shifts in the NMR spectrum (Itzhaki, Redfield, and Dobson, unpublished data). The results indicate, therefore, that the rate of exchange of the majority of amides cannot be rationalized merely by consideration either of the pattern of hydrogen bonding in the native structure or distance from the surface of the molecule alone. However, consideration of both the distribution of hydrogen bonds and burial of the amide from solvent can account for the exchange behavior of virtually all of the amides studied here.

Exchange Kinetics in Acid-Denatured CM^{6-127} Lysozyme

It is clear from Figure 2 that in the case of most amides in CM^{6-127} lysozyme the exchange rate is predicted rather well by a simple random coil model. This implies that, under these conditions, the differences in rate between different amides are dominated by the effects of side chains immediately adjacent in the sequence, with relatively small perturbations arising from conformational effects. This is in contrast to the case of the related protein α-lactalbumin. Under similar conditions, guinea pig α-lactalbumin exists in a compact denatured state in which a number of amides, in specific helical segments, are substantially protected from exchange.[13,20] Caution is needed in interpreting such differences, since hydrogen exchange is a rather insensitive method for detecting structural elements of marginal stability. Nonetheless we can conclude that there is, at least, no evidence for the persistence of substantial elements of residual structure in this denatured lysozyme, even under such mild conditions.

Mechanism of Hydrogen Exchange at Elevated Temperature

The distribution of exchange rates from the native state of lysozyme at high temperature is very narrow, resembling more closely that of the denatured protein rather than that of the native state at lower temperature. This suggests that the primary mechanism of exchange is fundamentally different at elevated temperature and that it is likely to be related to large-scale unfolding. The question then remains whether exchange occurs simply via overall denaturation of the molecule or whether other, subglobal fluctuations are important under these conditions of marginal stability. The simple model based on reversible global unfolding is supported by detailed analysis in terms of the independently measured kinetics of folding and unfolding. The apparent exchange rates from the denatured state, calculated

according to this model, are all comparable to or smaller than would be predicted for a random coil polypeptide and there is a clear, albeit imperfect, correlation with the predictions for exchange in a hypothetical random coil state (Fig. 2). Had there been a significant contribution from faster, subglobal unfolding processes we should have expected at least some of the exchange rates to have been faster than the global denaturation kinetics could account for; with the possible exception of Phe-3, this is evidently not the case. Overall, therefore, the evidence strongly suggests that transient global unfolding is the dominant exchange mechanism, demonstrating clearly the extreme cooperativity of folding under these conditions. Similarly, at elevated temperatures the exchange mechanism of ribonuclease A and BPTI is also thought to occur via global unfolding of the native molecules.[32,33] Thus, even though the two domains of lysozyme have been shown not to be stabilized simultaneously during the refolding of the protein under strongly native conditions,[19] such noncooperativity is not detectable in the unfolding fluctuations of the protein under conditions where the native state is marginally stable. This conclusion is also supported by coincidence of the rates of folding and unfolding measured by magnetization transfer NMR for a number of protons distributed through the structure.[25] This interpretation has enabled us to deduce from the exchange data the intrinsic rates of exchange from the denatured state under conditions where 90% of the protein is in the folded state.

Patterns of Exchange Kinetics in the Denatured States

The exchange rates measured in the acid-denatured state and deduced in the transient thermally unfolded state are nearly all within an order of magnitude of the values predicted from published data obtained from studies of small peptides.[28,29] This broadly supports the use of these data as a model of exchange behavior in unfolded proteins and suggests, in particular, that these denatured states of lysozyme do not retain extensive stable residual structure. Similar conclusions have been drawn in the cases of thermally unfolded BPTI[11] and ribonuclease A.[12,34] Nonetheless, discrepancies from the predicted values were measurable under both sets of conditions studied, suggesting either that there are limitations to the accuracy of the rates predicted from model compound data or that there is significant deviation from random coil behaviour in these denatured states.

The uncertainties involved in prediction of random coil rates could be significant, at least in interpreting the exchange kinetics of the denatured states, where protection factors are relatively small. In the case of the thermally denatured state, in particular, model compound data must be extrapolated over a large gap in temperature, which raises the question of how accurately the activation energies for the different catalytic processes are known.[28] In addition, the correction factors used to take account of sequence effects have only been measured experimentally for 9 of the 20 amino acids, factors for the remaining amino acids being estimates based on considerations of chemical similarity.[29] The validity of the latter approximation has been called into question in light of new measurements.[34] Thus, for example, the protection factors for the five Ala residues measured in the thermally denatured state are all approximately 1, while the three Val and two Ile residues all exchange significantly more slowly than predicted. Val and Ile side chains are assumed to have identical effects to Ala in the work of Molday et al.,[29] but if this were not the case then at least part of these discrepancies might be explained rather straightforwardly.

The uncertainty in predicting amide hydrogen exchange rates means that we are forced to be extremely cautious in attempting to attribute any perturbations to conformational effects, especially when such deviations are only small. A variety of experimental observations has suggested that there is deviation from random coil behavior in thermally denatured lysozyme.[35–38] The distribution of chemical shifts in proton NMR spectra suggests that there is a tendency for hydrophobic residues to cluster together in thermally denatured lysozyme.[39] Specifically, interactions with aromatic rings which, on average, appear to be much closer together than anticipated for a fully disordered state are thought to occur. In accord with this, photo CIDNP experiments have indicated that the side chains of Trp residues are apparently much less accessible to photoexcited flavin than expected for an unstructured polypeptide.[40] However, the rather limited shift perturbations observed in the proton NMR spectra suggest that these side chain interactions are nonspecific and highly averaged. It is not clear to what extent such structure would affect main chain hydrogen exchange rates. However, it is interesting in this regard that 9 out of 10 cases of protection factors greater than 5 in the thermally denatured state, and 8 out of 9 in the acid-denatured state, are for amides of hydrophobic residues. It is possible that partial exclusion of water in the vicinity of these residues could be associated with the observed rate retardations.

A possible correlation can also be discerned between diminished exchange rates and proximity to disulfide bridges in the denatured states. Thus, in the thermally denatured state 3 of the 10 amides with the highest protection factors are of cystine residues and four of the remainder are within two residues of a disulfide in the sequence. This is also consistent with the result of the chemical shift analysis described above[39] in which the importance of disul-

fide cross-links in stabilizing nonrandom interactions was clearly apparent.

Whatever the origin of the limited deviations from random coil predictions in the exchange kinetics of the denatured states, perhaps the most important conclusion is that there is no correlation between those amides which are significantly protected in the denatured state and the pattern of amide exchange or the presence of secondary structural elements in the native conformation. In addition, there is no obvious clustering of significantly protected amides, such as might be associated with the persistence of specific secondary structural features, native-like or otherwise. This supports the idea that any conformational preferences which do persist in denatured lysozyme are not related in any simple way to the native structure. This is in spite of our endeavors to find particularly mild conditions under which to study denatured lysozyme and differs markedly from observations on a number of compact denatured states of proteins, including apomyoglobin, cytochrome *c* and α-lactalbumin, which give rise to compact denatured states with well-defined secondary structure.[13–15] The difference in behavior of lysozyme and α-lactalbumin is particularly interesting since these are homologous proteins. The results presented in this paper for acid-denatured CM^{6-127} lysozyme were obtained under conditions virtually identical to those under which specific residual structural features have been observed in the acid denatured states of α-lactalbumin, CM^{6-120} α-lactalbumin,[41] and fully reduced α-lactalbumin.[42] This shows that the different levels of residual structure are an intrinsic property of the denatured states and not simply a consequence of the milder conditions under which α-lactalbumin has hitherto been studied.

The patterns of protection also contrast strongly with that observed for intermediate species formed in the early stages in the refolding of lysozyme.[19] In the latter case, the majority of the more rapidly protected amides are those residing in α-helices in the native protein. The denatured states studied here bear little trace of protection in these regions. On the other hand, three further amides (Trp-63, Cys-64, and Ile-78) which lie in a region of irregular structure in the native protein are protected surprisingly rapidly during refolding and these are also significantly protected in thermally denatured lysozyme and, with the exception of Cys-64, in CM^{6-127} lysozyme. The case of Ile-78 is especially intriguing since in the native structure it is located close to the surface, apparently protected only by a rather long tertiary hydrogen bond. This seems to be a relatively unlikely candidate for an intrinsically stable interaction in a native or a partially ordered state. In the light of the present data it is possible that the protection of this amide, at least, is associated with structure characteristic of the denatured rather than the native state. This highlights the care which must be exercised in interpreting hydrogen exchange in transient intermediates in terms of structure in the native state, particularly if the patterns of protection do not straightforwardly point to characteristic native-like structural elements, since there will inevitably be some ambiguity as to whether the structure responsible is native-like or not.

Overall we can conclude that lysozyme illustrates rather clearly the extent to which different levels of stable structure can be discerned from patterns of exchange kinetics. At low temperature the native state exchange profile is entirely different from that observed at high temperature. This reflects the change in the dominant exchange mechanism from one mediated by local structural fluctuations, reflecting the details of the highly ordered native conformation, to one mediated by transient global unfolding. The latter mechanism has been verified by independent measurement of the rate of fluctuation involved and by observation of a similar profile of exchange kinetics to that observed in acid denatured CM^{6-127} lysozyme at lower temperature. The exchange of amides from these denatured states differs significantly in only a few cases from that anticipated for an unstructured polypeptide. This emphasizes very clearly, at least in the cases of the lysozyme folding intermediate and the compact denatured state of α-lactalbumin,[13,19] the validity of inferring native-like elements of structure from their hydrogen-exchange profiles.

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