Effects of Proline Mutations on the Unfolding and Refolding of Human Lysozyme: The Slow Refolding Kinetic Phase Does Not Result from Proline Cis–Trans Isomerization

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ABSTRACT: The unfolding and refolding kinetics of six proline mutants of the human lysozyme (h-lysozyme) were carried out and compared to that of the wild-type protein. Our results show that the slow refolding phase observed in the h-lysozyme refolding kinetics cannot be ascribed to proline isomerization reactions. The h-lysozyme contains two proline residues at positions 71 and 103, both in the trans conformation in the native state. The refolding kinetics of the P71G/P103G mutant, in which both prolines have been replaced by a glycine, were found to be similar to those of the wild-type protein. The same slow phase amplitude of about 10% was found for both proteins, and the slow phase rate constants were also identical within experimental error. Other mutants such as P103G or P71G, in which only one of the two prolines has been replaced by a glycine, and A47P with its three prolines, gave identical slow refolding phases. The X-ray structure analysis and scanning microcalorimetric study of each protein (Herning et al., unpublished experiments) have confirmed that none of the considered mutations affects significantly protein structure and that no major changes in protein stability were brought about by these mutations. Therefore, comparison of the properties of the mutant and wild-type proteins is legitimate. Interestingly, the refolding kinetics of the V110P mutant, in which a proline residue has been introduced at position 110 (N-terminus of an α-helix), were clearly triphasic. For this mutant an additional very slow phase with properties similar to those expected from the proline hypothesis was detected. Equilibrium denaturation studies were conducted for each protein, and the refolding pathway of h-lysozyme is partly presented. We also discuss the effect of proline mutations on the energetics of the folding pathway of the h-lysozyme in water.

Although it is generally accepted that the information required for an unfolded protein to reach spontaneously its unique three-dimensional native structure is, in most cases, all contained in its amino acid sequence (Anfinsen, 1973), we do not fully understand the folding mechanism for any protein. The folding process is driven by noncovalent interactions and usually proceeds through transient intermediates that cannot be isolated for characterization due to their short lifetime (Dolgikh et al., 1985; Gast et al., 1986). Although application of indirect methods such as trapping of disulfide intermediates (Creighton, 1978) may sometimes provide valuable structural information on these short-lived intermediates (Kim & Baldwin, 1982, 1990), perturbations caused by the introduction of chemical groups in the protein cannot be neglected (Goto & Hamaguchi, 1979; Chavez & Scheraga, 1980). In the last decade, much attention has been dedicated to proteins such as α-lactalbumin (Kuwajima et al., 1976, 1989) or cytochrome *c* (Robinson et al., 1983) due to their ability to assume an equilibrium intermediate conformational state when placed under mildly denaturing conditions. Such stable intermediates have been analyzed by various spectroscopic techniques (Dolgikh et al., 1981, 1985; Izumi et al., 1983; Ikeguchi et al., 1986a,b) and were found to be nearly as compact as the native state and to have a significant secondary structure content and a fluctuating tertiary structure characterized by important intramolecular motions. Due to its physical properties, the intermediate state was termed the molten globule (Ohgushi & Wada, 1983). More importantly, it was shown that the α-lactalbumin molten globule was similar to or identical with an intermediate transiently accumulated at an early stage of refolding from the denatured state (Ikeguchi et al., 1986b). To date, such intermediates are thought to be of general occurrence on the refolding pathway of proteins.

Another factor that renders the folding pathway of many proteins difficult to establish is the coexistence under denaturing conditions of multiple unfolded forms of polypeptide chains (Kim & Baldwin, 1982, 1990). Such species are assumed to refold at different rates, giving rise to additional slow refolding kinetic phases.

The "three-species model" was proposed several years ago to interpret the folding kinetics of pancreatic ribonuclease A (Garel & Baldwin, 1973). The rapid unfolding reaction ($N \rightarrow U_F$) of the native protein (N) is followed by slow isomerization reactions of the denatured polypeptide chains, which lead to an equilibrium distribution of fast refolding (U_F) and slow refolding (U_S) species: $N \rightarrow U_F \rightleftharpoons U_S$. This model has since been applied to explain the folding kinetics of many other proteins (Kato et al., 1982; Ramdas & Nall, 1986; Kiefhaber et al., 1990a).

The proline hypothesis was formulated, providing a plausible explanation for the molecular nature of these isomerizations (Brandts et al., 1975). According to this hypothesis, slow refolding species are produced by the cis–trans isomerization around X–Pro peptide bonds in the denatured polypeptide chain after unfolding. These molecules thus contain incorrect proline isomers and refold slowly because reisomerization is

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an intrinsically slow process with a high activation energy. The proline hypothesis has been tested on several proteins such as hen egg white lysozyme (Kato et al., 1982), a mouse light-chain immunoglobulin (Goto & Hamaguchi, 1982), *Escherichia coli* thioredoxin (Kelley & Stellwagen, 1984), yeast iso-1-cyt *c* (Ramdas & Nall, 1986), yeast iso-2-cyt *c* (White et al., 1987), RNase T1, and porcine RNase (Kiefhaber et al., 1990a; Grafl et al., 1986). Different approaches have been introduced to provide a direct test of the proline hypothesis such as isomer-specific proteolysis (Lin & Brandts, 1985), site-directed mutagenesis (Wood et al., 1988; Nall et al., 1989; Kiefhaber et al., 1990b), and enzymatic catalysis of proline isomerization (Lang et al., 1987). Although for some proteins such slow isomerization of the denatured polypeptide was indeed observed (Nall et al., 1978; Goto & Hamaguchi, 1982; Hurle & Matthews, 1987), in refolding the correspondence with the proline hypothesis predictions was often found to be rather poor (Nall et al., 1978; Goto & Hamaguchi, 1982; Schmid et al., 1986). Another corollary of the proline hypothesis is that the amount of U_s species and the complexity of slow refolding should increase with the number of prolines in the protein. This prediction was first tested by comparing the refolding kinetics of homologous parvalbumins, each containing a different number of prolines (Lin & Brandts, 1978). The results were consistent with the proline prediction, but their interpretation was complicated by the strong dissimilarity of the individual folding reactions of the related proteins. In the case of pancreatic RNases with four to six prolines, the poor agreement between experimental data and theoretical predictions was explained by the assumption that not all the prolines were essential to folding (Krebs et al., 1983). Recently, the role of specific prolines for rate-determining slow steps in folding has been clarified for RNase T1 (Kiehaber et al., 1990b) using site-directed mutagenesis. However, previous similar studies on other proteins gave results difficult to interpret due to major changes in both stability and folding kinetics brought about by the mutations (Wood et al., 1988; Nall et al., 1989).

Although the proline hypothesis appears to interpret satisfactorily the presence of slow refolding kinetics for some proteins, in many cases there is a lack of direct evidence for proline involvement in slow refolding processes. The results presented in this report do not support the proline hypothesis in the case of the refolding of human lysozyme (h-lysozyme). The refolding kinetics of h-lysozyme were found to be biphasic. Using a protein engineering approach, we have analyzed separately the role of each proline contained in the wild-type h-lysozyme. None of them was found to influence the slow refolding mechanism.

The h-lysozyme is a single-domain protein (130 amino acids, 4 disulfide bonds) that contains only two proline residues at positions 71 and 103. From X-ray results (Artymiuk & Blake, 1981), both prolines are in the trans conformation in the native state.

In order to investigate the role of specific prolines in the folding of h-lysozyme, the following mutants were designed: P71G (Pro71 is replaced by Gly), P103G (Pro103 is replaced by Gly), and P71G/103G (Pro71 and Pro103 are replaced by Gly). Other mutants such as A47P, D91P, and V110P (Ala47, Asp91, and Val110 are replaced by Pro, respectively) were also constructed to extend our study. Only one proline remains in the P71G and P103G mutants, and the P71G/P103G mutant is devoid of proline, whereas the A47P, D91P, and V110P mutants contain three proline residues. In the wild type, Pro71 and Pro103 are located in loop regions, Asp91 and Val110 are located in α-helices near the N-terminus, and Ala47 is in a β-turn. The h-lysozyme Pro mutants used in the present study have been characterized in detail (Herning et al., unpublished experiments), including a thermodynamic characterization by scanning microcalorimetry of each mutant along with its structural analysis by circular dichroism and by X-ray structure analysis. None of these mutations were found to alter significantly the protein structural properties.

Materials and Methods

Materials. Restriction enzymes and T4 ligase were purchased from either New England Biolabs (Beverly, MA) or Takara Shuzo (Kyoto, Japan). Enzyme reactions were carried out under the conditions recommended by the suppliers. *Micrococcus lysodeikticus* cells and wild-type human lysozyme (h-lysozyme) were purchased from Sigma (St. Louis, MO). Guanidine hydrochloride, GuHCl, (specially prepared reagent grade) was purchased from Nakarai Tesque, Inc. (Kyoto, Japan), and used without further purification. All other chemicals were reagent grade.

Strain and Media. Saccharomyces cerevisiae AH22R$^-$ (*a, leu2, his4, can1, pho80*) (Miyanohara et al., 1983) was used for the host strain and cultivated in modified Burkholder minimal medium (Toh-e et al., 1973) supplemented with 8% sucrose.

Oligonucleotide-Directed Mutagenesis. Oligonucleotides were synthesized by the phosphoramidite method (Beaucage et al., 1981) on an Applied Biosystems DNA synthesizer, Model 380A, and purified by HPLC on a TSK gel ODS-120T (Tosoh, Tokyo) column. M13 derivatives containing desired mutations in the h-lysozyme gene were prepared as described previously (Taniyama et al., 1988). Mutated primers were as follows: P71G, 5′-ACAGGCGTTTACGGCACCGCC-AGTCTTGCC-3′; P103G, 5′-CTAATACCCTGCCCG-TCACGGAC-3′; A47P, 5′-GGTCGCCAGGGTTGTAA-TTGG-3′; D91P, 5′-TAGCGCAGGCAACAGCCGGG-GCAATGTTGTCCTGAAGC-3′, where underlinings indicate mismatches. The sequences of the mutated genes were confirmed by dideoxy sequencing. The gene of P71G/P103G was obtained by site-directed mutagenesis of P103G using the already described mutated primer for P71G. V110P has been introduced in a previous study (Kikuchi et al., 1988). The genes encoding the signal sequence and mutated h-lysozyme were combined with a *Xho*I–*Sma*I large fragment from pERI8602 (Taniyama et al., 1988) to construct the expression plasmids.

Purification. Secreted h-lysozyme mutants were purified as previously described (Kikuchi et al., 1988).

Equilibrium Experiments. GuHCl denaturation curves were determined by measuring the intrinsic fluorescence of h-lysozyme solutions (280-nm excitation, 350-nm emission) thermostated at 25 ± 0.1 °C in 2-mm optical path length quartz cuvettes with a Hitachi (Model F-4000) spectrofluorometer. Final protein concentrations in the fluorescence cuvette were in the range of 10 μM. All samples were fully equilibrated (overnight incubation) prior to the collection of spectra, and denaturation was completely reversible under the conditions reported here.

Kinetic Experiments. For kinetic measurements, protein unfolding and refolding reactions were initiated by GuHCl concentration jumps, and the subsequent time-dependent changes in fluorescence were monitored. Fluorescence stopped-flow experiments were carried out on a Photal RA-401 (Otsuka Electronics) stopped-flow spectrophotometer equipped with a mixing device of 10:1. Measurements were performed

by monitoring the protein intrinsic fluorescence at wavelengths longer than 300 nm using a cut filter UV30, after excitation at 280 nm with a slit width of 7 mm. The dead time for measurement was about 10 ms. For all the kinetic experiments, temperature was controlled at a precision of ±0.1 °C using a thermostated water bath, and measurements were carried out under nitrogen atmosphere. The kinetic data were fitted by the method of nonlinear least-squares with the equation: $A(t) - A(\infty) = \sum_i A_i \exp(-k_i t)$, where $A(t)$ and $A(\infty)$ are the parameters chosen to follow the kinetics at time t and infinite time, respectively; k_i is the apparent first-order rate constant of the ith kinetic phase, and A_i is the amplitude of the ith phase. The buffer used in all experiments was 40 mM glycine, pH 3.00. Protein concentration was determined by absorbance at 280 nm, taking $\epsilon_{280} = 37700$ (Kuroki, personal communication).

Results

Comparison of the properties of the wild-type and mutant h-lysozyme hinges on the assumption that amino acid replacements do not alter significantly the conformation of the protein. By CD spectroscopy and X-ray structure analysis, we have checked (Herning et al., unpublished experiments) the validity of this assumption for each mutant considered in this report. The three-dimensional structure of all the mutants was found to be similar to that of the wild type, and no major changes in protein stability were brought about by these mutations. The lytic activity against *M. lysodeikticus* cells of all mutant h-lysozymes, except V110P, was found to be close to that of the wild-type protein. V110P has almost no activity because position 110 is very close to the active site.

Denaturation at Equilibrium. Equilibrium unfolding transitions induced by GuHCl were followed by monitoring the changes in the intrinsic fluorescence (280-nm excitation, 350-nm emission) for the wild-type and mutant h-lysozymes. For each protein, fluorescence intensity was found to increase as a sigmoidal function of the denaturant concentration, and the concentration at the inflection or midpoint differed only slightly among the various mutants. The ratio of the calorimetric enthalpy to the van't Hoff enthalpy measured by scanning microcalorimetry (Herning et al., unpublished experiments) was found to be close to unity for the wild-type and mutant h-lysozymes, indicating that unfolding closely approaches a two-state mechanism (Kidokoro & Wada, 1987). Therefore, GuHCl-induced denaturation curves have been analyzed in the present report by fitting the data to the following equation for a two-state unfolding process N ⇌ D (Pace et al., 1990):

$$F_D = K_u/(1 + K_u) \quad (1)$$

F_D is the apparent fraction of unfolded proteins, defined as $F_D = (I - I_N)/(I_D - I)$, where I is the measured fluorescence intensity, I_N is the extrapolated value for the native state, and I_D is the extrapolated value for the denatured state. K_u is the unfolding equilibrium constant. The equations for I_N and I_D were obtained by a least-squares analysis of data in the pre- and posttransition regions.

GuHCl equilibrium unfolding curves for the wild-type h-lysozyme, P71G, P103G, and P71G/P103G are shown in Figure 1 and are representative of the data collected from equilibrium studies. For each protein the logarithm of K_u derived from eq 1 was found to vary linearly with GuHCl concentration. A least-squares analysis was used to fit the data to the equation (Matouschek et al., 1990)

$$\log K_u = \log K_u(H_2O) - m_e[\text{GuHCl}] \quad (2)$$

where $K_u(H_2O)$ is the equilibrium constant for unfolding in water and m_e is a constant that is proportional to the increase in degree of exposure to solvent of the protein on denaturation. At least two data sets were obtained for each protein, more often three or four. Values of midpoint concentration, slope m_e, and log $K_u(H_2O)$ consistently agreed to within 2, 2, and 5%, respectively. The equilibrium parameters of each mutant are summarized under Table I. For the wild type, denaturation curves monitored by recording ellipticity at 222 nm (Herning et al., unpublished experiments) gave parameters basically identical with those obtained from fluorescence measurements, as expected for a two-state unfolding transition.

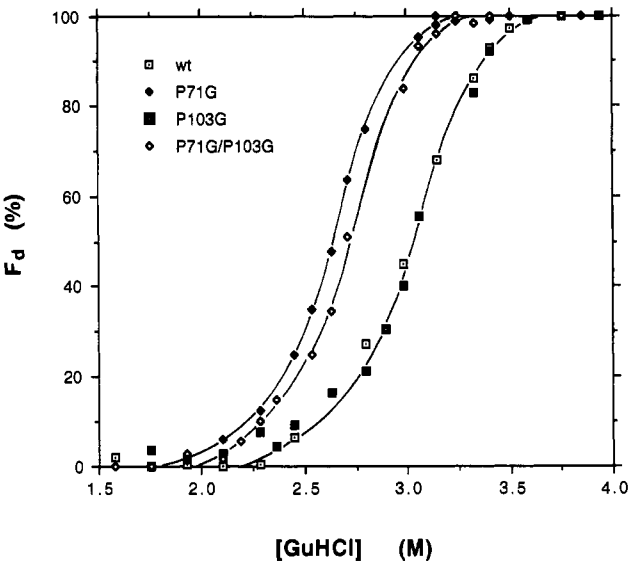

FIGURE 1: Dependence of the apparent fraction of unfolded protein, F_d, on the GuHCl concentration for the wild-type and proline mutant h-lysozymes at pH 3.00, 40 mM glycine hydrochloride buffer, 25 °C. Data were collected by measuring the fluorescence intensity at 350 nm after excitation at 280 nm.

Table I: Thermodynamic Parameters for the GuHCl-Induced Unfolding Transition of Wild-Type and Substituted h-Lysozymes[a]

protein	C_m (M)	m_e (M^{-1})	$K_u(H_2O) \times 10^8$
wild type	2.97 ± 0.06	5.52 ± 0.11	7.7
P71G/P103G	2.64 ± 0.05	5.48 ± 0.11	52.0
P71G	2.63 ± 0.05	5.43 ± 0.11	62.0
P103G	2.95 ± 0.06	5.55 ± 0.11	7.7
A47P	3.30 ± 0.07	5.50 ± 0.10	1.4
D91P	2.78 ± 0.06	5.42 ± 0.11	29.0
V110P	3.05 ± 0.06	6.69 ± 0.13	0.1

[a] Thermodynamic parameters were obtained by fitting equilibrium data to eq 2. Errors represent standard deviations. Equilibrium experiments were carried out at 25 °C in 40 mM glycine hydrochloride, pH 3.00.

Among the mutations considered in this report, the most destabilizing one is the replacement of Pro71 by a glycine. However, the destabilization brought about by this mutation is not dramatic considering the high stability of h-lysozyme in these conditions. The replacement of Pro103 by a glycine does not affect protein stability. In the P71G/P103G mutant, devoid of proline residue, the total destabilization corresponds to the summation of individual destabilizations of P71G and P103G.

Unfolding and Refolding Kinetics Monitored by Fluorescence. The unfolding of the wild-type and mutant h-lysozymes was induced by GuHCl concentration jumps, and the unfolding kinetics were subsequently monitored by recording the fluorescence intensity of the protein solution. Prior to [GuHCl] jumps, proteins were dissolved in 40 mM glycine buffer, pH 3.00, and final denaturant concentrations after the jump were

chosen within the concentration range above the transition zone. The temperature was 25 °C. For all proteins, unfolding kinetics were strictly single phase and were fitted by the monoexponential function $A(t) - A(\infty) = A_u \exp(-k_u t)$, where k_u is the apparent unfolding rate constant and A_u is the phase amplitude (see Materials and Methods).

Refolding kinetics were monitored in the same way by recording the fluorescence intensity changes of the refolding protein. Refolding was induced by reversed [GuHCl] jumps: proteins were first denatured by a 1-h incubation in 4.5 M GuHCl/40 mM glycine buffer, pH 3.00; the final GuHCl concentration after the jump fell into the concentration range below the transition zone. All experiments were carried out at 25 °C. Refolding kinetics of the wild type were biphasic, and experimental data were fitted to the double-exponential function $A(t) - A(\infty) = A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t)$, where A_1 and k_1 are the amplitude and refolding rate constant of the faster phase, and A_2 and k_2 refer to the slower kinetic phase (see Materials and Methods). It was pointed out by one of the reviewers that in our experiments the time range of the h-lysozyme slow refolding phase is rather short ($\tau \approx 1$ s), relative to the expected time range of most protein slow refolding phases ($\tau \approx 10$–10^3 s). Although it is correct, the terms "fast refolding phase" and "slow refolding phase" will be kept and used here for convenience when each of the observed phases in h-lysozyme refolding kinetics is being described.

The fast phase is the major phase, having, independently of the final GuHCl concentration, 90% of the fluorescence intensity change. The fast phase rate constant decreases quickly with increasing [GuHCl], whereas the slow phase rate constant decreases relatively more slowly with [GuHCl]. Biphasic refolding kinetics were also observed for hen egg white lysozyme and α-lactalbumin, two proteins homologous to h-lysozyme. The presence of two phases in their refolding kinetics was ascribed to the cis–trans slow isomerization of prolines in the unfolded state (Kato et al., 1982; Kuwajima, 1989).

A quite interesting result of the present work is that similar biphasic kinetics were also obtained for the h-lysozyme P71G/P103G mutant, which is devoid of any proline residue. The slow refolding phase of both the wild type and P71G/P103G mutant have, within experimental error, the same rate constant at a given final GuHCl concentration, and the relative amplitude of the slow phase, A_s, defined as $A_s = A_1/(A_1 + A_2)$, is equal to 10 ± 3% for both proteins. Representative refolding kinetic curves of the wild type and the P71G/P103G mutant are shown in parts a and b of Figure 2. Their semilogarithmic representations, showing for both proteins the same clear deviation from the single-phase behavior, are given in parts c and d of Figures 2. Biphasic behaviors were also observed for P71G and P103G, the mutants containing a single proline, as well as for A47P, the mutant containing three prolines (kinetics not shown). For all of these mutants, neither the slow refolding rate constant nor the relative slow phase amplitude was found to be significantly different from that of the wild type.

Parts a–c of Figure 3 report the plots of the logarithm of the apparent rate constants for unfolding and refolding versus the final GuHCl concentration of the concentration jumps. The wild-type h-lysozyme is used as a reference for data comparison. The unfolding side ([GuHCl] ≥ 3 M) in each figure reveals a strong dependency of the unfolding rate constant, k_u, on the final GuHCl concentration for all the proteins. Plots of log k_u versus [GuHCl] above the transition zone give good straight lines close to parallel. The unfolding rate constant in water, $k_u(H_2O)$, can be derived by fitting the data to the equation (Matouschek et al., 1990)

$$\log k_u = \log k_u(H_2O) + m_{ku}[\text{GuHCl}] \quad (3)$$

where m_{ku} is a proportionality factor. Values of $k_u(H_2O)$ and m_{ku} are given for each protein in Table II. Each kinetic measurement was carried out at least twice, and good reproducibility was obtained. The left-hand side of Figure 3a–c, the refolding side ([GuHCl] ≤ 3 M), shows the GuHCl concentration dependency of the fast and slow refolding rate constants for each mutant. Due its low amplitude, the slow phase had data that were slightly statistically dispersed. However, a great number of experiments have unambiguously shown that for P71G/P103G, P71G, P103G, and A47P, the slow refolding rate constant and the relative amplitude of the slow phase were basically identical with those of the wild-type protein at any given final GuHCl concentration. The fast refolding rates of P71G/P103G and P103G were identical with and slightly higher than that of the wild type (Figure 3a,b), whereas P71G and A47P have fast phases identical with that of the wild type (Figure 3b,c). These results show that the replacement of Pro103 by Gly increases the refolding fast kinetic rate of h-lysozyme, whereas the replacement of Pro71 by a Gly does not affect at all the refolding kinetics of the h-lysozyme. The refolding kinetics of D91P are also biphasic, and the relative amplitude of the slow phase is again close to 10%. However, for this mutant the refolding rates of both phases were found to be considerably reduced as shown in Figure 3c. For V110P, an additional very slow phase was clearly detectable; hence the refolding kinetics of this mutant were triphasic. The two phases (Figure 4a), similar in rate and amplitude to the two phases of the wild type, were followed by another very slow phase that was measured separately from the two faster phases due to the great difference of time scale (Figure 4b). The relative amplitudes of the three V110P refolding phases were 76%:8%:16% for the fast, slow, and very slow phases, respectively. We see in Figure 5 that the refolding rate of the additional very slow phase of V110P is almost independent of the final GuHCl concentration. We have summarized all the refolding kinetic parameters of each protein in Table III.

Table II: Kinetic Parameters for the GuHCl-Induced Unfolding of Wild-Type and Substituted h-Lysozymes[a]

protein	k_u(4 M) (s^{-1})	$k_u(H_2O) \times 10^3$ (s^{-1})	m_{ku} (M^{-1})
wild type	0.34	1.90	0.56
P71G/P103G	3.86	0.35	0.50
P71G	2.44	0.21	0.52
P103G	0.46	3.30	0.53
A47P	0.16	1.60	0.51
D91P	0.15	1.00	0.55
V110P	0.32	2.80	0.51

[a] k_u(4 M) is the unfolding rate constant when unfolding from 0 to 4 M GuHCl at 25 °C in glycine hydrochloride, pH 3.0. Values in water, $k_u(H_2O)$, and m_{ku} were obtained by fitting the data to eq 3.

Discussion

The aim of the present study is to analyze the effects of specific proline residues on h-lysozyme folding, especially the influence of proline isomerization on slow folding reactions. Using a protein engineering approach, we have investigated separately the individual role of each proline in h-lysozyme. The h-lysozyme is suitable for such study due to its low proline content. The wild type has two trans prolines in its native state, located at positions 71 and 103. Only Pro71 is conserved

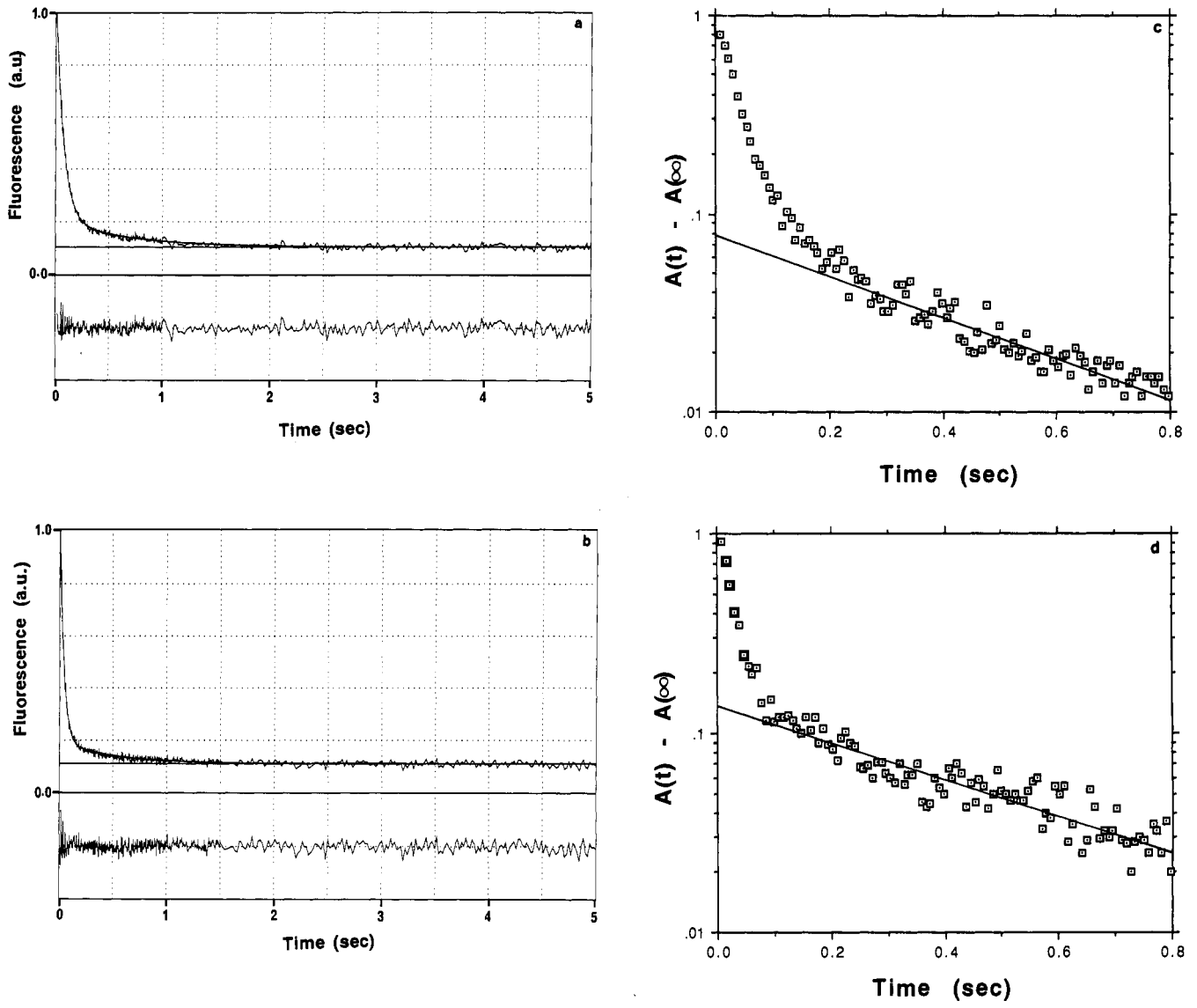

FIGURE 2: Kinetic progress curves for refolding of (a) the wild-type h-lysozyme and (b) P71G/P103G, as measured by fluorescence after a stopped-flow concentration jump from 4.5 to 0.5 M GuHCl in 40 mM glycine hydrochloride buffer, pH 3.00. The final protein concentration was 10 μM. Lower panels of kinetics show the residual root mean square deviation of the fitting procedure. Panels c and d show the semilogarithmic plots of the net fluorescence change calculated from the data shown in panels a and b, respectively.

Table III: Kinetic Parameters for the Refolding of Wild-Type and Substituted h-Lysozymes[a]

protein	k_1 (s^{-1})	k_2 (s^{-1})	k_3 (s^{-1})	A_1/A_T (%)	A_2/A_T (%)	A_3/A_T (%)
wild type	14.3	1.6		90	10	
P71G/P103G	32.6	2.0		88	12	
P71G	16.2	1.8		89	11	
P103G	25.0	1.9		91	9	
A47P	15.8	1.8		92	8	
D91P	2.0	0.2		90	10	
V110P	25.0	1.1	0.016	76	8	16

[a] Initial conditions were 4.5 M GuHCl and 40 mM glycine hydrochloride, pH 3.0. Refolding was carried out at 25 °C by 11-fold dilution to the final conditions of 1 M GuHCl, 40 mM glycine hydrochloride, pH 3.0. The resulting kinetics were measured by the decrease in fluorescence after excitation at 280 nm. k_1, k_2, and k_3 and A_1, A_2, and A_3 refer respectively to the rate constants and amplitudes of the fast, slow, and very slow refolding kinetic phases, and $A_T = A_1 + A_2 + A_3$.

among other lysozymes from different origins. Our approach involves the comparison of the properties of the wild-type and mutant proteins, which implies that amino acid replacements do not alter protein conformation and stability too much. This assumption was tested by circular dichroism, X-ray structure analysis, and scanning microcalorimetric measurements (Herning et al., unpublished experiments). No major changes in protein structure and stability were caused by these mutations. Therefore, it is likely that the general folding pattern remains the same for the wild type and the proline mutants considered here. In the remainder of this report, we shall review and discuss the effects of removal and introduction of proline residues on the folding process of h-lysozyme. The first part of the discussion deals with the effects of proline residues on the h-lysozyme slower refolding reactions. In the second part, we combine kinetic and equilibrium results to construct partly the folding pathway of h-lysozyme and discuss the effects of proline mutations on the free energy profile for folding in water. We emphasize the fact that a direct comparison of mutant free energy profiles for folding is arbitrary

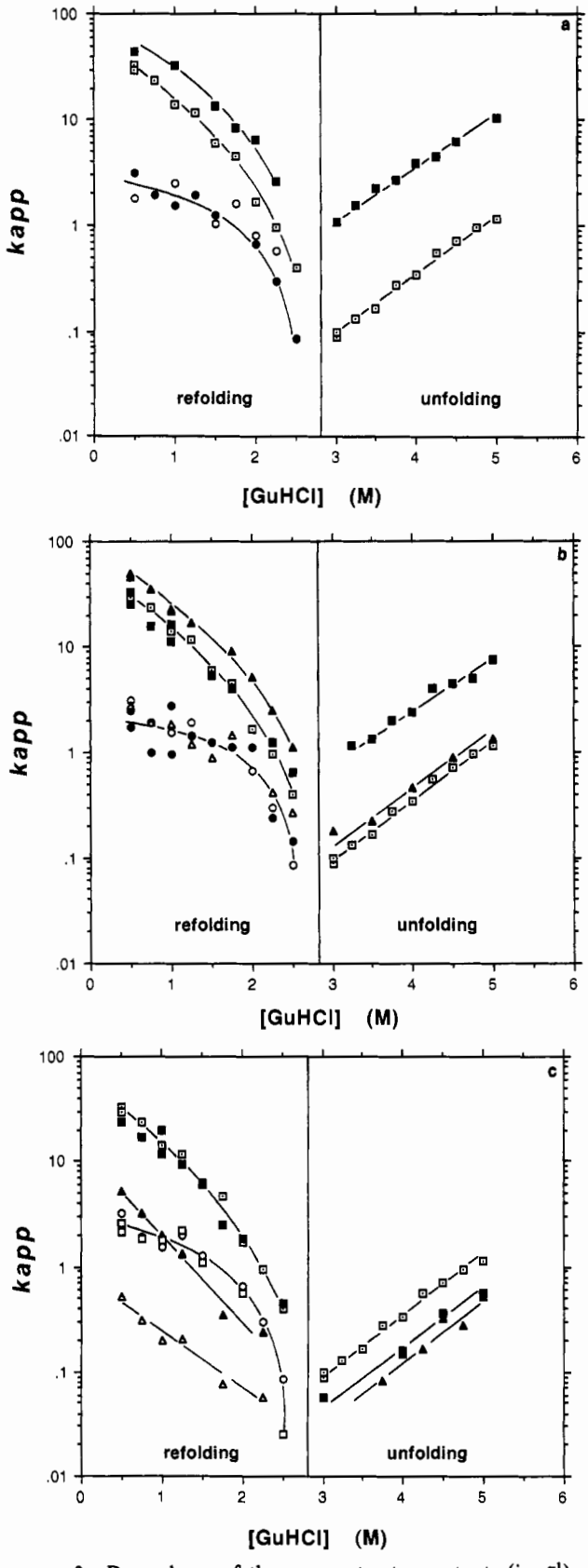

FIGURE 3: Dependence of the apparent rate constants (in s^{-1}) of refolding and unfolding on GuHCl concentration in the wild-type and proline mutant h-lysozymes. Experimental conditions were the same as those in Figure 2. (a) Unfolding and fast refolding for (⊡) the wild type and (■) P71G/P103G; slow refolding for (○) the wild type and (●) P71G/P103G. (b) Unfolding and fast refolding for (⊡) the wild type, (■) P71G, and (▲) P103G; slow refolding for (○) the wild type, (●) P71G, and (△) P103G. (c) Unfolding and fast refolding for (⊡) the wild type, (■) A47P, and (▲) D91P; slow refolding for (○) the wild type, (□) A47P, and (△) D91P.

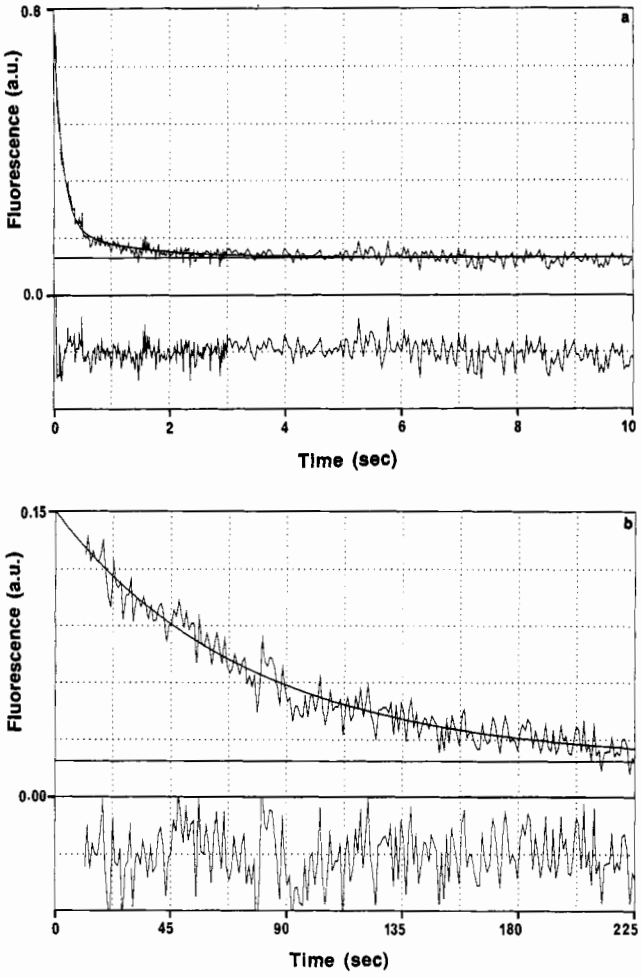

FIGURE 4: Kinetic progress curves for refolding of V110P on a (a) short and (b) long time scale after a stopped-flow concentration jump from 4.5 to 1.0 M GuHCl. Other experimental conditions were the same as those in Figure 2. Lower panels of kinetics show the residual root mean square deviation of the fitting procedure.

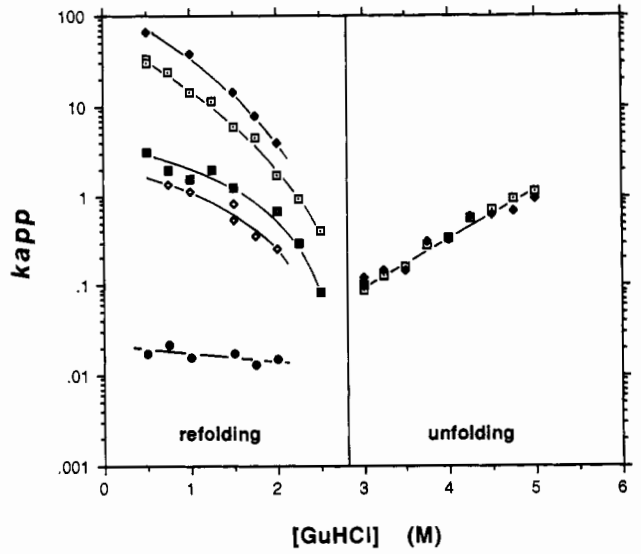

FIGURE 5: Dependence of the apparent rate constants (in s^{-1}) of refolding and unfolding on GuHCl concentration in the wild type and V110P. Unfolding and fast refolding for (⊡) the wild type and (◆) V110P; slow refolding for (■) the wild type and (◇) V110P; (●) very slow refolding for V110P. Experimental conditions were the same as those in Figure 2.

and limit our discussion to the effects of proline substitution on the folding profile energy differences.

The Human Lysozyme Slow Refolding Phase Has No Relation to Proline Isomerization. The refolding kinetics of wild-type h-lysozyme are biphasic, resembling observations with hen egg white lysozyme (Kato et al., 1982). The biphasic character of hen egg white lysozyme refolding kinetics was ascribed to proline slow isomerization due to the rather good agreement between the slow refolding phase properties and the theoretical predictions of the proline hypothesis. However, the measured relative amplitude of the slow phase, a value of 10%, was too small to be fully consistent with the theoretical prediction of 36% for a protein containing two prolines such as hen egg white lysozyme (Brandts et al., 1975). The discrepancy was explained by the possibility that some prolines were more "permissive" due to their direct environment in the protein (Kato et al., 1982).

The refolding kinetics of the wild-type h-lysozyme also show a major fast phase, corresponding to 90% of the fluorescence intensity change, followed by a 10% amplitude slower phase. However, the refolding kinetic results of the various proline mutants considered in this work do not support the assumption that the h-lysozyme slow refolding phase is related to proline isomerization. The refolding kinetics of the P71G/P103G mutant, which does not possess any proline, are also biphasic, and the rate constant and the amplitude of the slow phase are identical, within experimental error, with those of the wild type. The refolding kinetics of P71G and P103G mutants, which have a single proline, and A47P, with its three prolines, are also biphasic. Their slow phase amplitude is close to 10%, and their rate constant is identical with that of the wild type protein. These results show that neither Pro71 nor Pro103 has any relation with the slow refolding kinetic phase of h-lysozyme and that the biphasic character of h-lysozyme kinetics cannot be explained in terms of proline isomerization. Considering these experimental results, it may not be surprising that the refolding kinetics of phage T4 lysozyme were reported to be single phase in spite of the fact this protein contains three proline residues (Chen et al., 1988, 1989). Moreover, for T4 lysozyme mutants containing an extra proline residue, refolding kinetics were still single phase and identical with those of the wild-type protein (Chen et al., 1988). Although slow refolding phases have been proven unambiguously to be due to proline isomerization for several proteins such as RNase A (Lin & Brandts, 1978, 1985) or RNase T1 (Kiefhaber et al., 1990a), our results suggest that it would be worthwhile to go further in the molecular interpretation of the slow refolding kinetics of h-lysozyme homologous proteins such as hen egg white lysozyme or α-lactalbumin. However, our observations do not call into question the fact that slow phases originate from slow equilibria between spectroscopically equivalent forms of the polypeptide under denaturing conditions. Double-jump experiments carried out on hen egg white lysozyme (Kato et al., 1982) have shown that the relative amplitude of the slow phase was decreased to about 5% when refolding experiments were performed following a very short incubation period under denaturing conditions. The slow phase amplitude increased up to 10% when the incubation period under denaturing conditions was extended, indicating that on unfolding fast refolding species are produced before slow refolding species and that an equilibrium between them is reached eventually. The same phenomenon was observed in similar experiments with other proteins such as α-lactalbumin (Ikeguchi et al., 1986b). We do not, at this time, have any alternative molecular interpretation for the slow refolding kinetics measured on h-lysozyme. Although not fully satisfactory, the so-called "cluster model" (Kanehisa & Tsong, 1979) for protein folding proposed several years ago is worth being mentioned because it predicts multiphasic kinetics of the protein folding without relying on the proline isomerization hypothesis. In this model, the initial equilibrium distribution of the denatured state D appears to be separated into D_1 and D_2 in the final refolding conditions because of the change in position of the free energy barrier. This model also predicts single-phase unfolding kinetics. Although regarded as unlikely, an alternative molecular explanation for the slow phase would be that amino acids other than proline have to undergo isomerization to allow the protein to reach its final native conformation on refolding.

The refolding kinetics of D91P are biphasic and have a slow phase amplitude close to 10%. However, the kinetic rates of both phases are considerably reduced. Although the general pattern of folding seems to be conserved, the folding process appears to be quite affected by this mutation. In the wild-type protein, Asp91 is located in an α-helix which starts at Ala90 and ends at Val99. X-ray and circular dichroism studies (Herning et al., unpublished experiments) did not show any modification in the secondary structure of the D91P mutant. It is possible that refolding is delayed in this mutant because the formation of the α-helix 90–99, necessary for the stabilization of the tertiary structure, is delayed by the replacement of Asp91 by Pro.

The V110P mutant has interesting properties: its refolding kinetics are triphasic. An additional very slow phase was measured on a very long time scale (typically 250 s under the chosen conditions). The two faster phases (typically decayed within 10 s) are nearly identical with the fast and slow phases measured for the wild-type protein. The relative amplitudes of each phase are 76, 8, and 16% for the fast, slow, and very slow phases, respectively. The rate constant of the very slow phase does not depend on final GuHCl concentration and corresponds to an activation energy of about 18 kcal/mol at 25 °C according to the Eyring equation in the transition-state theory (Laidler, 1950). Such properties are typically those expected for a proline cis–trans isomerization reaction (Brandts et al., 1975). Therefore, it is likely that this additional very slow phase results from the cis–trans isomerization of Pro110. Pro110 is located at the N-terminus of an α-helix which formation may require Pro110 to be in the trans conformation. However, it has been shown above that one has to be cautious in the interpretation of slow phases measured on protein refolding, and verification of this hypothesis requires study of its own. We are planning to perform double-jump experiments in order to clarify whether the very slow phase of V110P is due to the isomerization of Pro110, and the results of these experiments will be described in a future publication.

Free Energy Profile for Folding: Effects of Proline Residues. This discussion concerns the fast and major refolding phase which corresponds to the folding of the fraction of protein belonging to the fast refolding species. If we assume that h-lysozyme folding involves a reversible transition between only two states, the denatured state and the native state, the refolding rate constant, k_f, of such a protein is expected to follow the simple law (Matouschek et al., 1990)

$$\log k_f = \log k_f(H_2O) - (m_e - m_{ku})[\text{GuHCl}] \qquad (4)$$

with $\log k_f(H_2O) = \log \{k_u(H_2O)/K_u(H_2O)\}$. m_e and m_{ku} have already been defined in eqs 2 and 3. Therefore, according to eq 4, $\log k_f$ may be calculated as a function of GuHCl concentration for a two-state transition by using the unfolding kinetic and equilibrium data. Figure 6 shows that the experimental values of $\log k_f$ at low denaturant concentration deviate by many orders of magnitude from those predicted by the kinetics of a two-state equation. Such a deviation, which

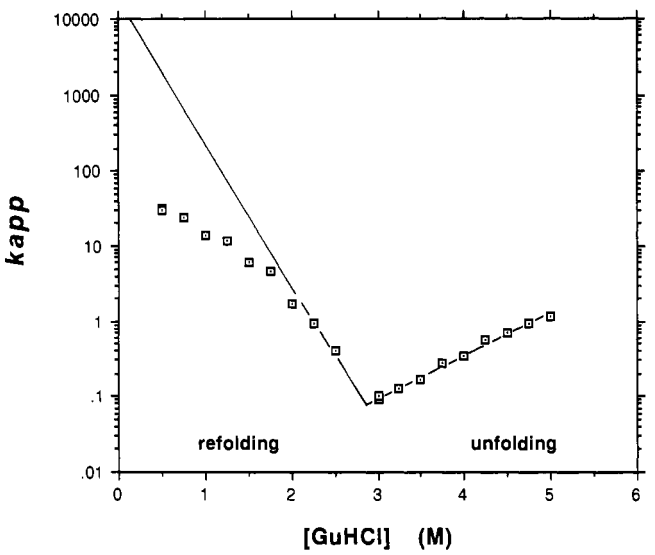

FIGURE 6: Dependence of the apparent rate constants (in s^{-1}) of refolding and unfolding on GuHCl concentration in the wild-type h-lysozyme. The solid curve is that calculated for a simple two-state transition. Experimental conditions were the same as those in Figure 2.

has also recently been reported for barnase refolding kinetics (Matouschek et al., 1990), indicates that a metastable intermediate is transiently populated on refolding and that the fast refolding phase actually reflects the transformation of the intermediate into the native protein. However, at higher denaturant concentrations ([GuHCl ≥ 2 M), the intermediate state is destabilized and the measured refolding rate constant follows the two-state transition behavior. Such an intermediate could be further characterized for h-lysozyme by monitoring the protein folding using circular dichroism in the peptide region (data not shown), according to the method established previously for the characterization, of the early folding intermediates of hen egg white lysozyme and α-lactalbumin (Ikeguchi et al., 1986b). CD refolding kinetics of h-lysozyme show the same two-stage behavior as for hen egg white lysozyme and α-lactalbumin, and the h-lysozyme kinetic intermediate has properties similar to those reported on hen egg white lysozyme and α-lactalbumin (Ikeguchi et al., 1986b): the h-lysozyme intermediate is formed within the measurement dead time at low GuHCl concentrations ([GuHCl] ≤ 2 M) and has a folded secondary structure but an unfolded tertiary structure. At higher GuHCl concentrations, the intermediate is destabilized and the tertiary structure is required for the stabilization of the secondary structure. Therefore, in this concentration range the formations of the secondary and tertiary structures follow the same kinetics.

We may then draw, at least partly, the folding pathway of h-lysozyme according to the reaction

$$D \rightleftharpoons \cdots\cdots \rightleftharpoons I \rightleftharpoons N$$

There may be many other intermediate states; however, they are not detectable as kinetic intermediates because they occur on the folding pathway after the rate-limiting step or because their free energy is higher than that of the initial state. Using a method previously established (Matouschek et al., 1990), the free energy profile for folding of the wild-type enzyme in water, shown in Figure 7, was calculated from the measured free energy of unfolding, and the rate constants $k_u(H_2O)$ and $k_f(H_2O)$, extrapolated at 0 M GuHCl, to give the energies of the folded state, transition state for unfolding, and the intermediate state relative to the unfolded state. Refolding and unfolding activation energies were calculated from the kinetic

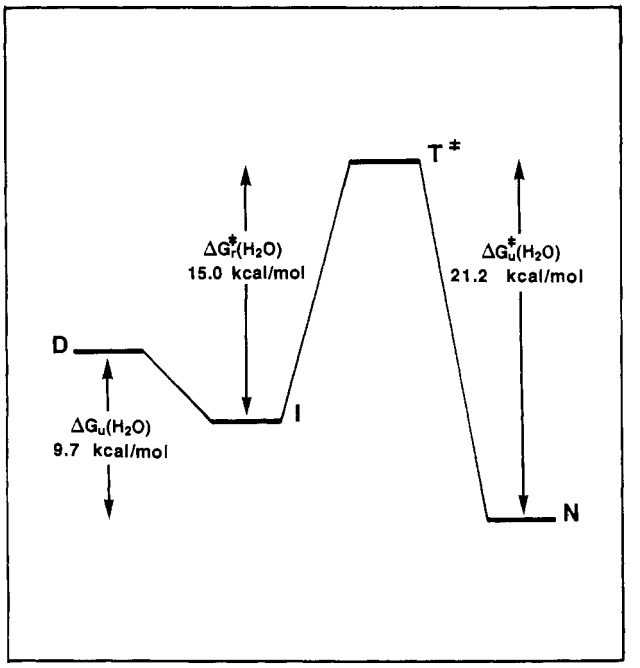

FIGURE 7: Free energy profile for folding of the wild-type h-lysozyme in water calculated from the measured free energy of unfolding and the rate constants $k_u(H_2O)$ and $k_f(H_2O)$, extrapolated at 0 M GuHCl to give the energies of the folded state (N), transition state (T^*) for unfolding, and the intermediate state (I) relative to the unfolded state (D).

Table IV: Unfolding Free Energy and Activation Energies for Unfolding and Refolding in Water for the Wild-Type and Mutant h-Lysozymes[a]

protein	$\Delta G_u(H_2O)$	$\Delta G_u(H_2O)^*$	$\Delta G_r(H_2O)^*$
wild type	9.7 ± 0.5	21.2 ± 0.1	15.04 ± 0.02
P71G/P103G	8.6 ± 0.4	19.5 ± 0.1	14.87 ± 0.02
P71G	8.5 ± 0.4	19.8 ± 0.1	15.04 ± 0.02
P103G	9.7 ± 0.5	20.9 ± 0.1	14.87 ± 0.02
A47P	10.8 ± 0.5	21.3 ± 0.1	15.04 ± 0.02
D91P	8.9 ± 0.5	21.6 ± 0.1	15.86 ± 0.02
V110P	12.1 ± 0.6	21.0 ± 0.1	14.87 ± 0.02

[a] $\Delta G_u(H_2O)$ is the free energy of unfolding in water, and $\Delta G_u(H_2O)^*$ and $\Delta G_r(H_2O)^*$ are the activation energies in water at 25 °C (pH 3.00) for unfolding and refolding, respectively. Energies are given in kilocalories per mole. Errors represent standard deviations.

data by using the transition-state theory via the Eyring equation (Laidler et al., 1950). The free energy profile for folding in water may be determined for each mutant as well, and the various energy values for each mutant are summarized in Table IV.

In a recent work on barnase (Matouschek et al., 1990), free energy profiles for folding of mutants and wild-type proteins were compared by assigning the unfolded state a standard energy of zero for all the proteins, which implies that the mutations do not significantly affect the free energy of the unfolded state. However, it has been reported (Nemethy et al., 1966; Matthews et al., 1987) that the entropic contribution to the unfolded state free energy depends on the nature of the amino acid side chains because the freedom of internal rotation of the backbone in the random-coil form is influenced by the length or bulkiness of the side chains. Such effect is particularly important in the case of proline substitutions because the pyrrolidine ring of proline restricts this residue to fewer conformations than are available to the other amino acids. For instance, at 25 °C, the backbone contribution to the entropy of unfolding of a proline relative to a glycine has been estimated to about −1.8 kcal/mol, and to about −1.2 kcal/mol for a proline relative to an alanine (Nemethy et al., 1966; Mat-

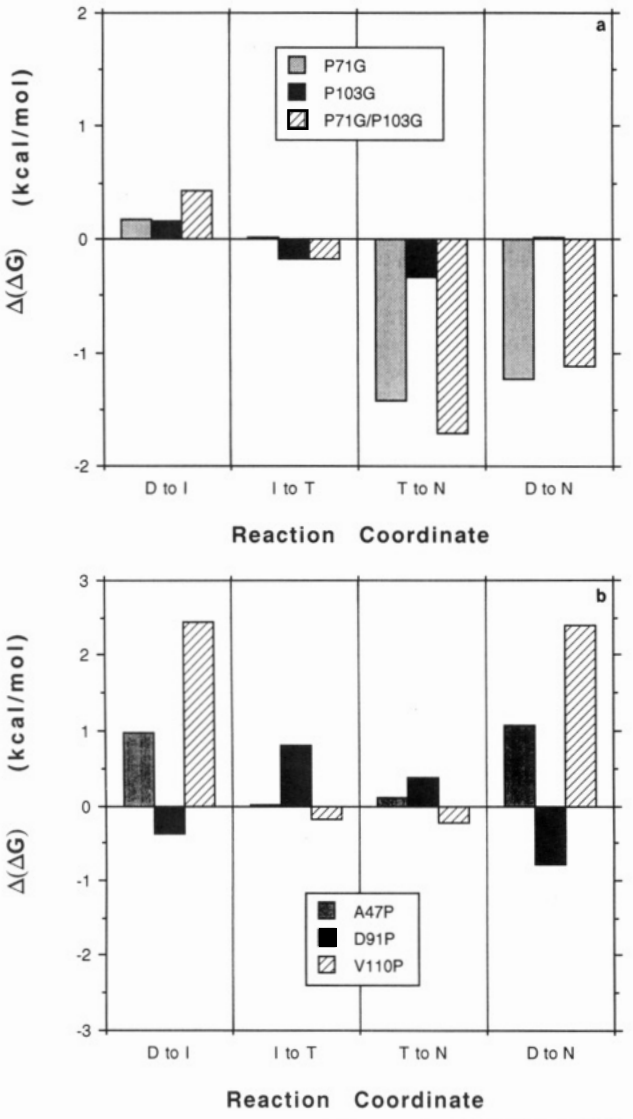

FIGURE 8: Differences of energy differences for (a) P71G, P103G, and P71G/P103G and (b) A47P, D91P, and V110P relative to the wild-type h-lysozyme. Values are presented in the form of a histogram for each step detected on the folding pathway. The differences in energy differences were calculated from the data in Table IV according to $\Delta(\Delta G_{ij}) = |\Delta G_{ij}'| - |\Delta G_{ij}|$, where the prime denotes mutants and (i,j) denotes two states of the folding pathway. D, I, T‡, and N refer to the denatured, intermediate, transition, and native states, respectively.

thews et al., 1987). A direct comparison of the mutant and wild-type folding free energy diagrams is somewhat arbitrary because no experiment provides any information concerning how the amino acid replacements influence the free energy of any of the states detected on the folding pathway. Nevertheless, one can discuss about how proline substitutions affect the energy difference between any two states of the folding pathway. Using the energy data in Table IV, the differences of energy differences, $\Delta(\Delta G_{ij})$, were calculated for each mutants relative to the wild type according to $\Delta(\Delta G_{ij}) = |\Delta G_{ij}'| - |\Delta G_{ij}|$, where the prime denotes mutants and (i,j) denotes two states of the folding pathway. Data are presented for all mutants in Figure 8a,b in the form of a histogram for each detectable step of the folding pathway. Parts a and b of Figure 8 show that all the amino acid substitutions of this study except Asp91 → Pro and Pro103 → Gly influence h-lysozyme stability according to the prediction that expects the replacement of a less flexible amino acid by a more flexible one to increase the configurational entropy of unfolding (histograms D to N).

Although an aspartate is considered more flexible than a proline, the substitution of Asp91 by Pro is destabilizing (i.e., the free energy of unfolding is decreased by about 0.8 kcal/mol). However, the calorimetric analysis (Herning et al., unpublished experiments) shows that the mutant D91P is destabilized due to a considerable decrease in the denaturation enthalpy (about 4 kcal/mol) brought about by the mutation. Actually the entropic effect led by the Asp91 → Pro substitution, indeed, corresponds to a stabilization of about 3 kcal/mol, but it is offset by the larger destabilizing enthalpic contribution.

The Pro103 → Gly substitution does not alter h-lysozyme stability, whereas the Pro71 → Gly replacement decreases the free energy of unfolding by about 1.2 kcal/mol. The accessible surface area values (ASA), calculated from the wild-type X-ray structure of h-lysozyme, are 146.05 Å^2 for Pro103 and 75.95 Å^2 for Pro71. The standard ASA value for a proline residue in a random-coil conformation was estimated to be 155.86 Å^2 (Go & Miyazawa, 1980). Using this value as a reference for the 100% random-coil character for a proline, Pro103 has a 93.7% random-coil character whereas Pro71 has only 48.7%. We suggest that the substitution of Pro103 by Gly equally stabilizes through entropic effects the unfolded and folded states of h-lysozyme. Therefore, no global destabilization results from this mutation. For the P71G mutant the unfolded state is entropically more stabilized than the folded state because position 71 which is more buried into the protein, is probably less sensitive to amino acid flexibility differences. Therefore, this mutation destabilizes h-lysozyme.

Another kind of information provided by the free energy difference histograms concerns the timing of the influence of the amino acid substitution on refolding. For instance, Figure 8a shows that the effects of the substitution Pro71 → Gly occur later on the folding pathway, between the transition (T‡) and native (N) states (Figure 8a, mutants P71G and P71G/P103G). No important changes are observed in the first two histograms, which correspond to the folding steps from the denatured (D) to the intermediate (I) states and from the intermediate (I) to the transition states (T‡), respectively. However, the T‡ to N step histogram is very much similar to the D to N histogram, which reflects the global effect of the substitution on h-lysozyme stability. Such a result indicates that the conformation around Pro71 has a rather random-coil character up to the transition state and that the denatured, intermediate, and transition states are approximately equally stabilized by entropic effects. It is only during the final step that the chain around position 71 acquires its native conformation which has only a 48.7% random coil character. The situation is different for mutations Ala47 → Pro, Asp91 → Pro, and Val110 → Pro (Figure 8b) because they are involved in secondary structure frameworks which are locked in the early stages of the folding reaction (Ala47 is located in a β-turn, and Val110 and Asp91 are located in α-helices). Therefore, the main effect on stability produced by these substitutions occurs in the first step of the folding (D to I), which has been shown to correspond to the formation of the secondary structure framework. It seems that if the mutation effect on protein stability has mainly an entropic origin, it may be possible from the analysis of these histograms to follow the "kinetics" of the loss of random-coil character around a specific amino acid on refolding and to get information on the timing of its involvement in a structured framework.

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REFERENCES

Anfinsen, C. B. (1973) *Science (Washington, D.C.) 181*, 223.
Artymiuk, P. J., & Blake, C. C. F. (1981) *J. Mol. Biol. 152*, 737–762.
Beaucage, S. L., & Caruthers, M. H. (1981) *Tetrahedron Lett. 22*, 1859–1862.
Brandts, J. F., Halvorson, H. R., & Brennan, M. (1975) *Biochemistry 14*, 4953–4963.
Chavez, L. G., Jr., & Scheraga, H. A. (1980) *Biochemistry 19*, 1005–1012.
Chen, B.-Lu, Baase, W. A., Nicholson, H., Schellman, C. G., & Schellman, J. A. (1988) *Biophys. J. 53*, 68a.
Chen, B.-Lu, Baase, W. A., & Schellman, J. A. (1989) *Biochemistry 28*, 691–699.
Creighton, T. E. (1978) *Prog. Biophys. Mol. Biol. 33*, 231–297.
Dolgikh, D. A., Gilmanshin, R. I., Brazhnikov, E. V., Bychkova, V. E., Semisotnov, G. V., Venyaminov, S. Yu., & Ptitsyn, O. B. (1981) *FEBS Lett. 136*, 311–315.
Dolgikh, D. A., Abaturov, L. V., Bolotina, I. A., Brazhnikov, E. V., Bychkova, V. E., Buchuev, V. N., Gilmanshin, R. I., Lebedev, Yu. O., Semisotnov, G. V., Tiktopulo, E. I., & Ptitsyn, O. B. (1985) *Eur. Biophys. J. 13*, 109–121.
Garel, J.-R., & Baldwin, R. L. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3347–3351.
Gast, H., Zirver, D., Welfe, H., Bychkova, V. E., & Ptitsyn, O. B. (1986) *Int. J. Biol. Macromol. 8*, 231–236.
Go, M., & Miyazawa, S. (1980) *Int. J. Pept. Protein Res. 15*, 211–224.
Goto, Y., & Hamaguchi, K. (1979) *J. Biochem. (Tokyo) 86*, 1433–1441.
Goto, Y., & Hamaguchi, K. (1982) *J. Mol. Biol. 156*, 891–910.
Grafl, R., Lang, K., Wrba, A., & Schmid, F. X. (1986) *J. Mol. Biol. 191*, 281–293.
Hurle, M. R., & Matthews, C. R. (1987) *Biochim. Biophys. Acta 913*, 179–184.
Ikeguchi, M., Kuwajima, K., & Sugai, S. (1986a) *J. Biochem. (Tokyo) 99*, 1191–1201.
Ikeguchi, M., Kuwajima, K., Mitani, M., & Sugai, S. (1986b) *Biochemistry 25*, 6965–6972.
Izumi, Y., Miyake, Y., Kuwajima, K., Sugai, S., Inoue, K., Iizumi, M., & Katano, S. (1983) *Physica B+C (Amsterdam) 120B*, 444–448.
Kanehisa, M. I., & Tsong, T. Y. (1979) *Biopolymers 18*, 2913–2928.
Kato, S., Shimamoto, N., & Utiyama, H. (1982) *Biochemistry 21*, 38–43.
Kelley, R. F., & Stellwagen, E. (1984) *Biochemistry 23*, 5095–5102.
Kidokoro, S., & Wada, A. (1987) *Biopolymers 26*, 213–229.
Kiefhaber, T., Quaas, R., Hahn, U., & Schmid, F. X. (1990a) *Biochemistry 29*, 3053–3070.
Kiefhaber, T., Grunert, H. P., Hahn, U., & Schmid, F. X. (1990b) *Biochemistry 29*, 6475–6480.
Kikuchi, M., Yamamoto, Y., Taniyama, Y., Ishimaru, K., Yoshikawa, W., Kaisho, Y., & Ikehara, M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9411–0415.
Kim, P. S., & Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51*, 459–489.
Kim, P. S., & Badwin, R. L. (1990) *Annu. Rev. Biochem. 59*, 631–660.
Krebs, H., Schmid, F. X., & Jaenicke, R. (1983) *J. Mol. Biol. 169*, 619–635.
Kuwajima, K., Nitta, K., Yoneyama, M., & Sugai, S. (1976) *J. Mol. Biol. 106*, 359–373.
Kuwajima, K., Mitani, M., & Sugai, S. (1989) *J. Mol. Biol. 206*, 547–561.
Laidler, K. J. (1950) in *Chemical Kinetics*, Chapter 3, McGraw-Hill, New York.
Lang, K., Schmid, F. X., & Fischer, G. (1987) *Nature 329*, 268–270.
Lin, L. N., & Brandts, J. F. (1978) *Biochemistry 17*, 4102–4110.
Lin, L. N., & Brandts, J. F. (1985) *Biochemistry 24*, 6533–6538.
Matouschek, A., Kellis, J. T., Serrano, L., Bycroft, M., & Fersht, A. R. (1990) *Nature 346*, 440–445.
Matthews, B. W., Nicholson, H., & Becktel, W. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6663–6667.
Miyanohara, A., Toh-e, A., Nozaki, C., Hamada, F., Ohtomo, N., & Matsubara, K. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1–5.
Nall, B. T., Garel, J. R., & Baldwin, R. L. (1978) *J. Mol. Biol. 118*, 316–330.
Nall, B. T., Zuniga, E. H., White, T. B., Wood, L. C., & Ramdas, L. (1989) *Biochemistry 28*, 9834–9839.
Nemethy, G., Leach, S. J., & Scheraga, H. A. (1966) *J. Phys. Chem. 70*, 998–1004.
Ohgushi, M., & Wada, A. (1983) *FEBS Lett. 164*, 21–24.
Pace, C. N., Laurents, D. V., & Thomson, J. A. (1990) *Biochemistry 29*, 2564–2572.
Ramdas, L., & Nall, B. T. (1986) *Biochemistry 25*, 6959–6964.
Robinson, J. B., Jr., Strottman, J. M., & Stellwagen, E. (1983) *J. Biol. Chem. 258*, 6772–6776.
Schmidt, F. X., Grafl, R., Wrba, A., & Beintema, J. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 872–876.
Taniyama, Y., Yamamoto, Y., Nakao, M., Kikuchi, M., & Ikehara, M. (1988) *Biochem. Biophys. Res. Commun. 152*, 962–967.
Toh-e, A., Ueda, Y., Kakimoto, S., & Oshima, Y. (1973) *J. Bacteriol. 113*, 727–738.
White, T. B., Berget, P. B., & Nall, B. T. (1987) *Biochemistry 26*, 4358–4366.
Wood, L. C., White, T. B., Ramdas, L., & Nall, B. T. (1988) *Biochemistry 27*, 8562–8568.