Role of Non-Native Aromatic and Hydrophobic Interactions in the Folding of Hen Egg White Lysozyme†

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ABSTRACT: The folding kinetics have been determined for hen egg white lysozyme and two mutants in which Trp-62 and Trp-108 have been individually replaced by tyrosine (Tyr-62-lysozyme and Tyr-108-lysozyme, respectively). An earlier study of wild-type lysozyme [Denton, M. E., Rothwarf, D. M., & Scheraga, H. A. (1994) *Biochemistry 33*, 11225−11236] had indicated that two transient intermediates were formed during the early stages of refolding. Both intermediates were characterized by substantial quenching of tryptophan fluorescence which suggested that, during the refolding process, Trp-62 and/or Trp-108 was involved in a non-native tertiary interaction(s). Both Tyr-108- and Tyr-62-lysozyme fold significantly faster than wild-type lysozyme (7- and 13-fold, respectively). These results indicate that the rate-limiting step in the folding of lysozyme arises not from any inherent slowness in the formation of the native structure but rather as a consequence of the formation of a highly stable intermediate which contains significant non-native structure which must be disrupted prior to, or in concert with, subsequent folding. The data suggest that aromatic and hydrophobic interactions play a pivotal role in the formation of the non-native intermediate. The general role that non-native interactions play in the folding process is discussed.

The problem of how proteins fold is a fundamental one in biochemistry. While it has long been known that all of the information necessary for a protein to fold is contained in the amino-acid sequence (Anfinsen, 1973), it is not well understood how the folding process occurs. It is generally accepted that, for proteins larger than ∼80 residues, the folding process is not a random one but must involve a specific pathway or set of pathways (Karplus & Šali, 1995). A pathway is uniquely defined by the intermediates and transition states which lie between the denatured and native states. Considerable effort using a variety of experimental approaches has been expended in numerous laboratories to identify and characterize transient folding intermediates and the rate-limiting transition state in an attempt to elucidate specific folding pathways (Matthews, 1993; Rothwarf & Scheraga, 1993; Evans & Radford, 1994; Roder & Elöve, 1994; Baldwin, 1995; Fersht, 1995a).

One protein whose refolding pathway has been studied extensively is hen egg white lysozyme. Lysozyme is a monomeric protein containing 129 residues and four intramolecular disulfide bonds (Figure 1). It consists of two structural domains (Janin & Wodak, 1983; McCammon et al., 1976; Miranker et al., 1991) which are the two halves of the active site cleft (Imoto et al., 1972a). One "domain" of the protein, the α-domain, consists of four α-helices along with a 3_{10} helix. The other "domain", the β-domain of the protein, contains a large triple-stranded antiparallel β-sheet, a small double-stranded anti-parallel sheet, a 3_{10} helix, and an irregular loop. Kinetic studies have monitored lysozyme refolding by a wide variety of methods, including near- and far-UV CD (Chaffotte et al., 1992; Radford et al., 1992), absorbance (Kato et al., 1981, 1982; Denton et al., 1994), inhibitor binding (Kato et al., 1981; Itzhaki et al., 1994), binding of 1-anilinonaphthalenesulfonic acid (ANS)[1] (Itzhaki et al., 1994), fluorescence (Denton et al., 1994; Itzhaki et al., 1994), and H/D exchange detected by both mass spectroscopy (Miranker et al., 1993; Hooke et al., 1995) and NMR (Radford et al., 1992).

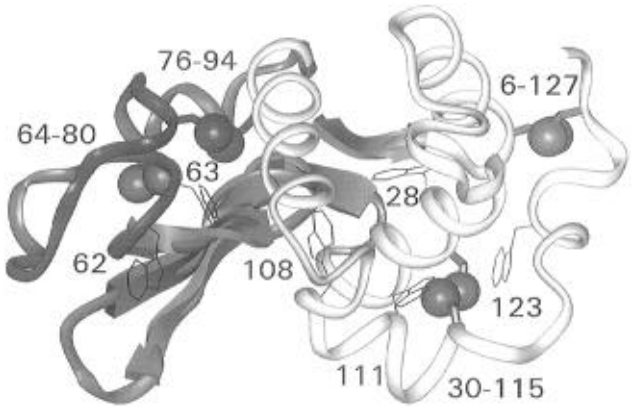

FIGURE 1: Structure of native lysozyme (Diamond, 1974). The α- and β-domains are shaded white and gray, respectively. The locations of the six tryptophan side chains and four disulfide bonds are indicated.

One of these earlier kinetic studies (Denton et al., 1994) had concluded, on the basis of fluorescence studies of wild-type lysozyme and a three-disulfide derivative of lysozyme (3SS-lysozyme), interpreted in conjunction with the H/D-

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[1] Abbreviations: Tyr-62-lysozyme, lysozyme mutant in which Trp-62 has been replaced by Tyr; Tyr-108-lysozyme, lysozyme mutant in which Trp-108 has been replaced by Tyr; 3SS-lysozyme, carboxymethyl(Cys^{6}, Cys^{127})lysozyme; ANS, 1-anilinonaphthalenesulfonic acid; GdnHCl, guanidine hydrochloride; BPTI, bovine pancreatic trypsin inhibitor; RNase A, bovine pancreatic ribonuclease A.

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exchange and CD-detected results, that the major refolding pathway of *unmodified* lysozyme could be expressed by the following sequential model:

$$U \xrightarrow{\text{burst}} I_1 \xrightarrow{\text{very-fast}} I_2 \xrightarrow{\text{fast}} N \qquad (1)$$

where U represents the unfolded protein, I_1 is an intermediate formed within the burst phase (the first 2 ms), I_2 is an intermediate formed during the "very-fast phase" (τ of ~40 ms), and N is the final native protein formed in the "fast phase" (τ of ~1 s). In this model, the first intermediate, I_1, contains significant native structure in the α-domain, much of which is metastable. The α-domain becomes stabilized in the very-fast phase, leading to the population of the second intermediate, I_2. I_2 is stabilized through the formation of non-native tertiary interactions. I_2 has a number of unusual properties. Its ellipticity when measured by far-UV CD (222 or 225 nm) is considerably more negative than the corresponding ellipticity of native lysozyme (Chaffotte et al., 1992; Radford et al., 1992). The Trp fluorescence in the I_2 intermediate is very highly quenched (Denton et al., 1994; Itzhaki et al., 1994). The intermediate is quite stable as suggested by the H/D-exchange protection factors (Radford et al., 1992) and by the fact that it is still formed even when refolding is carried out in 2 M GdnHCl (Denton et al., 1994). I_2 is estimated to be ~10 kcal/mol more stable than the unfolded state at 25 °C and pH 7.5 in the absence of denaturant (Parker et al., 1995). The final step in the folding process (the fast phase) involves the correct folding of the β-domain which coincides with the formation of the native structure.

It is important to emphasize that, in this sequential model, the intermediates I_1 and I_2 are defined primarily by the conformations present in the α-domain and are probably not unique homogeneous species but contain a distribution of conformations in the β-domain. This is consistent with H/D-exchange studies which have suggested the existence of multiple folding pathways based upon different rates of protection within the β-domain (Radford et al., 1992). The sequential model of eq 1 is roughly equivalent to the schematic pathway proposed by Radford and Dobson (1995). A model essentially identical to eq 1 has been proposed on the basis of the GdnHCl dependence of the folding and unfolding rate (Parker et al., 1995). A minor direct pathway has also been detected (Kiefhaber, 1995).

The mechanism in eq 1 suggests that the stabilization of the α-domain which occurs during the formation of I_2 could play a role in slowing the subsequent formation of the β-domain and formation of the native state. Earlier studies indicate that disruption of this intermediate by selective reduction of the 6−127 disulfide bond, which connects two regions of the α-domain (Figure 1), eliminates formation of I_2 and leads to more rapid formation of the native state (Denton et al., 1994) with synchronous formation of the α- and β-domains (Eyles et al., 1994). This is accomplished by greatly destabilizing the protein and the α-domain. The free energy of unfolding is 6−7 kcal/mol less for 3SS-lysozyme than for the wild-type protein (Denton & Scheraga, 1991; Cooper et al., 1992). One question which arises is "can an even greater acceleration in the refolding rate be achieved by disrupting only those interactions in I_2 which retard formation of the β-domain?" Earlier fluorescence studies (Denton et al., 1994) indicated that Trp-62 and/or Trp-108 was involved in the formation of I_2. We have, therefore, carried out refolding studies using Tyr-62-lysozyme and Tyr-108-lysozyme to investigate the role that these Trp residues play in directing the folding of wild-type lysozyme.

The contribution that Trp-108 and Trp-62 make to the structure and function of native wild-type lysozyme is well characterized. Trp-108 is located in the α-domain (Figure 1) and is very highly conserved in the hen-lysozyme family (Nitta & Sugai, 1989). In the native structure, it is present at the N terminus of an α-helix and is almost completely buried (Imoto et al., 1972a), making a significant contribution to the hydrophobic core and the stability of the native structure (Inoue et al., 1992). Trp-108 is in close contact with Trp-111 which is located in the same α-helix (Imoto et al., 1972a). It has been suggested that this type of interaction may play a role in stabilizing helix formation (Dobson et al., 1994; Albert & Hamilton, 1995). It has been further suggested that the significant helical structure in aqueous solution which has been observed in a peptide fragment containing residues 84−129 may be due in part to this interaction (Yang et al., 1995). Trp-108 is involved in substrate binding (Imoto et al., 1972a), and Tyr-108-lysozyme has been reported to have 17−37% of the enzymatic activity of wild-type lysozyme (Inoue et al., 1992). Tyr-108-lysozyme has also been reported to be about 1.3 kcal/mol less stable than wild-type lysozyme on the basis of GdnHCl denaturation studies (Inoue et al., 1992).

Trp-62 is located within the β-domain in an irregular loop region and is quite surface-exposed (Imoto et al., 1972a) (Figure 1). Its contacts in the native state are entirely within that loop and the rest of the β-domain, although there is evidence for energy transfer occurring between Trp-108 and Trp-62 (Imoto et al., 1972b; Formoso & Forster, 1975; Kuramitsu et al., 1978). Trp-62 is replaced by Tyr in homologous lysozymes from many species, including human (Nitta & Sugai, 1989). Trp-62 is involved in the recognition of substrate, as indicated by the observation that Tyr-62-lysozyme has a 4-fold larger K_m and a 2-fold larger k_{cat} than the wild-type enzyme (Maenaka et al., 1995). X-ray crystallographic analysis (at 1.8 Å resolution) of Tyr-62-lysozyme indicates that the overall structure is indistinguishable from that of wild-type lysozyme (Maenaka et al., 1995). The T_m of Tyr-62-lysozyme is only 1 °C less than that of the wild-type protein (P. Shih and J. F. Kirsch, personal communication).

MATERIALS AND METHODS

Materials. Hen egg white lysozyme (Sigma Chemical Co., grade I) was purified according to previously reported procedures (Denton & Scheraga, 1991). Tyr-62-lysozyme and Tyr-108-lysozyme were a generous gift from Phoebe Shih and Professor Jack F. Kirsch (University of California, Berkeley). GdnHCl, ultrapure grade, was obtained from ICN Biochemicals, Inc. Phosphate buffers were prepared using HPLC grade phosphoric acid (Fisher Scientific Co.).

Wild-type lyophilized lysozyme was dissolved in 4 M GdnHCl, 100 mM phosphate, pH 2.0 buffer. The two mutant proteins previously stored frozen at −20 °C in 66 mM phosphate buffer (pH 6.2) were exchanged into the 4 M GdnHCl buffer using a 3000 MW cutoff Centricon dialysis unit (Amicon). The concentration of wild-type lysozyme

was determined by using the extinction coefficient and equipment previously reported (Denton & Scheraga, 1991). The concentration of the mutants was determined by using the method of Edelhoch (Pace et al., 1995). This method yielded values for the wild-type and Tyr-62-lysozymes which were within 2% of the reported values [Denton and Scheraga (1991) and Kumagai and Miura (1989), respectively].

Stopped-Flow Absorbance and Fluorescence Single-Jump Folding Measurements. All measurements were carried out on equipment described previously (Denton et al., 1994) and were made at 15.0 °C. The mutants were refolded by a jump from the initial unfolding conditions (100 mM phosphate, 4 M GdnHCl, pH 2.0) to the final refolding conditions (100 mM phosphate, 0.5 M GdnHCl, pH 6.7) by a 1:10 dilution with 100 mM phosphate and 0.15 M GdnHCl at pH 7.1. Syringes were driven pneumatically with a gas pressure of 3 bar. The dead time of the instrument has been calculated to be 2.1 ± 0.2 ms (Houry et al., 1994). This value was taken into account in fitting the data.

Absorbance measurements were carried out at 250 nm, since this wavelength gave the largest refolding amplitudes for wild-type lysozyme (Denton et al., 1994).

Fluorescence measurements were made using an excitation wavelength of 280 nm, and the intrinsic fluorescence was monitored by using a 295−400 nm band-pass filter. The excitation path length was 10 mm, and the emission path length was 2 mm. Initial protein concentrations were 142 μM for wild-type lysozyme, 131 μM for Tyr-62-lysozyme, and 133 μM for Tyr-108-lysozyme. Since very-high accuracy kinetic refolding measurements of the wild-type protein had already been made under the same refolding conditions (Denton et al., 1994), the wild-type protein was used only to determine the final folded fluorescence value for direct comparison with the mutant proteins and to check that the instrument was working properly. Values obtained from these control experiments were in agreement with those reported previously (Denton et al., 1994) (data not shown).

Double-Jump Fluorescence Assay. To determine the rate of formation of the native state, a double-jump assay was carried out. In the first step, the protein was refolded using 1:10 dilution exactly as described above except that the initial protein concentrations used were 273 μM for wild-type lysozyme, 173 μM for Tyr-62-lysozyme, and 149 μM for Tyr-108-lysozyme. After a variable time delay, each protein was unfolded by a 2.5:1 dilution into 100 mM phosphate and 6.1 M GdnHCl at pH 1.0 to yield final conditions of 100 mM phosphate and 4.5 M GdnHCl at pH 2.0. The fluorescence amplitude of the unfolding process was measured using the same equipment as for the single-jump experiments except that a monochromator from Edinburgh Instruments Ltd. was used during the excitation. The band-pass was ∼15 nm. The shortest folding time that could be measured was 68 ms, and measurements were carried out at this folding time with both mutants. For wild-type lysozyme, the shortest folding time measured was 250 ms. The longest folding time measured for wild-type lysozyme and Tyr-62-lysozyme was 10 s. For Tyr-108-lysozyme, a 10 min time point was also taken to ensure that the protein was completely folded by 10 s and that we were not observing an intermediate.

The double-jump unfolding assays were repeated at least three times (in most cases four times) at each refolding time point. The refolding rate constant was determined from the fluorescence amplitudes. In order to minimize any systematic errors arising from lamp fluctuations and instrument drift, repeats of the data for each time point were not made consecutively but rather were made by acquiring the data at all of the time points that comprised the curve before repeating the measurements. In addition, since the final fluorescence value of the unfolded protein should be the same independent of the folding time, this value was used to correct each individual amplitude for fluctuations in lamp intensity and/or photomultiplier response.

Table 1: Relative Fluorescence Intensities[a]

lysozyme	unfolded/folded[b] (%)	folded/wild-type[c] (%)
Tyr-62	156 ± 8[d]	73 ± 8
Tyr-108	150 ± 6	73 ± 3
wild-type	122 ± 3[e]	100

[a] Experimental conditions were as follows: unfolding conditions, 4 M GdnHCl, pH 2; folding conditions, 0.5 M GdnHCl, pH 6.7. [b] Fluorescence intensity of protein under unfolding conditions expressed as the percent of protein fluorescence under folding conditions. [c] Fluorescence intensity of protein under folding conditions expressed as the percent of wild-type lysozyme fluorescence under folding conditions. [d] The error is calculated at the 95% confidence limit. [e] Obtained from Denton et al. (1994).

Data Analysis. The single-jump data were fit as described previously (Denton et al., 1994); however, the refolding of Tyr-108-lysozyme was fit using a sequential model. Each single-jump experiment was repeated a minimum of seven or more times, resulting in multiple data sets. Each data set was fit separately, and standard deviations were determined in the usual manner by averaging over the data sets. All errors are given at the 95% confidence limit.

The double-jump data were fit to a single exponential using the procedures described by Houry et al. (1994).

RESULTS

Before the data are presented, it is important to mention that these studies consider only the fast-folding phases of lysozyme. A slow phase which is believed to result from proline isomerization has been observed in earlier studies (Kato et al., 1981). Only ∼10% of the protein refolds through the slow phase. This phase corresponds to ∼10% of the total absorbance change and makes a much smaller contribution to the fluorescence-detected refolding. Consequently, it will have a negligible influence on the results presented here.

All of the fluorescence amplitudes reported here are normalized to the total fluorescence of the native protein under the final folding conditions. This value was determined directly from the refolding experiments and corrected for contributions from scattered light by baseline subtraction.

Relative Fluorescence Intensities of Lysozymes. The fluorescence intensities of unfolded Tyr-62-lysozyme, Tyr-108-lysozyme, and wild-type lysozyme relative to the fluorescence intensities of those species under folding conditions are shown in Table 1. This table also shows the fluorescence intensities of the folded mutant proteins relative to that of folded wild-type lysozyme. The values obtained for the two mutant lysozymes are the same within experimental error. These values (73%) are somewhat larger than the values of ∼50% obtained in earlier studies of oxidized derivatives of lysozyme (Teichberg & Sharon, 1970; Imoto

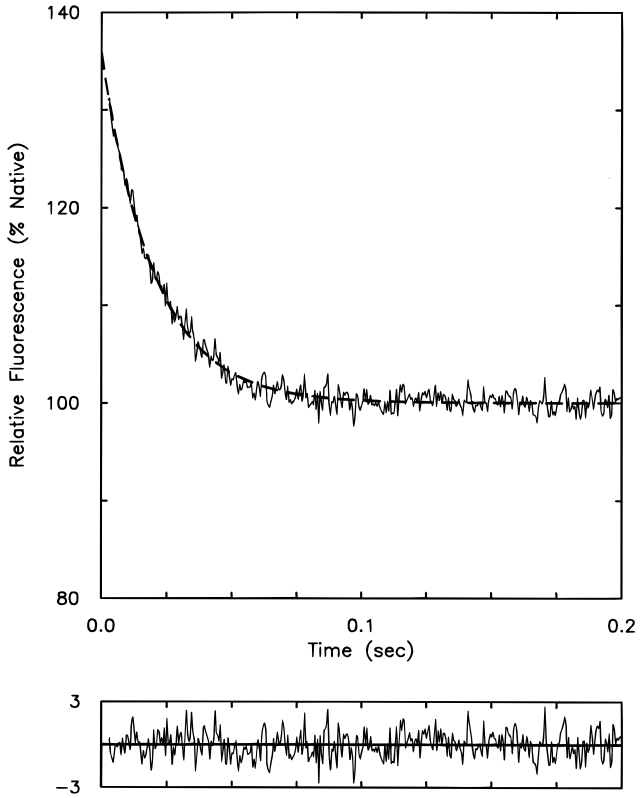

FIGURE 2: Typical data set showing the refolding of Tyr-62-lysozyme monitored by fluorescence emission between 295 and 400 nm at 15 °C. The excitation wavelength was 280 nm. Refolding was achieved by a 10:1 stopped-flow dilution and pH jump from 4 M GdnHCl (pH 2.0) to 0.5 M GdnHCl (pH 6.7) in 100 mM sodium phosphate buffer. The final protein concentration was 12 μM. The lower panel shows the residuals, the difference between the data and the fitted function (dashed line). The data are normalized to 100% for the native state. The fit of the calculated curve was judged by χ^2 analysis ($\chi^2 = 1.01$).

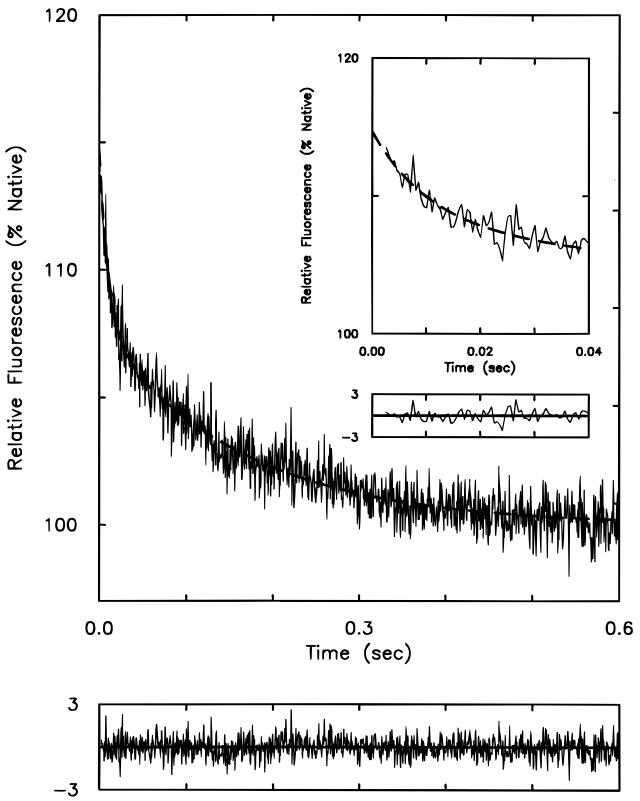

FIGURE 3: Typical data set showing the refolding of Tyr-108-lysozyme monitored by fluorescence emission at 15 °C. The inset shows a blowup of the early time regime. $\chi^2 = 1.02$. All other information is as described in Figure 2.

et al., 1972a,b). However, the results are in agreement with a previous fluorescence study of Tyr-62-lysozyme which found a value of 70% (Kumagai et al., 1992).

Fluorescence-Detected Refolding. Typical fluorescence-detected data sets for the refolding of Tyr-62-lysozyme and Tyr-108-lysozyme are shown in Figures 2 and 3, respectively. Both proteins were refolded in 0.5 M GdnHCl and 100 mM sodium phosphate at pH 6.7 and 15 °C. Figure 2 illustrates that the refolding of Tyr-62-lysozyme occurs in a single-exponential phase. The amplitude of this phase is rather large, 36.4% of the total fluorescence of native Tyr-62-lysozyme, and involves fluorescence quenching. In contrast, the refolding of Tyr-108-lysozyme is clearly biphasic (Figure 3) (fitting of the data to a single exponential results in a χ^2 of 1.4 and significant deviations from randomness in the residuals). The amplitudes of both phases are relatively small, approximately 8% of the total fluorescence of native Tyr-108-lysozyme, and involve the quenching of fluorescence. It is important to note that the fluorescence-detected refolding of both mutants lacks the presence of the highly quenched intermediate observed by Denton et al. (1994) during the refolding of wild-type lysozyme.

The fluorescence refolding data indicate the presence of a hidden phase that occurs during the dead time of the mixing. This dead time is approximately 2.1 ms (Houry et al., 1994). The amplitude of this phase was determined by measuring the fluorescence ratio of the unfolded protein relative to that of the folded state. These ratios are listed in Table 1, and the values of the burst phase amplitudes are shown in column 2 of Table 2. From Table 2, it can be seen that the burst phase amplitude for Tyr-108-lysozyme is much greater than that of Tyr-62-lysozyme but similar to that of wild-type lysozyme.

The time constants for the refolding phases are shown in columns 3 and 5 of Table 2. Both mutants refold significantly faster than wild-type lysozyme when monitored by fluorescence.

Absorbance-Detected Refolding. Figures 4 and 5 show refolding data sets for Tyr-62-lysozyme and Tyr-108-lysozyme, respectively, monitored by absorbance at 250 nm when the proteins are refolded at 15 °C, 0.5 M GdnHCl, 100 mM phosphate, and pH 6.7 (the same conditions as in Figures 2 and 3). The refolding of both lysozyme mutants is monoexponential, as contrasted with the absorbance-detected refolding of wild-type lysozyme which is a double-exponential process [see Table 3 and also Denton et al. (1994)]. The total absorbance changes upon refolding of the mutant and wild-type lysozymes are similar (Table 3), suggesting that Trp-62 and Trp-108 are not the optical probes being monitored by absorbance at 250 nm. The time constant for Tyr-108-lysozyme agrees well with the time constant of the slower phase monitored by fluorescence, suggesting that the two methods are monitoring the same process. This agreement between absorbance and fluorescence is observed for both phases in the wild-type protein. In the case of Tyr-62-lysozyme, refolding monitored by absorbance is much slower than the fluorescence-monitored refolding. Possible explanations for this will be presented in the Discussion.

Table 2: Kinetic Data for Fluorescence-Detected Refolding[a]

lysozyme	hidden amplitude (%)[b]	τ_1 (s × 10^3)	amplitude of τ_1 (%)	τ_2 (s)	amplitude of τ_2 (%)
Tyr-62	−19.3 ± 3.2[c]	21.1 ± 0.4	−36.4 ± 1.0	–	–
Tyr-108	−35.0 ± 6.0	9.3 ± 2.3	−7.8 ± 1.3	0.154 ± 0.015	−7.9 ± 0.4
wild-type[d]	−44.9 ± 3.0	36.9 ± 0.7	−36.1 ± 1.1	1.01 ± 0.01	59.1 ± 1.7

[a] Experimental conditions were as follows: initial conditions, 4 M GdnHCl, pH 2; final conditions, pH 6.7, 15 °C. [b] Amplitudes are normalized to the total fluorescence of the folded protein under the final folding conditions. A negative sign indicates that fluorescence quenching is occurring in that phase. [c] The error is calculated at the 95% confidence limit. [d] Obtained from Denton et al. (1994).

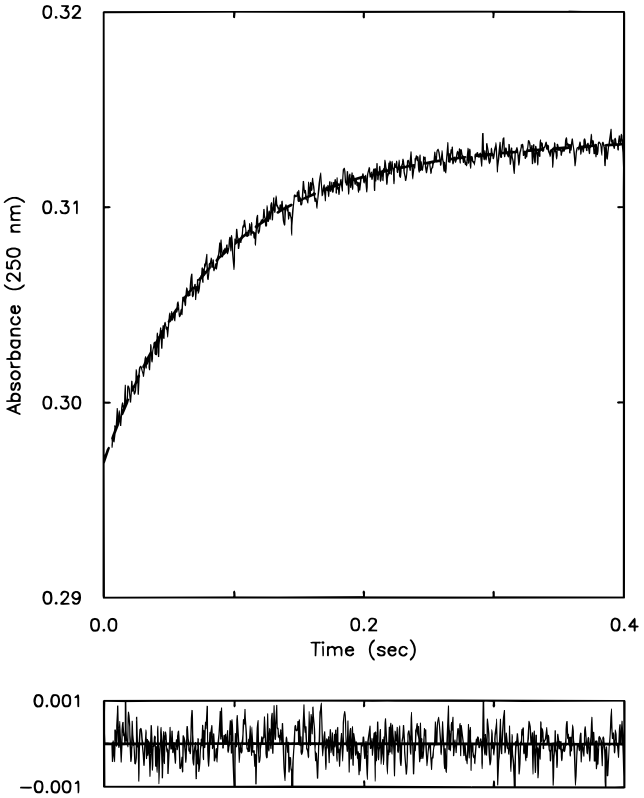

FIGURE 4: Typical data set showing the refolding of Tyr-62-lysozyme monitored by absorbance (observed OD) at 250 nm and 15 °C. $\chi^2 = 1.02$. All other information is as described in Figure 2.

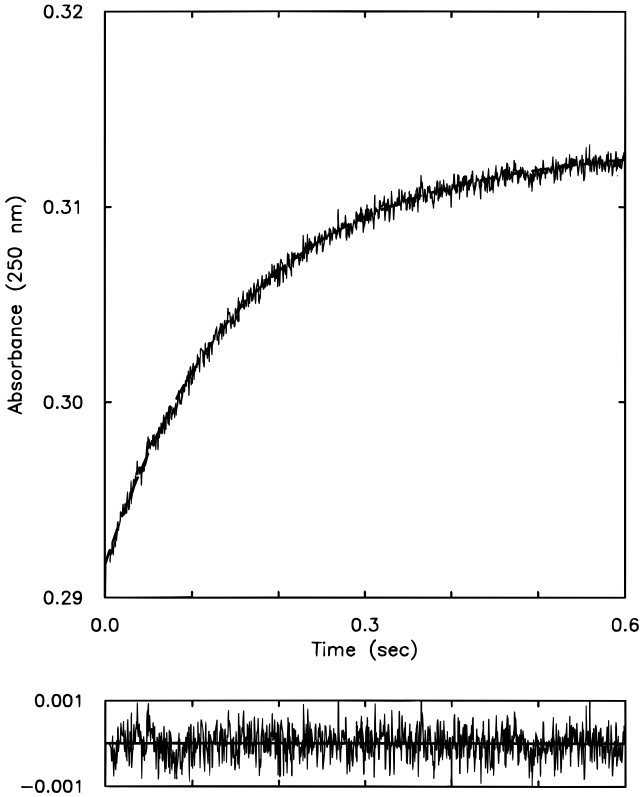

FIGURE 5: Typical data set showing the refolding of Tyr-108-lysozyme monitored by absorbance (observed OD) at 250 nm and 15 °C. $\chi^2 = 1.04$. All other information is as described in Figure 2.

Double-Jump Unfolding Assay. The double-jump unfolding assay monitors the formation of native protein during refolding. In these experiments, completely unfolded protein is jumped to folding conditions. At various times during the refolding, the solution is jumped back to unfolding conditions. Under these final conditions, any folding intermediates will unfold rapidly while the native state unfolds slowly. The amplitude of the slow-unfolding process is monitored, thereby providing a quantitative measurement of the amount of native protein formed at that refolding time. The data from the double-jump unfolding assay are shown in Figure 6, and the results obtained from fitting those data are given in Table 4. The time constants agree within experimental error with the slowest time constants monitored by absorbance in the single-jump refolding experiments, suggesting that the absorbance-detected refolding monitors the formation of the final folded state. The amplitudes for wild-type and Tyr-62-lysozyme are significantly less than 100%, suggesting that the formation of the native state may be biphasic with significant formation occurring at early times in the folding. Specifically, the results indicate that 12.9 ± 4.9% of wild-type lysozyme and 29 ± 14.2% of Tyr-62-lysozyme is formed in this rapid-refolding phase. Using this same type of double-jump assay, Kiefhaber (1995) found that ~14% of wild-type lysozyme refolds through a rapid-refolding phase.

The unfolding time constants at 4.5 M GdnHCl and pH 2.0 were 155 ± 12 s for Tyr-62-lysozyme, 219 ± 15 s for wild-type lysozyme, and 11.1 ± 0.4 s for Tyr-108-lysozyme (all errors are calculated at the 95% confidence limit). These values are consistent with the lower stabilities of the mutant proteins as discussed in the introductory section.

DISCUSSION

Formation of the Native Species. Whenever optical probes are changed by mutation, extreme care must be taken in comparing refolding rate constants. An apparent difference in the refolding rate of a mutant does not necessarily indicate any change in the folding pathway, since these differences could arise from alteration of the reporting groups. While it is generally assumed that absorbance measurements monitor the formation of native lysozyme, it is, however, essential to carry out experiments which measure the rate of formation of the native species unambiguously. The double-jump unfolding assay is such a procedure. Table 4 shows the results of these studies. Given the fact that the

Table 3: Kinetic Data for Absorbance-Detected Refolding at 250 nm[a]

lysozyme	τ_1 (s × 10^3)	amplitude of τ_1 (M^{-1} cm^{-1} × 10^{-2})	τ_2 (s)	amplitude of τ_2 (M^{-1} cm^{-1} × 10^{-2})
Tyr-62	–	–	78.1 ± 4.1[b]	13.1 ± 0.8
Tyr-108	–	–	151.0 ± 8.2	15.7 ± 0.6
wild-type[c]	36.7 ± 8.6	3.53 ± 1.26	1.00 ± 0.03	10.3 ± 0.4

[a] Experimental conditions were as follows: initial conditions, 4 M GdnHCl, pH 2; final conditions, pH 6.7, 15 °C. [b] The error is calculated at the 95% confidence limit. [c] Obtained from Denton et al. (1994).

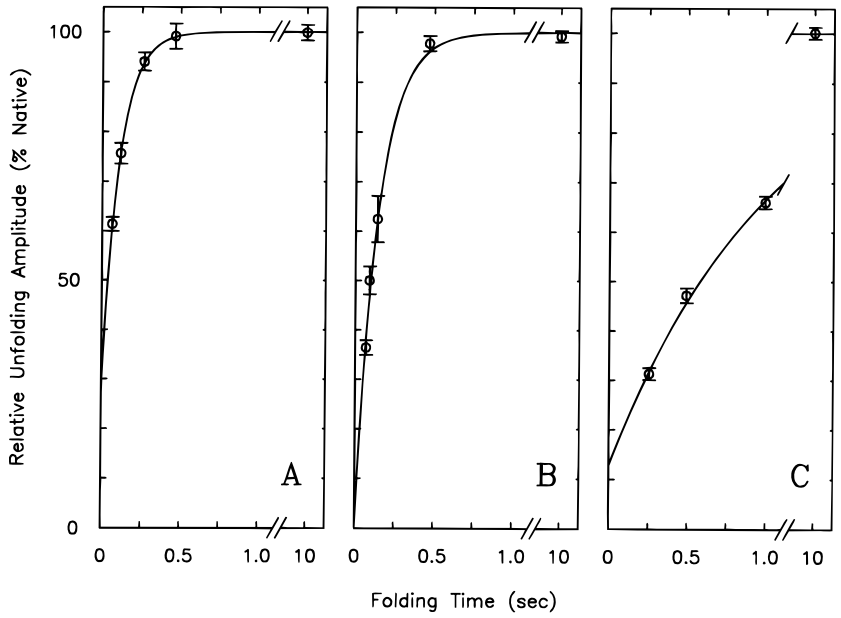

FIGURE 6: Change in the relative fluorescence amplitude upon unfolding the protein at 4.5 M GdnHCl, pH 2.0, and 15 °C: (A) Tyr-62-lysozyme, (B) Tyr-108-lysozyme, and (C) wild-type lysozyme. Double-jump experiments were carried out as follows. Protein was dissolved in 4.0 M GdnHCl (pH 2.0) and then refolded in 0.5 M GdnHCl (pH 6.7). After a certain folding time, the protein was unfolded at 4.5 M GdnHCl (pH 2.0) to determine the amount of folded protein. The solid curves show the theoretical fit to the data. Error symbols show the standard deviation of the individual measurements.

Table 4: Kinetic Data for Fluorescence-Detected Double-Jump Unfolding[a]

lysozyme	τ^b (s)	amplitude of τ^c (%)
Tyr-62	0.109 ± 0.032[d]	71.0 ± 14.2
Tyr-108	0.144 ± 0.013	99.9 ± 0.2
wild-type	1.04 ± 0.13	87.1 ± 4.9

[a] Data used are those of Figure 6. Double-jump experiments were carried out by first folding from 4.0 M GdnHCl (pH 2) to 0.5 M GdnHCl (pH 6.7) and then unfolding to 4.5 M GdnHCl (pH 2.0). The time of folding was varied. τ is the time constant for folding. [b] τ reflects the formation of native protein and corresponds to τ_2 values given in Tables 2 and 3. [c] Amplitudes are normalized to the total fluorescence of the folded protein under the final folding conditions. [d] The error is calculated at the 95% confidence limit.

time constants for the final absorbance-detected refolding phase agree within experimental error with the results from the double-jump assay, we will use the absorbance time constants which have much smaller errors when discussing the rate of formation of native protein.

Folding Rate. The rate of formation of the native structure is slowest for wild-type lysozyme. While Tyr-108-lysozyme and 3SS-lysozyme fold significantly faster (7- and 2-fold, respectively) than wild-type lysozyme, these derivatives are much less stable than the wild-type protein [~1.3 kcal/mol (Inoue et al., 1992) and 6−7 kcal/mol (Denton & Scheraga, 1991; Cooper et al., 1992), respectively]. Trp-108 and the 6−127 disulfide bond are involved in interactions primarily within the α-domain of the protein. On the other hand, Trp-62 is located in the β-domain portion of the protein, and the T_m of Tyr-62-lysozyme is only 1 °C less than that of the wild-type protein (P. Shih and J. F. Kirsch, personal communication). Tyr-62-lysozyme is the fastest-folding species, refolding 13 times faster than wild-type lysozyme.

While it could be argued that each of the lysozyme species has separate folding pathways and we are observing separate rate-enhancing processes, it seems more likely that the acceleration in folding rate in each of the derivatives occurs by the same mechanism, i.e. the destabilization of the I_2 intermediate (eq 1). The formation of a stable intermediate will in general serve to slow the folding process (Sosnick et al., 1994; Fersht, 1995b; Houry et al., 1995). In support of the importance of the stability of I_2 to the folding rate is the failure to observe this intermediate during the refolding of any of the mutant lysozymes. Since I_2 contains a largely native-like α-domain structure, any mutations that destabilize the α-domain in the native state are likely to destabilize I_2. It has recently been suggested that helical structure in a peptide that corresponds to residues 84−129 of lysozyme is stabilized by side chain interactions involving Trp-108 (Yang et al., 1995). In addition, in the native state, Trp-108 is buried within the hydrophobic core of the protein. Similarly, the 6−127 disulfide bond is localized in the α-domain (see Figure 1) and connects the C- and N-terminal portions of lysozyme. On the basis of the supposition that the stability of I_2 slows refolding, both of these derivatives would be expected to refold more rapidly than the wild-type protein, which they do.

Structure of the I_2 Intermediate. From the data presented here and data from other studies, a picture of the structure of the I_2 intermediate emerges. The rapid refolding of Tyr-62-lysozyme cannot be explained in terms of the stability of the α-domain in the native protein. Trp-62 is located in the β-domain and has no direct contacts with residues in the α-domain in the native state. Trp-62 is also fairly surface-exposed in the native state and does not contribute significantly to the hydrophobic core or the conformational stability of wild-type lysozyme. Given the arguments in the preceding section, this would suggest that Trp-62 is stabilizing I_2 presumably by participating in non-native tertiary interactions. Earlier studies described in the introductory section had suggested that non-native tertiary interactions were involved in stabilizing I_2 and that either Trp-62 and/or Trp-108 was able to monitor this process. Clearly, the enhanced refolding rates of Tyr-62-lysozyme and Tyr-108-lysozyme suggest that those two Trp residues are not simply probes of the formation of the intermediate but are active participants in its formation.

The involvement of Trp-62 in formation of I_2 is further supported by H/D-exchange studies which show that the neighboring residues Trp-63 and Cys-64 are protected with the same time constants as the α-domain (Radford et al., 1992). Protection of Trp-62 in I_2 cannot be determined because its amide proton is not protected from exchange in the final native state.

Additional structural information can be obtained from near-UV CD measurements which are a traditional method of ascertaining the existence of specific tertiary contacts involving Trp and Tyr residues. Studies of the refolding of wild-type lysozyme (Chaffotte et al., 1992) at pH 7.8, 20 °C, and 0.075 M GdnHCl reveal that there is a sizeable change in the CD at 289.5 nm occurring on a time scale consistent with the formation of I_2. Radford et al. (1992) have also reported observing a phase with a comparable time constant at 289 nm at pH 5.2, 20 °C, and 0.54 M GdnHCl. Curiously, the amplitudes of the phase reported by the two groups were of opposite magnitude. Recently, Itzhaki et al. (1994) have been unable to detect this phase under the solution conditions used by Radford et al. (1992) and suggest that the observation by Radford et al. (1992) was artifactual. The apparent absence or presence of this phase may be due to the different pH and/or GdnHCl concentrations at which the measurements were made. Only two groups in native wild-type lysozyme titrate appreciably over the pH range of 5.2−7.8. They are His-15 and Glu-35 (Imoto et al., 1972a). Both residues are in the α-domain, and Glu-35 is found in a nonpolar environment in van der Waals contact with Trp-108 (Imoto et al., 1972a). This would seem to suggest that Glu-35 is in some way involved in the I_2 intermediate and would be consistent with the view that the structure in the α-domain in I_2 is quite native-like.

H/D-exchange results (Radford et al., 1992) indicate that three residues in the β-domain—Trp-63, Cys-64, and Ile-78—are protected in I_2. Lysozyme has a disulfide bond between residues 64 and 80. It, therefore, seems likely that Cys-80 and Pro-79 are also involved. Because these residues are not protected in the native protein, this cannot be confirmed. The structure of I_2 can best be described as an essentially native α-domain forming a non-native hydrophobic/aromatic cluster with residues in the irregular loop region of the β-domain (see Figure 1). This structure probably includes Trp-62, Trp-63, Cys-64, Ile-78, Pro-79, and Cys-80 and contains specific non-native tertiary contacts. The contribution of aromatic interactions will be discussed in greater detail below.

One point of interest is that, from the above discussion, there is no evidence for any significant contribution to I_2 from the residues found in the triple-stranded β-sheet in the native structure (see Figure 1). This would seem to suggest that creation of a lysozyme derivative with extensive destabilizing mutations made within the β-sheet region would result in I_2 being the energetically most stable state. Creation of such a mutant would permit detailed NMR structural studies to be carried out.

Structure of the I_1 Intermediate. The burst phase fluorescence amplitude of wild-type lysozyme has previously been interpreted as reflecting formation of fluctuating helical structure within the α-domain (Denton et al., 1994). On the basis of this, one would expect that the replacement of Trp-108, which is in the α-domain, with Tyr would have a greater effect on the burst phase than replacement of Trp-62 with Tyr. As shown in column 2 of Table 2, this is not the case. The burst phase amplitude of Tyr-108-lysozyme is almost twice that of Tyr-62-lysozyme. This difference is not due to differences in the quantum yields of either the folded or GdnHCl-denatured state, as shown in Table 1. This result suggests that the intermediate formed in the fluorescence-detected burst phase not only contains fluctuating structure within the α-domain but also must involve Trp-62. This is consistent with the results from H/D-exchange studies (Radford et al., 1992) which show that the residues adjacent to Trp-62 are protected coincidentally with the α-domain. The amides at these sites are, however, quite labile in I_1 and achieve protection factors comparable to those of the α-domain only after formation of I_2. This would suggest that the I_1 intermediate (eq 1) is probably very similar to I_2 in structure but is much less stable, lacking specific tertiary contacts (possibly arising from aromatic interactions as discussed in the next section). This interpretation is supported by recent studies of the GdnHCl dependence and stabilities of the I_1 and I_2 intermediates which suggest that only minor reorganization occurs in the formation of I_2 from I_1 (Parker et al., 1995).

Aromatic Interactions. Interactions involving aromatic residues make considerable contributions to the three-dimensional structure of proteins (Burley & Petsko, 1985, 1988; Hunter et al., 1991; Serrano et al., 1991). Their role in protein folding is less defined since, in most proteins, the interactions are between Phe and Tyr and the magnitude of $\pi-\pi$ interactions for such small π-systems is on the order of 1 kcal/mol (Burley & Petsko, 1985, 1988). The enthalpic contributions from π stacking in heterocyclic ring systems such as Trp should be significantly greater (Burley & Petsko, 1988; Hunter & Sanders, 1990). However, because of the relative scarcity of Trp in proteins, very little information is available.

The involvement of aromatic interactions in the major refolding pathway of lysozyme is suggested by several experimental observations. As discussed above, lysozyme has six Trp's, all of which appear to be contributing to the stability of I_2. Florescence quenching experiments using potassium iodide have been used to evaluate the solvent accessibility of the Trp residues during refolding (Itzhaki et al., 1994). Those results indicate that there is significant

reduction in the solvent accessibility of Trp's in I_1, with the final and major change occurring during formation of I_2. No significant alterations in the solvent accessibility of the Trp residues occur after formation of I_2. This protection from solvent is not the result of a general hydrophobic collapse since ANS binding studies indicate that there is still significant exposed hydrophobic surface in I_2 (Itzhaki et al., 1994). Aromatic interactions (particularly ring stacking) also have been implicated in making large negative contributions to the CD spectrum at 222 nm (Arnold et al., 1992). These contributions are over 40-fold greater than those observed in studies of tryptophan amide (Adler et al., 1973) and are of the appropriate magnitude to account for the very large negative ellipticity at 222 nm exhibited by I_2 in kinetic refolding studies (Chaffotte et al., 1992; Radford et al., 1992). Moreover, such ring-stacking interactions result in fluorescence quenching (Longworth, 1966) which is also in excellent agreement with the fluorescence properties of I_2, in which the intrinsic Trp fluorescence is almost completely quenched (Denton et al., 1994; Itzhaki et al., 1994). Aromatic interactions have very little effect on absorbance spectra (Hunter & Sanders, 1990), in agreement with the very small absorbance changes observed upon formation of I_2 [see Table 3 and Denton et al. (1994)].

Fluorescence-Detected Refolding. While the time constants for the fluorescence-detected refolding of wild-type lysozyme are in agreement with the absorbance-detected measurements, the time constants of the mutants are different when monitored by the two optical techniques. The refolding of Tyr-62-lysozyme is approximately 4-fold faster when monitored by fluorescence than when monitored by absorbance, clearly indicating that different processes are being observed. Given that four of the remaining five Trp residues are in the α-domain, it is very likely that the fluorescence measurements are monitoring the formation of structure within the α-domain. Moreover, given the absence of any subsequent phase corresponding to formation of the final native structure, this would suggest that the fluorescence-detected structure is predominantly native-like.

The biphasic folding behavior of Tyr-108-lysozyme by fluorescence is more difficult to explain. The slower phase clearly corresponds to the formation of the native species, and the faster phase must correspond to formation of an intermediate. Whether this intermediate is native-like cannot be determined from the existing data, and H/D-exchange studies are probably required to answer this question.

Multiple Phases. It has recently been reported (Kiefhaber, 1995) that the native species of lysozyme is formed in two phases, not one as had been generally presumed. By carrying out double-jump unfolding studies very similar to those performed here, but over a wider range of refolding times, Kiefhaber (1995) has been able to show that, in addition to the folding process that is observed spectroscopically, there is a second more rapid and direct folding phase (∼50 ms) in which ∼14% of the protein refolds. These results can be compared with the results of the double-jump unfolding studies that we have presented here. However, since the first refolding time that we have monitored for wild-type lysozyme (Figure 6C) is 253 ms, we cannot comment on the time constant for this faster phase but only on the amplitude. We find that the amplitude of this faster phase (assuming that it is much faster than 253 ms) is 12.9 ± 4.9% (Table 4). A phase comparable to this has been observed by H/D exchange detected by both mass spectroscopy (Miranker et al., 1993) and NMR (Radford et al., 1992). These H/D-exchange results have been interpreted in terms of formation of an intermediate rather than native structure, on the basis of inhibitor binding studies (Itzhaki et al., 1994). The inhibitor-binding-detected refolding very clearly shows that no direct rapid formation of native lysozyme is occurring. It has, however, been suggested that the failure to observe any rapid phase by inhibitor binding may, in part, be due to the slow time constant for the binding of native lysozyme to the inhibitor (5−10 ms) (Kiefhaber, 1995).

It is important to note that the results of the double-jump unfolding assay for Tyr-108-lysozyme suggest that there is no measurable rapid phase [99.9 ± 0.2% refolds through the major pathway (Table 4)]. This would suggest that replacement of Trp-108 with Tyr, in addition to destabilizing I_2 and accelerating the major refolding pathway, also decelerates (or eliminates) the direct rapid-folding pathway.

Interestingly, the results of the double-jump unfolding assay for Tyr-62-lysozyme suggest that a larger rapid phase occurs in its folding than in the case of wild-type lysozyme, 29.0 ± 14.2% (Table 4). Since the earliest refolding time measured was 64 ms (Figure 6A), either the refolding is much faster than the ∼50 ms time constant reported for wild-type lysozyme by Kiefhaber (1995) or the amplitude shown in Table 4 is much larger than the true amplitude; i.e. more native protein forms through a rapid phase. However, the uncertainty in the amplitude of this phase is quite large. If we fit the data using the time constant for refolding obtained from the absorbance data (78.1 ms from Table 3), then the amplitude becomes 12%, virtually the same value as observed for wild-type lysozyme.

Now it must be asked, "if there are two folding phases leading to formation of native structure, shouldn't they be observable by optical spectroscopy (absorbance, fluorescence, and CD)?" Unfortunately, in the case of wild-type lysozyme, I_2 gives rise to large signals in all optically detected measurements obscuring the much smaller amplitude expected for the rapid direct-folding process. Refolding of Tyr-62-lysozyme as shown in Figures 2 and 4 is clearly a single-exponential process. Given that there is a 2.1 ms dead time of mixing and significant mixing artifacts in absorbance-detected refolding which persist for 6−12 ms beyond that, it is unlikely that this small amplitude would be detectable by absorbance unless its time constant were greater than ∼30 ms. This suggests that the direct rapid folding pathway for Tyr-62-lysozyme is more rapid than the corresponding phase for the native protein.

In contrast to absorbance-detected folding, fluorescence-detected folding is not affected by any significant mixing artifacts (this is primarily due to the much larger absolute amplitudes of the fluorescence-detected folded phases and the much shorter effective observation path length used in the fluorescence measurements). However, no evidence for a rapid-refolding phase is observed in the fluorescence-detected folding of Tyr-62-lysozyme. This is largely due to the observation, as discussed above, that the fluorescence-detected refolding of Tyr-62-lysozyme does not appear to report on the formation of the native species but rather on the formation of an intermediate.

It is important to stress that there are only a small number of time points taken in the double-jump refolding assay, and the earliest time point is already almost equal to the refolding

time constant. Consequently, any systematic errors that are unaccounted for in the individual values could easily lead to a large error in the refolding amplitudes shown in column 3 of Table 4. As discussed above, the main reason for carrying out the double-jump unfolding assay was to confirm that the absorbance-detected refolding was providing an accurate measurement of the rate of formation of the native state.

Homologous Lysozymes. A number of detailed folding studies have been carried out on homologous lysozymes, especially human lysozyme. The refolding properties of these homologous lysozymes are often compared to those of hen egg white lysozyme. Seventy-eight of the 129 residues are identical in human and hen egg white lysozyme. Human lysozyme refolds about 4 times more rapidly than hen egg white lysozyme (Hooke et al., 1994). The refolding of human lysozyme exhibits none of the unusual optical properties observed during the refolding of hen egg white lysozyme (Herning et al., 1991; Hooke et al., 1994). Hooke et al. (1994), studying the refolding of human lysozyme using a variety of probes, including H/D exchange, made the very reasonable suggestion that the differences in the refolding properties of the two lysozymes arise as a result of differences in the efficiency of packing of the hydrophobic core of the two proteins. However, one of the differences between the two proteins is that human lysozyme has a Tyr at residue 62. Given the results of the Tyr-62-lysozyme refolding studies presented here, it seems likely that many of the differences in folding between human and hen lysozymes are a result of this single side chain substitution. Consequently, it is the stability of non-native structure rather than the efficiency of packing that dictates the relative refolding rates of the two proteins.

Lysozyme-Folding Model. The results presented here support the sequential mechanism shown in eq 1 and suggest that both native and non-native hydrophobic and aromatic interactions stabilize I_1 and I_2. The results of double-jump unfolding assays [presented here and by Kiefhaber (1995)] suggest that, in addition to the major pathway described by eq 1, a direct pathway exists. These two pathways arise from a kinetic partitioning that probably occurs very early in the folding process. This is supported by H/D-exchange studies (Radford et al., 1992; Miranker et al., 1993) which indicate that a species with native-like protection in both the α- and β-domains forms with a time constant of 5−10 ms. Moreover, the analysis presented above suggests that I_1 contains non-native interactions which presumably would hinder direct formation of N. This is also consistent with the results of Parker et al. (1995), who found that I_1 is ∼7−8 kcal/mol more stable than the unfolded species.

The question may be asked if there is any evolutionary advantage to the population of I_1 and I_2. One possibility is that the non-native structures formed in the intermediates are important in the physiological folding process in which the disulfide bonds are formed. Stabilization of the α-domain could direct the correct formation of specific disulfide bonds, thereby accelerating the oxidative refolding process. It is interesting to note that the rate of the in vitro oxidative refolding of wild-type hen egg white lysozyme (Saxena & Wetlaufer, 1970) is considerably faster than that of other well-studied proteins such as ribonuclease A (RNase A) (Rothwarf & Scheraga, 1993) and bovine pancreatic trypsin inhibitor (BPTI) (Creighton & Goldenberg, 1984). In line with this suggestion is the experimental observation that human lysozyme regenerates more slowly than hen egg white lysozyme (Dubois et al., 1982).

Implications for Protein Folding. The results presented here highlight a general problem in studying protein folding. The rate-limiting step(s) in the folding process does not necessarily involve exclusively the progressive formation of native structure but may include significant contributions arising from the need to disrupt existing interactions. The role that structure plays in slowing the folding process is becoming increasingly clear as experimental studies (Jackson & Fersht, 1991; Houry et al., 1994, 1995; Sosnick et al., 1994; Kragelund et al., 1995; Villegas et al., 1995; Schindler et al., 1995) demonstrate that protein folding is an inherently very rapid process, perhaps sub-millisecond, if stable intermediates could be eliminated. While most of these rapidly folding proteins are much smaller than lysozyme, in studies of two proteins of comparable size, cytochrome *c* (Sosnick et al., 1994) and RNase A (Houry et al., 1994), refolding has been shown to occur on the millisecond time scale once non-native interactions were eliminated. These non-native interactions arose from heterogeneity within the unfolded state, e.g. cis/trans proline isomerization. In the case of lysozyme, considerable evidence suggests that its major folding pathway is not affected by any residual structure in the unfolded state (Kotik et al., 1995).

The disruption of structure during refolding has also been demonstrated in the oxidative refolding of BPTI (Weissman & Kim, 1992) and has been suggested to be a common occurrence in disulfide-linked folding (Weissman & Kim, 1995). A similar observation has been made in the case of the refolding of ribonuclease T1 where the rate of cis/trans proline isomerization is slowed by structure formation (Kiefhaber et al., 1992; Mücke & Schmid, 1994). However, in these proteins, the structure formed is native-like structure.

The folding pathway of lysozyme would, therefore, appear to be somewhat unique in that it requires the disruption of non-native structure that does not arise from heterogeneity in the unfolded state. However, since it is very difficult to detect non-native structure by conventional experimental procedures (Creighton, 1991), the presence of non-native interactions may be quite common.

In studies of lysozyme refolding, it has been possible to detect the presence of the non-native tertiary contacts because Trp residues are directly involved in the formation of the non-native structure. Most proteins do not have as high a Trp content as lysozyme, and it is, therefore, much more difficult to detect the formation of non-native structure. Mutations that destabilize an intermediate and thereby accelerate the folding process provide a clear indication that the intermediate is slowing the folding process. However, the interpretation of data from such mutants is rarely straightforward because mutations will in general make contributions to the intrinsic folding rate. We have been very fortunate in this regard. The mutation of Trp-62 to Tyr was propitious, since that residue makes no significant contribution to the structure and stability of the native species. The Trp-108 to Tyr mutation was equally fortuitous in that Trp-108 has the majority of its native contacts formed in I_2, so that the mutation destabilizes the intermediate more than the native species or the transition state.

Recently, experimental attempts have been made to avoid this problem by choosing proteins that refold very rapidly

and without the population of intermediates (Schindler et al., 1995; Villegas et al., 1995). However, it is very difficult to exclude the presence of intermediates. It is generally assumed that a linear dependence of the log of the rate constant on denaturant concentration and the absence of a burst phase amplitude is sufficient evidence for the absence of intermediates. Unfortunately, this assumption is not correct. For example, the major conformational folding intermediate populated during the folding of RNase A is undetectable by these methods and was detected only by detailed kinetic studies of the pH and GdnHCl dependence of the refolding process (Houry et al., 1995).

It appears that protein folding is intrinsically fast and that slow conformational folding processes involve the disruption of structure within intermediates formed along the folding pathway. This structure can be native-like as in the case of the oxidative refolding of BPTI (Weissman & Kim, 1995) where premature formation of native structure delays the attainment of the final native fully oxidized state. Alternatively, this structure may involve non-native interactions such as those that occur during the refolding of wild-type lysozyme. Given the experimental difficulties in identifying the presence of non-native structure, it is likely that the slow rate of folding observed in most proteins results from the formation of non-native structure during the refolding process.

CONCLUSIONS

The results presented here clearly show that the rate-determining step in the major refolding pathway of wild-type lysozyme involves the disruption of non-native structure as suggested by the earlier studies of Radford et al. (1992), Denton et al. (1994), and Itzhaki et al. (1994). This structure is stabilized by formation of non-native tertiary contacts among tryptophan residues. Disruption of these contacts by replacement of Trp-108 or Trp-62 with Tyr greatly accelerates the rate of refolding. Trp-108 and Trp-62 are located in separate domains of the protein, the α- and β-domains, respectively. These results, interpreted in conjunction with previous studies of lysozyme, suggest that the non-native structure consists of a hydrophobic and aromatic cluster involving a native-like α-domain and the irregular loop region of the β-domain which contains Trp-62. It is further suggested that the disruption of non-native structure may be the rate-determining process in the folding of most proteins.

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REFERENCES

Adler, A. J., Greenfield, N. J., & Fasman, G. D. (1973) *Methods Enzymol. 27*, 675−735.
Albert, J. S., & Hamilton, A. D. (1995) *Biochemistry 34*, 984−990.
Anfinsen, C. B. (1973) *Science 181*, 223−230.
Arnold, G. E., Day, L. A., & Dunker, A. K. (1992) *Biochemistry 31*, 7948−7956.
Baldwin, R. L. (1995) *J. Biomol. NMR 5*, 103−109.
Burley, S. K., & Petsko, G. A. (1985) *Science 229*, 23−28.
Burley, S. K., & Petsko, G. A. (1988) *Adv. Protein Chem. 39*, 125−189.
Chaffotte, A. F., Guillou, Y., & Goldberg, M. E. (1992) *Biochemistry 31*, 9694−9702.
Cooper, A., Eyles, S. J., Radford, S. E., & Dobson, C. M. (1992) *J. Mol. Biol. 225*, 939−943.
Creighton, T. E. (1991) *Curr. Biol. 1*, 8−10.
Creighton, T. E., & Goldenberg, D. P. (1984) *J. Mol. Biol. 179*, 497−526.
Denton, M. E., & Scheraga, H. A. (1991) *J. Protein Chem. 10*, 213−232.
Denton, M. E., Rothwarf, D. M., & Scheraga, H. A. (1994) *Biochemistry 33*, 11225−11236.
Diamond, R. (1974) *J. Mol. Biol. 82*, 371−391.
Dobson, C. M., Evans, P. A., & Radford, S. E. (1994) *Trends Biochem. Sci. 19*, 31−37.
Dubois, T., Guillard, R., Prieels, J. P., & Perraudin, J. P. (1982) *Biochemistry 21*, 6516−6523.
Evans, P. A., & Radford, S. E. (1994) *Curr. Opin. Struct. Biol. 4*, 100−106.
Eyles, S. J., Radford, S. E., Robinson, C. V., & Dobson, C. M. (1994) *Biochemistry 33*, 13038−13048.
Fersht, A. R. (1995a) *Philos. Trans. R. Soc. London, Ser. B 348*, 11−15.
Fersht, A. R. (1995b) *Proc. Natl. Acad. Sci. U.S.A. 92*, 10869−10873.
Formoso, C., & Forster, L. S. (1975) *J. Biol. Chem. 250*, 3738−3745.
Herning, T., Yutani, K., Taniyama, Y., & Kikuchi, M. (1991) *Biochemistry 30*, 9882−9891.
Hooke, S. D., Radford, S. E., & Dobson, C. M. (1994) *Biochemistry 33*, 5867−5876.
Hooke, S. D., Eyles, S. J., Miranker, A., Radford, S. E., Robinson, C. V., & Dobson, C. M. (1995) *J. Am. Chem. Soc. 117*, 7548−7549.
Houry, W. A., Rothwarf, D. M., & Scheraga, H. A. (1994) *Biochemistry 33*, 2516−2530.
Houry, W. A., Rothwarf, D. M., & Scheraga, H. A. (1995) *Nat. Struct. Biol. 2*, 495−503.
Hunter, C. A., & Sanders, J. K. M. (1990) *J. Am. Chem. Soc. 112*, 5525−5534.
Hunter, C. A., Singh, J., & Thornton, J. M. (1991) *J. Mol. Biol. 218*, 837−846.
Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C., & Rupley, J. A. (1972a) in *The Enzymes* (Boyer, P. D., Ed.) 3rd ed., Vol. 7, pp 665−868, Academic Press, New York.
Imoto, T., Forster, L. S., Rupley, J. A., & Tanaka, F. (1972b) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1151−1155.
Inoue, M., Yamada, H., Yasukochi, T., Kuroki, R., Miki, T., Horiuchi, T., & Imoto, T. (1992) *Biochemistry 31*, 5545−5553.
Itzhaki, L. S., Evans, P. A., Dobson, C. M., & Radford, S. E. (1994) *Biochemistry 33*, 5212−5220.
Jackson, S. E., & Fersht, A. R. (1991) *Biochemistry 30*, 10436−10443.
Janin, J., & Wodak, S. J. (1983) *Prog. Biophys. Mol. Biol. 42*, 21−78.
Karplus, M., & Šali, A. (1995) *Curr. Opin. Struct. Biol. 5*, 58−73.
Kato, S., Okamura, M., Shimamoto, N., & Utiyama, H. (1981) *Biochemistry 20*, 1080−1085.
Kato, S., Shimamoto, N., & Utiyama, H. (1982) *Biochemistry 21*, 38−43.
Kiefhaber, T. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 9029−9033.
Kiefhaber, T., Grunert, H.-P., Hahn, U., & Schmid, F. X. (1992) *Proteins: Struct., Funct., Genet. 12*, 171−179.
Kotik, M., Radford, S. E., & Dobson, C. M. (1995) *Biochemistry 34*, 1714−1724.
Kragelund, B. B., Robinson, C. V., Knudsen, J., Dobson, C. M., & Poulsen, F. M. (1995) *Biochemistry 34*, 7217−7224.
Kumagai, I., & Miura, K. (1989) *J. Biochem. (Tokyo) 105*, 946−948.
Kumagai, I., Sunada, F., Shigeki, T., & Miura, K. (1992) *J. Biol. Chem. 267*, 4608−4612.
Kuramitsu, S., Kurihara, S., Ikeda, K., & Hamaguchi, K. (1978) *J. Biochem. (Tokyo) 83*, 159−170.
Longworth, J. W. (1966) *Biopolymers 4*, 1131−1148.
Maenaka, K., Matsushima, M., Song, H., Sunada, F., Watanabe, K., & Kumagai, I. (1995) *J. Mol. Biol. 247*, 281−293.
Matthews, C. R. (1993) *Annu. Rev. Biochem. 62*, 653−683.
McCammon, J. A., Gelin, B. R., Karplus, M., & Wolynes, P. G. (1976) *Nature 262*, 325−326.

Miranker, A., Radford, S. E., Karplus, M., & Dobson, C. M. (1991) *Nature 349*, 633−636.
Miranker, A., Robinson, C. V., Radford, S. E., Aplin, R. T., & Dobson, C. M. (1993) *Science 262*, 896−900.
Mücke, M., & Schmid, F. X. (1994) *J. Mol. Biol. 239*, 713−725.
Nitta, K., & Sugai, S. (1989) *Eur. J. Biochem. 182*, 111−118.
Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., & Gray, T. (1995) *Protein Sci. 4*, 2411−2423.
Parker, M. J., Spencer, J., & Clarke, A. R. (1995) *J. Mol. Biol. 253*, 771−786.
Radford, S. E., & Dobson, C. M. (1995) *Philos. Trans. R. Soc. London, Ser. B 348*, 17−25.
Radford, S. E., Dobson, C. M., & Evans, P. A. (1992) *Nature 358*, 302−307.
Roder, H., & Elöve, G. A. (1994) in *Mechanisms of Protein Folding* (Pain, R. H., Ed.) pp 26−54, Oxford University Press, New York.
Rothwarf, D. M., & Scheraga, H. A. (1993) *Biochemistry 32*, 2671−2679.
Saxena, V. P., & Wetlaufer, D. B. (1970) *Biochemistry 9*, 5015−5023.
Schindler, T., Herrler, M., Marahiel, M. A., & Schmid, F. X. (1995) *Nat. Struct. Biol. 2*, 663−673.
Serrano, L., Bycroft, M., & Fersht, A. R. (1991) *J. Mol. Biol. 218*, 465−475.
Sosnick, T. R., Mayne, L., Hiller, R., & Englander, S. W. (1994) *Nat. Struct. Biol. 1*, 149−156.
Teichberg, V. I., & Sharon, N. (1970) *FEBS Lett. 7*, 171−174.
Villegas, V., Azuaga, A., Catasús, Ll., Reverter, D., Mateo, P. L., Avilés, F. X., & Serrano, L. (1995) *Biochemistry 34*, 15105−15110.
Weissman, J. S., & Kim, P. S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 9900−9904.
Weissman, J. S., & Kim, P. S. (1995) *Nat. Struct. Biol. 2*, 1123−1130.
Yang, J. J., Buck, M., Pitkeathly, M., Kotik, M., Haynie, D. T., Dobson, C. M., & Radford, S. E. (1995) *J. Mol. Biol. 252*, 483−491.

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