Kinetics of Secondary Structure Recovery during the Refolding of Reduced Hen Egg White Lysozyme*

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We have shown previously that, in less than 4 ms, the unfolded/oxidized hen lysozyme recovered its native secondary structure, while the reduced protein remained fully unfolded. To investigate the role played by disulfide bridges in the acquisition of the secondary structure at later stages of the renaturation/oxidation, the complete refolding of reduced lysozyme was studied. This was done in a renaturation buffer containing 0.5 M guanidinium chloride, 60 μM oxidized glutathione, and 20 μM reduced dithiothreitol, in which the aggregation of lysozyme was minimized and where a renaturation yield of 80% was obtained. The refolded protein could not be distinguished from the native lysozyme by activity, compactness, stability, and several spectroscopic measurements. The kinetics of renaturation were then studied by following the reactivation and the changes in fluorescence and circular dichroism signals. When bi- or triphasic sequential models were fitted to the experimental data, the first two phases had the same calculated rate constants for all the signals showing that, within the time resolution of these experiments, the folding/oxidation of hen lysozyme is highly cooperative, with the secondary structure, the tertiary structure, and the integrity of the active site appearing simultaneously.

Since Anfinsen’s work on the in vitro renaturation of unfolded ribonuclease A (1), it is commonly accepted that all the information required for a protein to fold properly is contained in its amino acid sequence. However, the code that allows the formation of a fully folded protein from its amino acid sequence has not yet been deciphered. Three models are currently proposed to describe this process. The framework model is a sequential model in which secondary structure elements form first followed by a tighter packing of the molecule (2). Another model assumes that the polypeptide chain undergoes a rapid collapse driven by hydrophobic forces that would yield an intermediate close to the molten globule (3). In the puzzle model (4) structural elements form at different sites on the polypeptide chain, and their formation induces further folding of the whole protein.

According to experimental data, it is not yet possible to determine which model most accurately describes the folding mechanism. However, some general features of protein folding have arisen. Stopped-flow circular dichroism studies showed that a large amount of secondary structure is formed in the dead time (milliseconds time range) of the observation. This has been observed for proteins such as α-lactalbumin and lysozyme (5), dihydrofolate reductase (6), and holocytochrome c (7). On the other hand, when using pulsed proton exchange followed by NMR identification of the protected protons, the formation of stable secondary structure elements could be detected over a slower observable time range (8, 9). Using both techniques to observe the folding of the same protein, as was the case for cytochrome c (7), lysozyme (10, 11), and interleukin-1β (12), secondary structures could be observed by CD at a stage where pulsed proton exchange/NMR failed to detect protected protons. This apparent contradiction suggested that secondary structure elements could form without providing an efficient protection against proton exchange. This was confirmed by studies on a model peptide that mimics early folding intermediates, the C-terminal F2 domain of the F2 subunit of Escherichia coli tryptophan synthase (13, 14). These studies showed that in isolated F2 the secondary structure elements were in such a fast equilibrium that their protons were only weakly protected from exchange. Hence the initial secondary structures that are present in very early folding intermediates appear to be poorly stabilized.

A major question in solving the folding problem is to understand the relative roles of local versus long range interactions in controlling the folding process. While both types of interactions are obviously linked energetically throughout the folding pathway, and both determine the stabilities of all the folding intermediates (15), the predominance of one over the other is at the basis of the distinction between the framework, the jigsaw puzzle, and the hydrophobic collapse models.

The propensity of short natural amino acid sequences to spontaneously form native-like secondary structures through local interactions has been the focus of much attention and has lent much support to the framework model (16). Yet, several well documented cases are described in the literature where the protein fails to show any detectable secondary structure when long range interactions are not formed. Thus, in the absence of long range interactions between the heme and the polypeptide chain, apocytochrome c remains completely unfolded and shows no secondary structure under conditions where the holoprotein refolds completely (17). It thus appears that native tertiary contacts play an important role not only in the late stages of the folding but also to initiate or stabilize the formation of the secondary structure.

A similar conclusion was reached for lysozyme, based on the following observations. When the native disulfide bonds are maintained in the denatured state, the protein recovers the majority of its secondary structure in less than 4 ms (11). In contrast, the species that are present after 4 ms of refolding of
was equilibrated and eluted with the renaturation buffer. The column flow rate was 0.5 ml/min. Native and denatured lysozyme were incubated for 24 h in renaturation buffer. Both samples were then centrifuged at 10,000 × g for 30 min, to eliminate large aggregates, and 200 μl of each supernatant were loaded on the column.

Analytical Ultracentrifugation—Analytical ultracentrifugation was performed in a Beckman XLA ultracentrifuge, equilibrated at 20 °C, using standard double sector cells. Both native and renatured lysozyme were dialyzed against the same renaturation buffer for 20 h and were centrifuged at 55,000 rpm. The lysozyme concentration was around 0.1 mg/ml. Once the final speed was reached, radial scans at 280 nm were recorded over 20-min intervals. After completion of the seventh scan, the apparent sedimentation coefficients were calculated using the second moment method of the XLA-Vel program provided with the XLA-Data Analysis Software (Beckman Instruments, Palo Alto, CA).

CD and Fluorescence Spectra—For recording the CD and the fluorescence spectra, the protein was resuspended in 10 mM KPi, pH 7, at a concentration of 0.1 mg/ml. The instruments were the same as those used for the kinetic analysis (see below). All the spectra were the average of three scans and were corrected by subtracting the spectrum of the buffer recorded under the same conditions just before analyzing the protein. The CD spectra were recorded between 190 and 260 nm, with a spacing of 1 nm, and an integration time of 2 s in a 5-mm optical path cell. The fluorescence spectra were recorded with an integration time of 1 s and an interval of 0.5 nm. The emission and pass was 2.125 nm, and the excitation bandwidth was 4.25 nm. The excitation wavelength was 295 nm, and the spectra were recorded between 310 and 380 nm, using a cuvette with a 4-mm excitation pathlength and a 1-cm emission pathlength.

Mass Spectrometry Experiments—The mass spectrometry experiments were performed on a Platform spectrometer coupled with an electrospray source (Fisons Instruments, Manchester, UK). The flow rate was 5 μl/min, and the proteins were dissolved in a 1:1 mixture of acetonitrile and water containing 0.2% formic acid.

ºH NMR Experiments—Three different samples were used for the NMR experiments as follows: one corresponding to the native lysozyme, one corresponding to the native lysozyme incubated in the renaturing buffer, and the renatured lysozyme sample. All the samples were dialyzed extensively against dilute HCl, pH 3, before use and freeze-dried. The lyophilized powder was dissolved in 40 μl of D2O (Eurosp-Top). The lysozyme concentration, as measured from UV absorbance at 280 nm, was 6.2 mg/ml for the native lysozyme, 0.9 mg/ml for the native lysozyme subjected to the renaturing buffer, and 2.8 mg/ml for the renatured lysozyme. °H NMR experiments, using a Nano-NMR probe (Varian) were run on a Bruker 85.5 MHz on a Varian Unity spectrometer with an on-line Sun Sparc 2 workstation. The Nano-NMR probe provides high resolution spectra from liquid samples of only 40 μl (21) as a result of high resolution magnetic susceptibility matching probe technology used in conjunction to magic angle spinning (22). The experimental data were processed using the VNMR 5.1B program. The spectral width was 7200 Hz and the spinning rate around 2 kHz. Spectra were referred to the water signal at 4.68 ppm at 35 °C (relative to 3-trimethylsilyl-(2,2,3,3-2H4)-propionate, the external reference). Quadrature detection was employed in all experiments with the carrier frequency always maintained at the solvent resonance. The two-dimensional °H NMR spectra were recorded in the phase-sensitive mode (23) with 3200 data points in the δ2 dimension and 440 τ1 increments. 8, 48, or 64 scans were acquired for the NOESY experiments depending on the concentration with a single mixing time of 150 ms allowing a direct comparison with previously published data (24). Zero filling was applied prior to Fourier transformation, and data were processed with shifted sine bell window functions in both dimensions. Low power selective irradiation during the recycling delay and, for NOESY spectra, during the mixing period was used to suppress the residual water peak.

Microcalorimetry Experiments—The microcalorimetry experiments were performed on a VP-DSC microcalorimeter from MicroCal Inc (Northampton, MA). The renatured and the native lysozymes were dialyzed overnight against 20 mM glycine, pH 2.5, and centrifuged for 30 min at 10,000 × g. The sample concentrations were 0.20 and 0.21 mg/ml, respectively, for the renatured and the native proteins. The samples were equilibrated at 25 °C, introduced into the cell, and incubated for 15 min at 25 °C and 27 °C (i.e., before starting the up scan). The temperature range scanned was 25–80 °C, the rate of temperature change was 40 °C/h for both the up scans and the down scans, and the filter period was 20 s. A 1-min pause was introduced at the end of the up scan before starting the down scan. Three independent up scan/down scan experiments were performed with each protein. Each scan with a protein sample was preceded and followed by an identical scan with the column.

EXPERIMENTAL PROCEDURES

Proteins and Chemicals—Hen egg white lysozyme (HEWL) and oxidized glutathione (GSSG) were purchased from Boehringer Mannheim GmbH, Germany, reduced dithiothreitol (DTT) and Micrococcus lysodeikticus from Sigma, and guanidine hydrochloride (GdnHCl) and urea from ICN Biomedicals.

Preparation of Reduced/Denatured Lysozyme—Reduced/denatured lysozyme was prepared as described by Goldberg et al. (20).

Preparation of Reduced/Denatured Lysozyme—The lyophilized reduced/denatured lysozyme was dissolved at 10 mg/ml in 6 M urea from ICN Biomedicals. The protein sample was preceded and followed by an identical scan with the column.

Enzyme Assays—Lysozyme activity was measured by mixing 20 μl aliquots of lysozyme solution (0.1 mg/ml) with 0.980 ml of a M. lysodeikticus solution (0.25 mg/ml) in 66 mm monobasic potassium phosphate (KPom), pH 6.2, equilibrated at 25 °C. The samples were mixed by repeatedly inverting the cuvette for 15 s. The slope of the linear part of the decrease in turbidity, monitored at 450 nm, was taken as the lytic activity. One unit of activity corresponds to an absorbance decrease of 0.0026/min. The concentrations of lysozyme were measured by absorbance at 280 nm using extinction coefficients of 2.62 cm−1 mg−1 for the native form and 2.37 cm−2 mg−1 for the reduced/denatured form (20).

Characterization of the Refolded Lysozyme: Sample Preparation—Reduced/unfolded lysozyme (0.1 mg/ml) was incubated in the renaturation buffer at 25 °C for 24 h. The protein was then dialyzed against 10 mM KPm, pH 5.5, for 48 h and against 50 mM ammonium acetate, pH 5.5, for another 48 h. The ratio between the lysozyme solution volume and the dialysis buffer volume was about 1/100, and the dialysis buffer was changed every 12 h. At that point, the lysozyme was concentrated about 10-fold using an Amicon DIAFLO cell and a YM3 membrane. After subsequent centrifugation at 10,000 × g for 30 min, the supernatant was lyophilized, and the protein was kept at −20 °C.

Gel Filtration—Gel filtration was performed on a Superdex-75 HR 10/30 column hooked up to a Pharmacia FPLC system. The column was 29.5 cm long, 7.8 mm in diameter, and 0.5 μm in effective pore size. It was eluted with a 1:1 mixture of acetonitrile and water containing 0.2% formic acid.

1 The abbreviations used are: HEWL, hen egg white lysozyme; DTT, dithiothreitol; GdnHCl, guanidine hydrochloride; KPom, potassium phosphate; NOESY, nuclear Overhauser effect spectroscopy.
buffer to establish the base line. After each scan, the corresponding buffer base line was subtracted, and both the up scan and down scan were analyzed using the MicroCal-Origin software for VP-DSC supplied with the machine.

**Kinetics of Refolding of Reduced/Denatured Lysozyme—**Refolding kinetics were studied by CD at three different wavelengths (220, 222, and 228 nm) using a Jobin-Yvon (Longjumeau, France) CD6 spectropolarimeter. The observation cuvette was a cylindrical, 5-mm-path cell, and the sample holder was thermostated at 25 °C. The dead time needed to mix the solutions, fill the cuvette, and start the scan, was about 80 s and was taken into account for data processing. The integration time was 5 s, and the time interval was 10 s. Each experiment was repeated at least 3 times. The files were converted into ASCII files by the ISA/hda ASCII conversion subroutine of the CD6 software, and data processing was achieved by means of the program Fig.P version 2.7 for windows (Biosoft, Cambridge, UK).

The signal of the denatured protein in renaturation buffer was calculated as indicated above for the reduced lysozyme. The fluorescence of the denatured protein in the renaturation band pass was 2.125 nm, and the emission band pass was 4.25 nm.

**CD and Fluorescence Spectrum of the Refolded Lysozyme—**The denatured protein was unfolded under non-reducing conditions in the presence of 6 M GdnHCl as follows: The dependence of the signal on the GdnHCl concentration was accounted for by incubating HEWL in renaturation media containing GdnHCl at concentrations between 3.5 and 6 M, where the protein is completely unfolded. The ellipticities of these solutions were plotted versus the concentration of denaturant and fitted by a linear regression. The extrapolation of the fit to the GdnHCl concentration in the renaturation buffer was used as the signal of the denatured protein under these conditions.

The fluorescence kinetics were recorded in a SPEX (Edison, NJ) Fluoromax spectrophotometer. The recording time was 2–3 h, with a sampling interval of 10 s and an integration time of 5 s. 1 × 1-cm cuvettes were placed in a cell holder thermostated at 25 °C. The excitation band pass was 2.125 nm, and the emission band pass was 4.25 nm. The fluorescence of the denatured protein in the renaturation buffer was calculated as indicated for the CD experiment.

**Refolding of the Oxidized Lysozyme—**The lysozyme was unfolded under non-reducing conditions in the presence of 6 M GdnHCl and 0.1 M acetic acid, pH 2.5, and its concentration was adjusted to 8 mg/ml by addition of the same buffer. The CD stopped-flow apparatus used was as described by Chaffotte et al. (11). The stopped-flow module (reservoirs, syringes, mixers, and observation cell) was thermostated at 25 °C. The recording time was 2 s with a sampling period and an integration time of 1 ms. Renaturation was initiated upon mixing of 15 μl of denatured protein (small syringe) with the 585 μl of renaturation buffer (two large syringes). The mixing time was 75 ms, and the final concentration of lysozyme was 0.1 mg/ml. The experiment was repeated 50 times, and the accumulated kinetic data were averaged and analyzed as indicated above for the reduced lysozyme.

**RESULTS**

**Optimization of the Renaturation Conditions—**The renaturation buffer used by Goldberg et al. (20) for renaturing reduced lysozyme at 25 °C was progressively optimized, taking into account the two following requirements. (i) Aggregation should be minimized at a lysozyme concentration (0.1 mg/ml) sufficient to give a detectable far UV CD signal. (ii) The disulfide exchange catalysts should not absorb too much light in the far UV so as to permit CD measurements in the 220-nm region. Based on the initial observation by Orsini and Goldberg (25), we used moderate concentrations of solubilizing or denaturing agents to minimize aggregation. Although urea and non-detergent sulfobetaines indeed drastically minimized aggregation during the refolding/reoxidation of reduced HEWL (26), they could not be used because they absorb light strongly in the far UV. In contrast, GdnHCl absorbs much less in the wavelength range used for far UV CD measurements. Similarly, the classical thiol-disulfide couple described by Saxena and Wetlauffer (19) for the refolding of lysozyme (GSSG/GSH) strongly absorbs light in the far UV. Since GSH, the reduced form of glutathione, is in a 10-fold excess over GSSG, we replaced it by DTT which absorbs light much less in the far UV. By varying the GdnHCl, reduced DTT, and GSSG concentrations, we found that the optimal refolding buffer was 0.1 M Tris-HCl, pH 8.2, 1 mM EDTA, 0.5 M GdnHCl, 60 μM GSSG, and 20 μM DTT, in which the yield of active HEWL was about 80% after 24 h of renaturation.

**Activity of the Refolded Lysozyme—**The protein refolded under the conditions described above was characterized and compared with the native enzyme, using the following methods. Both native and refolded lysozyme were centrifuged to eliminate large aggregates and tested for activity. All the activity present in the renatured HEWL preparation (i.e., 74% of the initial activity of the native protein) was recovered in the supernatant, indicating that the aggregates contained only inactive molecules. Moreover the protein in the supernatant and native HEWL had similar specific activities (30,750 units/mg for the native and 29,310 units/mg for the renatured lysozyme in the supernatant). Therefore the renatured mixture contained about 25% of inactive aggregates that were removed by centrifugation and 75% of soluble fully active protein.

**Hydrodynamic Studies of the Refolded Lysozyme—**To characterize further the refolded protein, the samples were analyzed on a gel filtration column (see “Experimental Procedures”). The two proteins had the same elution profile, with a major peak at a volume of 14.5 ml, which contained the protein, and a minor peak at 18 ml, which had an absorbance spectrum similar to that of glutathione. Hence, both native and renatured HEWL eluted in a peak at 14.5 ml, which indicated that they had the same Stokes radius. The proteins were also analyzed by analytical ultracentrifugation. The sedimentation profiles of the two proteins were similar, and the observed sedimentation coefficients were, within experimental error, identical, $2.03 \pm 0.05$ s, for the native lysozyme, and $1.92 \pm 0.05$ s, for the refolded lysozyme. Since both proteins also had identical Stokes radii, it could be concluded that the refolded protein is monomeric, like the native one, and has the same hydrodynamic properties.

**CD and Fluorescence Spectrum of the Refolded Lysozyme—**The renatured lysozyme was further characterized by CD and fluorescence. The CD spectra of the denatured and the native lysozyme were recorded between 190 and 260 nm. The two spectra were indistinguishable, with a minimum at around 208 nm. Hence the two enzymes have the same content in secondary structures. The emission fluorescence spectra recorded with an excitation wavelength of 295 nm were also superimposable and showed a maximum of intensity at 340 nm.

**Mass Spectrometry—**To ascertain that no chemical change was introduced during denaturation or renaturation, samples of lysozyme at different stages of the unfolding/refolding process were analyzed by mass spectrometry. The mass spectra of native untreated lysozyme, native lysozyme incubated in renaturation buffer, reduced/denatured lysozyme, and refolded lysozyme had the same features. They all showed a major peak whose intensity was arbitrarily set to 100%, and minor peaks whose intensities were between 10 and 20%. Most of these minor peaks did not change in all the spectra. One, however, had the same mass increase upon reduction as the major peak. Its mass of 14,502 Da could not correspond to a lysozyme from which a part only of the signal sequence would have been removed. It might be a modified form of the enzyme that copurified during the industrial purification of HEWL. The mass of the major peak was 14,305 Da for the two native and the renatured lysozyme and 14,311 for the unfolded lysozyme. These values are similar to those expected for the oxidized (14,306 Da) and the reduced (14,314 Da) form of lysozyme, respectively. These results indicate that no chemical change was introduced upon denaturation/renaturation.

**1H NMR Experiments—**The spectra obtained in D2O for the three samples exhibit similar features. 63 slowly exchanging amide protons were identified in the renatured lysozyme in agreement with previously published work on native lysozyme (24). The NOESY spectrum is comparable to that obtained for
the native lysozyme in the same conditions in terms of temperature and mixing time. Fig. 1 displays the $d_{NN}$ connectivities identified in the NOESY spectra for the renatured lysozyme. Extended region of $d_{NN}$ connectivities are observed for residues 8 to 14/15, 27–38, and 92–100 in agreement with the presence of regular $\alpha$-helices. Shorter stretches of $d_{NN}$ connectivities are also observed for residues 40–42, 55–57, 75–78, 82–84, 111–112, and 123–125. These regions correspond either to non-regular helices or tight turns. Extended regions of $d_{aN}$ sequential connectivities are also observed from residues 42–47, 50–55, and 57–60 in agreement with the presence of triple-stranded anti-parallel $\beta$-sheet (data not shown).

Microcalorimetry Experiments—The stability of the protein was tested using microcalorimetry. Whereas preliminary experiments made with native lysozyme at a scanning rate of 60 °C/min showed a significant difference (1.6 °C) between the transition temperatures observed for the up scan and the down scan, this difference was reduced to less than 1 °C at a scanning rate of 40 °C. With this slower rate, the transition curves of the renatured and the native lysozymes were practically superimposable. Thus, the transition temperatures obtained from the up scans were 58.5 ± 0.1 and 58.4 ± 0.1 °C for native and renatured lysozyme, respectively. The transition temperatures for the down scans were 57.5 ± 0.1 and 57.4 ± 0.1 °C, respectively, and the denaturation enthalpies were 107.6 ± 2.0 and 100.8 ± 1.8 kcal/mol, respectively. Although the differences in the denaturation enthalpies might seem experimentally significant, they could be accounted for by a 3% difference between the real and the measured relative protein concentrations of the two samples, which is well within the experimental error. Altogether, these results indicate that the thermodynamic properties of the renatured lysozyme are indistinguishable from those of the native enzyme, which confirms the identity of the conformations of both proteins. Nonetheless, it should be noted that the values found here for the $T_m$ are lower than the one found in the literature ($T_m = 64, 1$ °C, see Ref. 27). This difference could be due to an uncertainty in the pH value since the $T_m$ of lysozyme increases by 20 °C between pH 2 and pH 3 (28).

Kinetics of Reduced Lysozyme Refolding—The reactivation curve, obtained by plotting the fraction of recovery of specific activity versus refolding time, showed an initial lag time (see inset to Fig. 2) followed by an exponential increase reaching a plateau at about 0.8 (see Fig. 2), indicating an 80% yield of active protein. The curve obtained was fitted with a three-step model in which the first phase had a zero amplitude to describe the lag phase. The time constants thus obtained were: $k_1 = 0.055 \pm 0.274$ min$^{-1}$, $k_2 = 0.059 \pm 0.297$ min$^{-1}$, and $k_3 = 0.0002 \pm 0.020$ min$^{-1}$. The errors in the determination of these constants are very large due to the difficulty in describing the lag phase. However, as the number of data points increased, the errors decreased, and the values of the constants did not significantly change showing that they qualitatively describe the refolding.

Refolding of the Reduced Lysozyme Studied by Fluorescence—Next, the refolding of the lysozyme was studied by fluorescence at two different wavelengths, 340 nm, corresponding

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to the maximum of emission of the native HEWL, and 355 nm, around the maximum of the difference spectrum between unfolded and native HEWL. At 340 nm, the renaturation curve was biphasic for the 1st h. After reaching a plateau, the signal then followed an additional very slow decreasing drift which could not be described by a higher order model. At 355 nm, the totality of the curve could be described with a sequential three-phase model with $k_1 = 0.051 \text{ min}^{-1}$, $k_2 = 0.051 \text{ min}^{-1}$, and $k_3 = 0.002 \text{ min}^{-1}$. When the refolding sample was not illuminated continuously, the drift seemed to be reduced, suggesting that photobleaching is interfering with the measurements. Successive wavelength scans of the fluorescence emission of the refolding sample allowed the kinetics of the change of the maximum wavelength of emission to be followed. The latter could be described by a sequential two-phase model that reached a slowly decreasing plateau in about 2 h. In addition, the fluorescence of the unfolded protein in the renaturation buffer was determined at 340 and 355 nm, and the values were coincident with the initial values of the fluorescence renaturation curves. Thus no fluorescence burst phase was detected at the beginning of the folding of reduced HEWL.

**Refolding of the Reduced Lysozyme Studied by CD—**The refolding of denatured/reduced lysozyme was followed by circular dichroism (CD) at three different wavelengths, 220, 222, and 228 nm. As expected from the spectra of reduced/unfolded lysozyme and native lysozyme (18), the refolding is reflected by an overall decrease of the far UV ellipticity (see Fig. 3). Each curve was fitted by a two-step model which, at each wavelength, provided similar rate constants for both phases (see Table I). The values of these rate constants were also very similar for the kinetics at the three wavelengths. The relative amplitudes of the first phase compared with the total amplitude were 10% at 228 nm, 17% at 222 nm, and 20% at 220 nm. For all the experiments, the dead time due to the hand mixing, introduction of the sample into the cuvette, and starting the recording was determined to be about 80 s. Hence the initial point of the kinetics was calculated by extrapolating the fitted curves to time $\sim 80$ s. These calculated values were very close to the ellipticities of the denatured lysozyme in renaturation buffer at the same wavelengths (see Table I). Thus, there was no burst phase in the CD signal during the refolding of reduced lysozyme.

**Kinetics of Refolding of the Oxidized HEWL—**To compare the refolding of reduced and oxidized HEWL under exactly the same experimental conditions, we followed the recovery of ellipticity of the oxidized unfolded lysozyme in the renaturation buffer used in this study. This was done in a CD stopped-flow apparatus at 222 and 228 nm. Although the buffer had a significantly different composition to that used by Chaffotte et al. (11), in particular that it contained a redox system, the kinetics were very similar to those reported previously. At both wavelengths the ellipticity first rapidly decreased to a value below that of the native enzyme and then slowly increased. The kinetics could be described by a double exponential. At 222 nm, the fast rate constant was $k_1 = 69.9 \pm 3.4 \text{ s}^{-1}$, and the slow rate constant was $k_2 = 2.74 \pm 0.07 \text{ s}^{-1}$. At 228 nm the fast constant was $k_1 = 63.8 \pm 1.7 \text{ s}^{-1}$, the slow rate constant being $k_2 = 2.82 \pm 0.03 \text{ s}^{-1}$. As previously observed (11), the extrapolation of the fit to zero gave a value for the ellipticity considerably lower than that of the unfolded protein, showing that a burst phase occurred during the dead time of the experiment.

**DISCUSSION**

Detailed studies on the mechanism of the renaturation of reduced lysozyme have been hampered by the fact that at the concentrations needed for most physical-chemical analysis (above 50 $\mu$g/ml), the protein heavily aggregates (20). By adding GdnHCl at a 0.5 M concentration, we could not only improve the refolding yield but also increase the lysozyme concentration to a range where even CD measurements became possible. A yield of 80% was reached at a lysozyme concentration of 100 $\mu$g/ml. Similar yields were observed by Raman et al. (29), using high concentrations of the redox pair, but these also strongly interfere with optical measurements. By changing both the concentration and the composition of the redox pair, we suc-
Kinetics of Reduced Lysozyme Refolding

| λ (nm) | Extrapolation (mdeg) | k₁ (min⁻¹) | k₂ (min⁻¹) | Amplitude of 1st phase (%) |
|-------|----------------------|-------------|-------------|--------------------------|
| 220   | -18.2                | 0.053 ± 0.461 | 0.054 ± 0.466 | 20                       |
| 222   | -13.5                | 0.056 ± 0.068 | 0.058 ± 0.073 | 17                       |
| 228   | -11.7                | 0.058 ± 0.118 | 0.059 ± 0.122 | 10                       |

a The ellipticities of the reduced unfolded protein in the renaturation buffer were calculated by extrapolation to the concentration of GdnHCl used in the refolding buffer of the ellipticity of the reduced lysozyme at various denaturing concentrations of GdnHCl (see "Experimental Procedures").

b The initial value of the kinetics were calculated by back-extrapolating the fitting equation to a time t – dead time (see "Experimental Procedures").

c The kinetic constants were calculated by fitting a sequential two-exponential model to the data.

d mdeg, millidegree.

Kinetics data on the refolding of reduced/denatured lysozyme

where U indicates the unfolded state, and N_CD indicates a state which has recovered the native CD signal. The sequential model, with both rate constants 
k₁ and 
k₂ equal to 0.05–0.06 min⁻¹, provided the best description of the experimental data. Our results showed that the relative amplitudes of the two phases and the calculated rate constants were the same at the three wavelengths used. It had been shown previously that the contribution of the disulfides is maximum at 228 nm in the far UV region, and that at 220 nm the signal reflects only the contribution of the peptide backbone (11). Therefore, the fact that the CD changes at 220 and 228 nm obeyed the same kinetics showed that within the time resolution of our measurements, the acquisition of the secondary structure is concomitant with the change in the contribution of the disulfide bonds. Further conclusions about the rate of appearance of the disulfide bridges cannot be made since this contribution takes into account not only the signal due to the formation of the disulfide bonds but also the signal due to the configuration of these bonds. Moreover, it is not known whether all the disulfides contribute to the ellipticity or whether their contribution is of the same intensity. However, the similarity of the kinetics of the CD recovery at 228 nm and at 220 nm is consistent with the results of a discontinuous quantitation of the disulfide bridges that showed an increase in the number of disulfide bonds formed during the refolding (30, 31).

The renaturation of the lysozyme analyzed by measuring the recovery of the activity showed a lag phase, as previously observed by Anderson and Wetlauffer (30). In contrast to the recovery of far UV CD, the recovery of activity could not be fitted by a two-step model. A model with at least three phases, corresponding to the three possible mechanisms shown below, had to be used to account for the kinetics of reactivation (Models 3–5).

![Diagram of Models 3-5]

where I indicates an intermediate state and N indicates the native state. Model 5 could not describe the curve at all. When trying to fit Model 4 to the experimental data, the value obtained for 
k₃ was close to 0, and the model could not describe the final increasing drift. In contrast, Model 3 could describe all the features of the reactivation curve with fitted values for the first two rate constants 
k₁ and 
k₂ close to 0.06 min⁻¹. These rate constants are very close to those observed for the two kinetic phases of far UV CD recovery.

The results of the fluorescence kinetic study of lysozyme refolding are difficult to analyze. Indeed it seems that besides the refolding itself, some photobleaching is occurring (see "Results"). Moreover, in the renaturation buffer, the maximum of emission of both the native and the renatured protein is not the same as in a buffer without the redox pair and the GdnHCl (data not shown). Hence it appears that the renaturation mixture induces some reversible changes in the lysozyme. These changes, detected only in fluorescence, are slightly larger for the renatured protein than for the native one. Moreover, the formation of aggregates as shown above probably interferes with the recording of the fluorescence signal. All these factors are likely to interfere essentially during the last stage of the folding since the first two phases that were found when the fitting was possible at 355 nm had the same rate constants as those of recovery of far UV CD and as those of activity recovery. Because it was ascertained that no change in the fluorescence signal takes place in the dead time of the observation, it can be concluded that the recovery of fluorescence occurs in three phases, the first two coinciding with those observed in far UV CD and in enzymatic activity.

Thus, according to the above results, we propose Model 6 for the renaturation of the reduced lysozyme.
where I₁ is inactive and has recovered about 20% of the native secondary structure (CD at 220 nm), and where I₂ is partially active (about 70%) and corresponds to N_CD (i.e., the species that has recovered the ellipticity at all the observed wavelengths). Therefore, the third phase corresponds only to minor changes not detectable in our CD experiments. This model is compatible with the observations of Sawano et al. (32) showing that three out of the four possible three-disulfide derivatives of HEWL have specific enzymatic activities ranging between 70 and 80% of that of natural HEWL and have far-UV CD spectra very similar to that of HEWL. Thus, it seems likely that intermediate I₂ might be an incompletely oxidized form of HEWL with only three disulfide bonds and that the slow phase of activity recovery might correspond to the formation of the last disulfide bond.

The difference in the amplitudes of the first phase between the kinetics of the CD at 220 nm (20%) and at 228 nm (10%) might not be significant due to the error introduced in the calculation of the parameters of the fitting. However, if significant, this difference would suggest that the intermediate species I₁ either contains disulfide bridges that are in a chiral conformation different to that in the native enzyme and has not yet formed all the native disulfide bridges. Studies on the kinetics of appearance of the native disulfide bond during the oxidative refolding of reduced lysozyme will have to be made to answer this question, and to know whether or not I₂ indeed corresponds to a three-disulfide species.

The results reported here confirm that the presence of native disulfide bonds greatly accelerates not only the complete folding but also the appearance of stable secondary structure during the renaturation of lysozyme. Indeed, in the same buffer as that used for refolding reduced lysozyme, the oxidized form recovered its native ellipticity spectrum in less than 2 s, while it took about 2 h for the reduced form to do so. Moreover, the rapid recovery of secondary structure that takes place in the 4-ms dead time of the stopped-flow in the case of the oxidized form did not occur in the case of the reduced form. This confirms a previous report showing that no secondary structure was detected after 4 ms of folding of reduced lysozyme (18). In addition the studies reported here demonstrate that the secondary structure forms very slowly, with kinetics similar to the recovery of activity and probably with the formation of native S–S bonds. These differences between the oxidized and the reduced proteins suggest that the disulfide bridges play a major role in the formation of the secondary structure throughout the whole refolding process. Another difference between these two species is the absence of an overshoot in the CD signal for the reduced form. This is in agreement with the conclusion that the overshoot observed with the oxidized HEWL was due to non-native constraints on pre-existing disulfide bridges (11). In the present study the S–S bridges could be easily reduced and oxidized due to the pH and the presence of a reduct couple. Hence it can be assumed that disulfide bridges might get rapidly reshuffled rather than constrained. Another explanation could be that, in the reduced form, a disulfide bond is made and remains oxidized only when the cysteines are placed in such position that the bridge is already in a conformation close to the native one. From these considerations it appears that, during the oxidative folding of reduced lysozyme, the formation of tertiary contacts (disulfide bonds) inside the lysozyme, and the acquisition of the secondary structure, are strongly coupled throughout the folding process. Further investigations are in progress to determine whether only native disulfide bonds are effective in promoting secondary structure elements and to determine how many of these contacts are needed to lead to the properly folded form.

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