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Comparison of the kinetics of S-S bond, secondary structure, and active site formation during refolding of reduced denatured hen egg white lysozyme

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Abstract

To investigate the role of some tertiary interactions, the disulfide bonds, in the early stages of refolding of hen lysozyme, we report the kinetics of reoxidation of denatured and reduced lysozyme under the same refolding conditions as those previously used to investigate the kinetics of regain of its circular dichroism (CD), fluorescence, and activity. At different stages of the refolding, the oxidation of the protein was blocked by alkylation of the free cysteines with iodoacetamide and the various oxidation states present in the samples were identified by electrospray-mass spectrometry. Thus, it was possible to monitor the appearance and/or disappearance of the species with 0 to 4 disulfide bonds. Using a simulation program, these kinetics were compared with those of regain of far-UV CD, fluorescence, and enzymatic activity and were discussed in terms of a refined model for the refolding of reduced hen egg white lysozyme.

Keywords: folding; kinetics; lysozyme; mass spectroscopy; oxidation; secondary structure

Although it is well established that all the information needed for a protein to acquire its three-dimensional structure is contained in its amino acid sequence (Anfinsen, 1973), it is not yet understood how this information is translated into the native conformation of the polypeptide chain. One approach that has been extensively used to investigate the mechanisms of protein folding has been to carry out renaturation studies on isolated, unfolded proteins in vitro, using techniques that monitor the evolution of a variety of signals reflecting changes in the conformation of the protein, and thus can detect and characterize intermediates on the folding pathway(s). Among the proteins that were investigated by such an approach, hen egg white lysozyme (HEWL) has certainly been one of the most extensively studied. This monomeric protein of 129 amino acid contains eight cysteines that form four disulfide bonds. The structure of the native protein has been determined (Blake et al., 1965; Phillips, 1966; Smith et al., 1993) and shows two structural domains, α and β, on either side of the active site cleft. The α-domain consist of four α-helices along with a 3_{10}-helix, while the β-domain contains a triple- and a double-stranded antiparallel β-sheet, a 3_{10} helix, and an irregular loop. The refolding of the protein, starting from a denatured state in which the four native disulfide bonds were kept intact (oxidized/unfolded lysozyme), has been extensively studied by several techniques using stopped-flow technology due to the rapidity of the process. It involves steps occurring in the millisecond time range and is completed in about a second (Miranker et al., 1991; Chaffotte et al., 1992; Radford et al., 1992; Dobson et al., 1994; Matagne et al., 1997). Studies carried out to identify and characterize several intermediates, and to follow the progressive building up of the secondary and tertiary structure, demonstrated that denatured lysozyme uses parallel pathways to reach its native state (Radford et al., 1992; Wildegger & Kiefhaber, 1997).

In contrast with the oxidized protein, the renaturation of lysozyme starting from the reduced protein (i.e., the unfolded protein in which the disulfide bonds were reduced) takes several minutes, or even hours (Saxena & Wetlaufer, 1970; Fischer et al., 1993), thereby suggesting a crucial role of the disulfides in the acquisition of the tertiary structure. Moreover, stopped-flow studies on the

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Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GSSG, oxidized glutathione; CAM, carboxyamidomethyl; GdnHCl, guanidine Hcl; CD, circular dichroism; ESMS, electrospray mass spectrometry; LCMS, liquid chromatography mass spectometry; HEWL, hen egg white lysozyme; IAM, iodoacetamide; NEM, N-ethyl maleimide.

far-UV ellipticity changes during the folding of both nonreduced and reduced lysozyme showed that, after 4 ms of refolding, a native-like secondary structure content was regained for the oxidized protein (Chaffotte et al., 1992), while no secondary structure could be detected in this time range in the case of the reduced/unfolded protein (Goldberg & Guillou, 1994). This striking difference in the kinetics of regain of the secondary structure demonstrated that disulfides are involved not only in the "refinement" or the stabilization of the final refolded structure, but also at very early stages of the folding process. To better understand the role of disulfide bonds during the folding of HEWL, it seemed important to correlate the kinetics of oxidation of the polypeptide chain with the kinetics of appearance of intermediates detected by far-UV CD in the folding/oxidation pathway of reduced/denatured HEWL. Studies on the kinetics of oxidation during the renaturation of reduced HEWL had already been carried out before, and the assignment of the disulfides formed at various stages of the kinetics could even be achieved in some cases (Acharya & Taniuchi, 1976; Anderson & Wetlaufer, 1976). However, these data could not be correlated with the recovery of structural signals. Indeed, structural signals like CD in the far-UV could not be monitored under the renaturation/oxidation conditions then used, because of the strong propensity of reduced lysozyme to aggregate. In a previous paper (Roux et al., 1997), conditions have been described under which this difficulty could be circumvented. Thus, the recovery of the far-UV CD signal, the intrinsic fluorescence and the enzymatic activity could be monitored throughout the folding/oxidation of the reduced/denatured lysozyme. The analysis of these data led the authors to propose a three-step sequential model involving the formation of two intermediates. Finding out how many and which disulfide bonds are formed at each of these three steps should bring a clear view of the role of the disulfides in the folding process. The aim of the present study was to monitor, as a function of time during the renaturation/oxidation of reduced HEWL, the concentrations of species with one, two, three, and four disulfide bonds, and compare them with the concentrations of the species identified by spectroscopic and enzymatic methods.

The most convenient method to determine the states of oxidation present at different refolding times is chemical labeling of the intermediates followed by mass spectrometry (Torella et al., 1994; Ruoppolo et al., 1996). Aliquots of the renaturation mixture withdrawn at different times during the refolding process are alkylated with a thiol-blocking reagent such as iodoacetamide (IAM), iodoacetate, or N-ethyl-maleimide (NEM). The alkylation reaction traps the free SH groups and increases the molecular mass of the intermediates by a fixed amount for each reacted free SH group, thus allowing the identification by mass spectometry of intermediates containing different numbers of disulfide bonds. This method not only determines all the oxidation states present at a given time, but it also provides the quantitative distribution of the molecules among these states.

As a preliminary step before the identification of the individual disulfide bonds present at each stage of the renaturation of reduced HEWL, the present paper describes the use of mass spectrometry to monitor the kinetics of appearance and/or disappearance of the species with zero to four disulfide bonds during folding/oxidation of reduced/denatured lysozyme. The kinetics thus observed will be compared with those of regain of far-UV CD, fluorescence, and enzymatic activity obtained under the same refolding conditions (Roux et al., 1997) and will be discussed in terms of a refined model for the refolding of reduced HEWL.

Results

Alkylation of refolding aliquots

To analyze the oxidation state of HEWL throughout its renaturation from the reduced unfolded state, we first looked for suitable labeling and desalting conditions using the approach described by Torella et al. (1994). The optimum labeling conditions were found to be a 20 s incubation in the renaturation/reoxidation buffer (0.5 M GdnHCl, 0.1 M Tris HCl, pH 8.2, 1 mM EDTA 20 μM DTT, 60 μM GSSG) at a final iodoacetamide concentration of 1 M. For desalting prior to mass spectrometry, dialysis could not be used since, at the low protein concentrations used, most of the reduced and alkylated protein got adsorbed on the dialysis membrane. Rather, gel filtration on a PD-10 column prequilibrated with 0.1 M acetic acid was used, since it was shown that both native and denatured/reduced lysozyme were quantitatively recovered from the column (see Materials and methods). Using these labeling and desalting procedures, reduced HEWL was shown to carry eight carboxyamidomethyl groups while native HEWL was not labeled to any significant extent.

Kinetics of oxidation of reduced lysozyme during renaturation

Samples of reduced and denatured HEWL incubated for various times at 25 °C were labeled and desalted as described above. A different refolding sample was prepared for each refolding time because of the necessity, already pointed out previously (Goldberg et al., 1991), of having a very efficient mixing when initiating the renaturation by dilution, which could be easily achieved only with samples of limited volume. Because of this constraint, each sample represented a different refolding experiment, which might explain the somehow large dispersion of some data (see caption to Fig. 1). Each refolding time was sampled at least in triplicate and the data reported are the averages of independent experiments. Since the reactivation kinetics had already been described (Roux et al., 1997), the activity of a few samples was measured just before alkylation to check the reproducibility of the reactivation kinetics. It was indeed observed that the activity regain followed the same kinetics as previously observed.

After labeling and desalting, the samples were analyzed by ESMS. At different times, five different populations of disulfide intermediates were detected. They were identified on the basis of their accurate molecular mass. A typical spectrum is shown in Figure 1A and the measured molecular masses, the expected mass values, and the identification of the various intermediates are shown in Table 1. Since one carboxyamidomethylation of a free cysteine was expected to induce a mass increase of 57 Da, the peaks on the mass spectra could easily be attributed to a specific oxidation state of the protein. Each population of trapped intermediates was therefore characterized by a different number of intramolecular disulfide bonds (indicated as nS in Table 1) and carboxyamidomethyl (CAM) groups. The number of CAM groups corresponds to the number of free thiols present in the refolding intermediates, and it is therefore indicated as nH. It is important to emphasize that each observed molecular mass corresponds to a population of isomers, in which the total number of disulfides and free thiols is defined, whereas the positions of the effective cysteine residues involved are not identified. The number of possible isomers within each population is indicated in Table 1. No mixed disulfide species

Fig. 1. Time course analysis of the reoxidation of HEWL. **A:** Samples of denatured and reduced lysozyme were allowed to refold for various lengths of time. The free cysteines were reacted with iodoacetamide and lyophilized, as described in Materials and methods. The samples were analyzed by ESMS. The spectrum shown corresponds to a sample oxidized and renatured for 1 h. The masses corresponding to each peak were 14,770.7 ± 2.0 Da for the peak labeled 0 S-S, 14,653.3 ± Da for the 1 S-S peak, 14,537.3 ± 2.8 for the 2 S-S peak, 14,420.6 ± 2.6 Da for the 3 S-S peak, 14,363.8 ± 1.5 Da for the 3 S-S* peak, and 14,303.7 ± 2.6 Da for the 4 S-S peak. **B:** The oxidation state of the protein corresponding to each peak in the mass spectra was deduced from the corresponding mass and the relative amplitudes of each mass peak provided the relative amounts of the different oxidation states. The relative amount of each oxidized species, expressed as a percentage of the total proteins, is shown as a function of the refolding time. Each point represents the average of at least three independent experiments. For the sake of clarity, the error bars between the triplicates are not represented. For the vast majority of samples, they were about ±1–3% and never exceeded ±7%, expressed in percent of the total population of protein molecules in the sample. (●) 0 S-S, (◇) 1 S-S, (▲) 2 S-S, (□) 3 S-S, (♦) 4 S-S.

could be detected, unlike the situation reported for ribonuclease A and ribonuclease T1 (Torella et al., 1994; Ruoppolo et al., 1996). This was probably due to the unusually low concentrations of the redox components used in the present study (see Discussion).

In some samples, particularly those corresponding to folding/oxidation times of about 2 h, an additional peak could be observed in the mass spectra. It corresponded to molecular species carrying a single carboxyamidomethylation per polypeptide chain. Such a

Table 1. *Mass values of the different species observed during reoxidation of reduced HEWL*

nSS[a]	nSH[b]	Number of isomers[c]	Predicted mass[d] (Da)	Experimental mass[e] (Da)
0	8	1	14,769.5	14,765–14,774
1	6	28	14,653.5	14,650–14,657
2	4	210	14,537.5	14,533–14,541
3	2	420	14,421.5	14,418–14,424
4	0	105	14,305.5	14,302–14,309

[a]nSS is the number of intramolecular disulfide bonds.
[b]nSH is the number of free thiols.
[c]The number of isomers for each oxidation state was calculated using the formula niso $= N!/(P! * 2^P * (N - 2 * P)!)$ where N is the number of cysteines and P the number of disulfides.
[d]The predicted mass is calculated by adding 57 Da per alkylation to the mass of the lysozyme.
[e]The experimental mass gives the range of values found on the different spectra for that species.

result was not originally expected, but could be interpreted in three ways: an interchain disulfide bond leaving a "bachelor" free cysteine residue in each polypeptide chain, or an over-carboxyamidomethylation of the four disulfide containing species, or an under-carboxyamidomethylation of the three disulfide containing species. The first hypothesis was immediately ruled out, because disulfide bonds are stable under the conditions of our mass spectrometry experiments and an S-S bonded dimer would have given rise in the mass spectrum to a peak at a doubled molecular mass. The second hypothesis was ruled out and the third demonstrated by a double-labeling experiment (see Materials and methods) based on the following rationale. The thiols accessible at a given time of renaturation were reacted with iodoacetamide. Then, the protein was denatured, reduced, and incubated with N-ethyl maleimide (NEM). Thus, under these conditions, the protein was fully extended and all the cysteines that had not reacted with IAM were labeled with NEM. The resulting doubly labeled sample was analyzed by mass spectrometry. The species previously observed as a lysozyme with one CAM group, appeared as a species with one CAM group and seven NEM. Hence, it corresponded to a 3 S-S species in which one of the thiols was not accessible to IAM. Thus, this species will be considered as a 3 S-S species.

The relative amount of each population of intermediates present at various times of lysozyme renaturation was quantified by measuring the total ion current produced in the mass spectrometer by each species. This quantitative interpretation of the mass spectra was based on the assumption that all the species present in the mixture are endowed with comparable ionization capabilities (Ruoppolo et al., 1996; Ruoppolo et al., 1997). This seemed a reasonable hypothesis since all the species of interest were lysozyme molecules that differed only in their numbers of modified cysteines, a modification not likely to significantly change the ionization properties of the molecule. This was demonstrated in a previous work on the refolding of snake toxins (Ruoppolo et al., 1998), where the reduced, denatured, and carboxyamidomethylated toxin and the native oxidized toxin were shown to yield similar ion currents. The same conclusion was reached in the case of RNase A using LCMS, which also confirmed that there was no ionisation suppression in the mixture, thus ensuring that all species present were quantitatively detected by ESMS (F. Vinci, M. Ruoppolo, P. Pucci, R. Freedman, & G. Marino, in prep.). Figure 1B shows the curves obtained experimentally for the accumulation and/or disappearance of each oxidation state. This figure shows that the population of completely reduced molecules disappeared rapidly while 1 S-S and 2 S-S species got formed. The 3 S-S and the 4 S-S species appeared only later with a 3 min and 20 min lag, respectively. After 2 h, only the 3 S-S and the 4 S-S species were still present but their relative amounts went on changing with time, showing a slow enrichment in the 4 S-S species.

Characterization of the 3 S-S species

The persistence of 3 S-S species at the end of the renaturation process was unexpected since results obtained earlier showed that the mass spectrum of the refolded lysozyme corresponded to that of the fully reoxidized protein (Roux et al., 1997). Several possibilities could account for this discrepancy. The persistent 3 S-S species might consist in soluble aggregates that were not removed prior to the mass spectroscopy analysis in the present study, while they were removed by centrifugation in our earlier work (Roux et al., 1997). Or the protein obtained in our previous study might have been a mixture of 3 S-S and 4 S-S species, but the very small mass difference (only 2 Da) between the 3 S-S and the 4 S-S unlabeled species might have been missed by the mass spectrometry analysis. Or the 3 S-S species present at the end of the oxidation process might result from an equilibrium between the 3 and 4 S-S species. Or the persistent 3 S-S species might correspond either to a stable off-pathway conformation, or to an intermediate trapped by wrong S-S bond(s) that would be unable to get reshuffled.

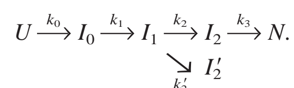
To sort between these alternative possibilities, the following experiments were carried out. Samples after 24 h of renaturation were split into two fractions. One was labeled as described, the other was first centrifuged for 60 min at 10,000 *g* and then labeled. Both samples had exactly the same mass spectra, thereby demonstrating that the 3 S-S species was soluble in the regeneration buffer and did not form large aggregates. To test for the presence of 3 S-S species in the renatured lysozyme that was previously characterized as "native," lysozyme was regenerated, dialyzed against 10 mM potassium phosphate at pH 5.5, then against 50 mM ammonium acetate, and then centrifuged to remove aggregates, as previously described (Roux et al., 1997). It was then lyophilized, redissolved in the regeneration buffer, labeled with IAM, and analyzed by mass spectrometry. Only the 4 S-S species could be observed. This indicated that the persistent 3 S-S species consisted in incompletely or misfolded molecules, which, though soluble in the renaturation buffer (see above), form aggregates when guanidine is removed from the solvent upon dialysis. To test for an equilibrium between the 3 and 4 S-S species, native lysozyme was incubated for 24 h at 25 °C in the renaturation buffer containing the disulfide interchange catalyst, labeled with iodoacetamide, desalted, and submitted to mass spectrometry. No 3 S-S species could be detected, ruling out the existence of an equilibrium between the 3 and 4 S-S species. The existence of a trapped intermediate with S-S bond(s) that would be unable to get reshuffled seemed plausible because of the very low reducing power of the redox mixture used in this study (see Discussion). To test this possibility, reduced lysozyme was incubated for 24 h in the renaturation buffer and separated in three aliquots. One was immediately labeled for 20 s with iodoacetamide. The second was incubated in the same buffer for 24 additional hours and labeled with iodo-

acetamide. The third was supplemented with 3 mM reduced glutathione and 3 mM oxidized glutathione, incubated again for 24 h, and labeled with iodoacetamide. The three samples were desalted on PD-10 columns and analyzed by mass spectrometry. The two first samples showed very similar mass spectra with about 20–30% 3 S-S and 70–80% 4 S-S species, confirming the presence of the persistent 3 S-S species and indicating that the reaction was already complete after the first 24 h of incubation. The mass spectrum of the third sample showed a unique peak corresponding to the 4 S-S species, and no detectable trace of the 3 S-S species. Thus, increasing the concentrations of the redox agents rescued the trapped 3 S-S species and lead to its conversion to the 4 S-S species by allowing for an efficient reshuffling of the S-S bonds within the 3 S-S species.

Simulations

To correlate the kinetics of oxidation established in this study with previous data on the recovery of spectroscopic signals and activity, we simulated the refolding of reduced/denatured lysozyme, using a sequential model. Such a model was chosen for two reasons. First, the oxidation process is more likely to be a process in which there is a progressive increase in the number of disulfide bonds per molecule, rather than a process in which several disulfide bonds form simultaneously. Moreover, previous studies on protein reoxidation pathways showed this type of behavior (Anderson & Wetlaufer, 1976; Creighton, 1978; Chatrenet & Chang, 1993; Ruoppolo et al., 1996). Second, the recovery of the secondary structure and of the activity had already been shown to be best described by a sequential model (Roux et al., 1997). We therefore tried to compare the previously proposed sequential model with the sequential building up of an increasing number of disulfide bonds during the renaturation of reduced HEWL.

However, in the case of the reoxidation, we could follow the appearance and/or disappearance of five different species while our previous model included only four species. A fourth step was therefore added to the previous sequential model to completely describe the folding/oxidation process. Since the disappearance of the completely reduced species was much faster than any of the three phases proposed in the previous study, this additional step was introduced at the beginning of the refolding, and the first rate constant k_0 was approximated by the value obtained by fitting a single exponential to the decay of the fully reduced (0 S-S) species. An additional, off-pathway step was also introduced to account for the presence of the persistent 3 S-S species (see above). The simplest, sequential model that could account for the coexistence of the 3 S-S and the 4 S-S species at the end of the refolding is



$$U \xrightarrow{k_0} I_0 \xrightarrow{k_1} I_1 \xrightarrow{k_2} I_2 \xrightarrow{k_3} N.$$
$$I_1 \xrightarrow{k_2'} I_2'$$

One could have imagined that the kinetic partitioning between I_2 and I_2' might occur at the 3 S-S stage, with I_2 converting either to I_2' or to N. This is, however, not compatible with our observation that increasing the concentration of oxidized glutathione by a factor 4 failed to affect the relative amounts of N and I_2'. Indeed, if this assumption were correct, increasing the concentration of oxidant should have accelerated the I_2 to N oxidation step (see Discussion) and not the isomerization between I_2 and I_2', thus favoring the N over the I_2' species. On the contrary, in the model depicted above, the apparent oxidation rate constants k_2 and k_2' would have been increased by the same factor, a prediction compatible with the observed invariance of the partitioning between I_2' and N upon increasing the glutathione concentration. The model with the kinetic partitioning between I_2 and I_2' occurring at the 2 S-S bond stage will therefore be preferred.

This model was used to construct simulations of the kinetics of formation and/or disappearance of U, I_0, I_1, I_2, and N. The rate constants were initially set as follows. As indicated above, k_0 was calculated by fitting a single exponential process to the decay of the 0 S-S species. The other constants were set to the values found in the previous study for k_1, k_2, and k_3, i.e., $k_1 = k_2 = 0.06$ min^{-1} and $k_3 = 0.002$ min^{-1}. The value of k_2' was chosen to keep k_2/k_2' equal to 2, so that $N/I_2' = 2$ which was the final value of the 4 S-S/3 S-S ratio. These rate constants were then adjusted "visually" to obtain the best fit between the kinetics of evolution of the various oxidation states shown in Figure 1B and the simulated kinetics of evolution of the U, I_0, I_1, I_2, and N species. The values used for the best simulation are presented in Table 2, and the corresponding curves are shown in Figure 2. These curves were found to match quite well the profiles obtained experimentally for the 1 S-S, the 2 S-S, the 3 S-S, and the 4 S-S species, the 0 S-S species being assimilated to the unfolded state. Moreover, the values obtained from this visual fitting procedure for k_1, k_2, and k_3 after adjustment remained close to their original values, which were those obtained from the kinetics of far-UV ellipticity, fluorescence, and enzymatic activity experiments, suggesting a close parallel between the intermediates postulated in the model proposed previously (Roux et al., 1997) and specific extents of oxidation.

Discussion

Following previous studies on the kinetics of reactivation and secondary structure recovery during the renaturation of reduced lysozyme (Roux et al., 1997), the present work reports the kinetics of its oxidation, using the method developed by Torella et al. (1994) in which the free cysteines are reacted with iodoacetamide and the resulting samples analyzed by mass spectrometry. We shall first discuss a practical difficulty that was encountered when using this method in the present study and that had already been pointed out by States et al. (1984). For the quantitative kinetic analysis of S-S bond formation, it is essential that all the free cysteines should be modified by the labeling procedure. This should not be a problem at early times of the refolding, when the protein is still unfolded

Table 2. *Values of the four different time constants used for the simulation of the appearance/disappearance of refolding intermediates*

Rate constant	Value (min^{-1})
k_0	0.148
k_1	0.065
k_2	0.032
k_3	0.005
k_2'	0.016

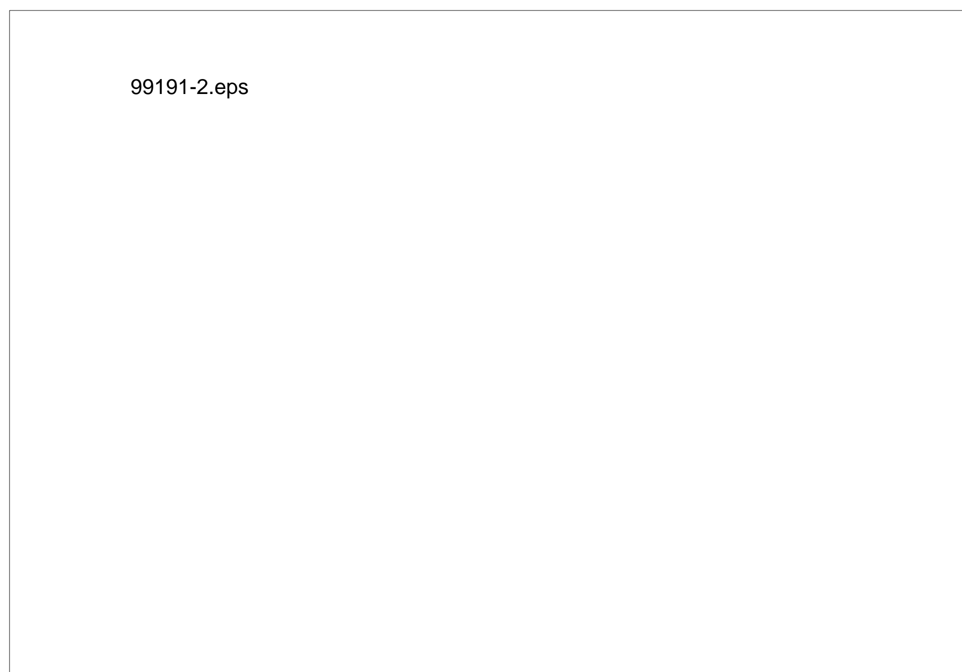


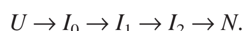
Fig. 2. Comparison of experimental results and simulation of the appearance and disappearance of intermediates. The experimental data points (open diamonds) correspond to the results of Figure 1B. The continuous curves represent the simulation of the variation with the refolding time of the different intermediates corresponding to the model proposed in Results. The values used for the rate constants are shown in Table 2.

enough for all its cysteines to be accessible to iodoacetamide. Indeed, during the renaturation in vitro of reduced hen lysozyme, samples labeled during the first hour of refolding showed essentially only even numbers of carboxyamidomethylation. In some experiments, minor peaks corresponding to species carrying 1, 3, 5, 7, and 9 labels could also be observed at this stage of the folding process, suggesting that the reaction had modified not only all the free cysteines but also an other residue. This residue was likely to be histidine 15 since iodoacetamide is known to react preferentially with cysteines but also, at a much lower rate, with histidines (Gurd, 1972). However, at later stages in the refolding process (i.e., between about 1 and 2 h of renaturation), the mass spectra showed a significantly larger peak corresponding to a species with one modification. The double labeling experiment with iodoacetamide and NEM, demonstrated that this species resulted from an incomplete labeling by iodoacetamide of molecules with 3 S-S bonds. This observation clearly indicated that, at some specific stages in the folding, a free cysteine becomes less accessible to iodoacetamide, presumably because it is shielded from the solvent in the interior of some transient folded 3 S-S bond species. This conclusion prompts some caution in the interpretation of this type of experiments. Indeed, completion of the labeling should be checked, and the experimental conditions eventually adjusted throughout the refolding to ascertain that all free cysteins are exposed to the reagent. While the existence of a problem with the labeling conditions was obvious in our experiments because of the presence of an odd number of chemical modifications, underlabeling might remain unnoticed in some systems if odd numbers of cysteines become shielded at some stages of the folding process. Adding a denaturing agent before the carboxymethylation, as in the second step of our double labeling experiments, should reduce this problem, provided the resulting denaturation is faster than the labeling.

A second difficulty, which arose in the interpretation of the kinetics of oxidation of hen lysozyme, was the presence of species with only 3 S-S bonds after 24 h of renaturation, a time when the renaturation was expected to be completed (Roux et al., 1997). The hypothesis of an equilibrium between the 3 S-S and the 4 S-S species was ruled out by the absence of any detectable 3 S-S species after incubation of native lysozyme in the renaturation buffer for 24 h. Rather, we showed that the persistent 3 S-S species, while soluble in the renaturation buffer containing 0.5 M guanidine, could no longer be detected when the protein was submitted to centrifugation after removal of the guanidine by dialysis. This strongly suggests that the 20–30% of the molecules that remain as 3 S-S species at the end of the renaturation–oxidation process are misfolded polypeptide chains. The presence of 20–30% of misfolded inactive molecules would account well for the 70–80% yield in activity obtained at the end of the renaturation. The residual 3 S-S species was shown to disappear and get converted to the 4 S-S species when the concentrations of reduced and oxidized glutathione were increased to 3 mM each. Thus, the amount of oxidant in our renaturation mixture might have been too low to oxidize all the protein thiols. Indeed, as shown by Raman et al. (1997), increasing the amount of oxidant in the renaturation buffer provides higher refolding yields for the same concentration of HEWL. Alternatively, the amount of reducing agent might have been too low to allow complete reshuffling of incorrect disulfide bonds. The latter hypothesis seems quite likely because, as discussed below, DTT is essentially oxidized and reduced glutathione is present in the renaturation mixture at a concentration of only 40 μM. Reduced glutathione being a weak reductant, there will be

little tendency for protein disulfides to be reduced under these conditions. Hence, there might exist 3 S-S species that cannot reach the fully oxidized state because they are trapped by (presumably) wrong disulfides in a conformation where the relative positioning of the two free cysteines precludes their oxidation into a fourth disulfide.

Taking into account this artificially irreversible formation of 3 S-S species, it was possible to plot the kinetics of appearance and disappearance of the different oxidation states of lysozyme and to correlate them with the previously reported kinetics of reactivation and recovery of secondary structure. The data obtained by mass spectrometry for each oxidation state (Fig. 2) were found to be in fair agreement with the simulation of the concentrations of the various intermediates in the sequential model proposed in Results. The main features of this model can be summarized as follows. First, the oxidation of HEWL (in terms of number of disulfide bonds formed) is sequential. At the beginning of the refolding, only 1 S-S species are detected, then 2 S-S species appear, then 3 S-S, and later 4 S-S. Although the experiments were carried out in a different buffer, our results are in agreement with those of Anderson and Wetlaufer (1976) showing that the two first disulfides get formed more rapidly than the third, and especially the fourth, ones. The second important feature of this model is that the rate constants that provided the best simulation for the last three steps were close to those found by fitting the CD and reactivation data. This indicates that the populations of intermediates I_1 and I_2 identified by CD and activity measurements correspond to those with 2 and 3 S-S bonds, respectively, characterized in the present study. Leaving aside the abortive formation of the persistent 3 S-S dead-end species, this leads to the following sequential model for the productive folding of lysozyme:



$$U \rightarrow I_0 \rightarrow I_1 \rightarrow I_2 \rightarrow N.$$

In this model, I_0 has no activity and no secondary structure but contains one disulfide. I_1 contains two disulfides, shows 20% of the native CD signal, but has no enzymatic activity. I_2 contains 3 S-S bonds, has 100% of the native CD and 80% of the native activity. N is the native, fully oxidized conformation with 100% activity and 4 S-S bonds. It is noteworthy that the intermediate I_2 with 3 S-S bonds has properties quite similar to those reported earlier for previously described native mutants of HEWL, each lacking one of the disulfide bonds of wild-type lysozyme (Sawano et al., 1992), whose ellipticities at 220 nm are close to that of the native wild-type molecule, whose specific activities range between 70 to 110% of that of the native wild-type, and which also contain 3 S-S bonds. It is also to be noted that the model we propose is the simplest that can describe our experimental data. In particular it does not take into account "side phenomena" such as aggregation, about which the methods we have been using provided no direct information.

One should emphasize that, at the present stage of our investigations, it is not possible to go into a more detailed interpretation of the oxidation kinetics, and hence of the folding mechanism. Indeed, we still have no information about the homogeneity of each of the populations defined by their number of S-S bonds. These populations could contain, in addition to productive intermediates, some dead end species that will fail to complete the folding-oxidation process. This seems quite likely in particular for the 3 S-S population, which, under our experimental conditions, is still present at the end of the folding process. Moreover, nothing is known about which disulfides are present in the different populations. It would be particularly interesting to know at which stage of the folding-oxidation process the native-like disulfide bonds start to prevail over non-native ones. In particular, a decisive step in understanding the coupling between S-S bond and secondary structure formation would be to know whether the far-UV CD signal (about 20% of the native ellipticity) of the I_1 intermediate corresponds to a partial, heterogeneous folding of all the molecules present in the 2 S-S species, or is produced by a subpopulation (20% of the 2 S-S molecules) that carry some specific native-like disulfide bonds and exhibit a native-like secondary structure.

We shall now discuss some of the properties of the unusual redox mixture used in this work, the composition of which was chosen empirically (Roux et al., 1997). DTT was initially introduced in the renaturation mixture as the reducing agent. However, reduced DTT rapidly equilibrates with oxidized glutathione and, because of its high equilibrium constant (about 200 M^{-1}) to form an internal disulfide bond (Chau & Nelson, 1991; Rothwarf & Scheraga, 1992), it gets practically entirely oxidized. The redox mixture present during the renaturation process therefore consisted essentially in 20 μM oxidized DTT and 40 μM each of reduced and oxidized glutathione. Thus, mixed disulfides with the protein thiols could be formed either with DTT or with glutathione. However, the mixed disulfide involving dithiothreitol is so unstable that it never accumulates substantially, and protein disulfide formation using oxidized DTT as disulfide reagent is proportional to its concentration and is slow (Creighton, 1986). Therefore, because of its low concentration in our experiments, oxidized DTT should not contribute significantly to the oxidation of HEWL. Hence, only glutathione should act as the oxidant. Thus, the kinetics of formation and exchange of protein disulfides were essentially controlled by 40 μM each of reduced and oxidized glutathione. From the value ($\sim$1,200 min^{-1} M^{-1}) of the second order rate constant of reaction of an individual protein thiol with oxidized glutathione at pH 8.7 (Creighton, 1986), and considering that the reactive species in this reaction is the thiolate ion and that the average pK of the thiols in the unfolded protein is 8.3, one can estimate the pseudo-first order rate constant at pH 8.2 and 40 μM glutathione to be about 0.03 min^{-1}. Taking into account the number of cysteins present in lysozyme at each stage of its oxidative folding, the predicted rate constants for the formation of a mixed disulfide in the reduced, the 1 S-S, the 2 S-S, and the 3 S-S species would be 0.24, 0.18, 0.12, and 0.06 min^{-1}, respectively. Together with the observation that 40 μM reduced glutathione is a very poor reductant, these considerations lead to the following conclusions:

(1) The low concentration of reduced glutathione we used, by greatly minimizing the back reactions on the folding pathway, accounts for the striking qualitative difference between our results with lysozyme and those reported by Sheraga and his coworkers for ribonuclease A (Rothwarf & Scheraga, 1993; Rothwarf & Scheraga, 1998). In the case of ribonuclease A, the distribution of the reduced and intermediate species reached a steady state equilibrium distribution, while for lysozyme the oxidation of each species into the next one can be considered as irreversible (see model above and fit curves in Fig. 2). This was experimentally verified for the persistent 3 S-S species, which could be driven to the native state only by increasing the concentrations of redox components. That the oxidation of lysozyme folding intermediates is practically irreversible under our experimental conditions leads to the important prediction that nonnative disulfides formed during

the folding process must undergo intramolecular exchange with free cysteinyl side chains of the same polypeptide chain.

(2) The composition of our redox mixture accounts for the absence of observable mixed disulfides between lysozyme and DTT, as was already noted for ribonuclease A where mixed disulfides could be detected when glutathione was used during the regeneration, but not when DTT was used (Rothwarf & Scheraga, 1993).

(3) The renaturation/oxidation conditions used in this study (low concentrations of redox components and presence of residual GuHCl) renders the kinetics of formation of disulfide bonds slow compared to the duration of the iodoacetamide pulse, thus preventing any significant perturbation of the degree of oxidation of the protein by the chemical modification.

(4) The values estimated for the pseudo-first order rate constants of formation of a mixed disulfide in the unfolded protein (see above) are sufficiently close to those observed experimentally (see Table 1) to support the assumption that, in our experimental conditions, the formation of a mixed disulfide is the rate limiting step in the formation of each of the four internal disulfide bonds of lysozyme. This is compatible with the report that increasing the amount of oxidant in the renaturation buffer improves the folding of HEWL (Raman et al., 1997). It also accounts for our failure to detect any mixed disulfides with glutathione in the mass spectrum of lysozyme folding intermediates, which indicates that the intramolecular step of disulfide exchange leading to the formation of a protein disulfide was faster than that of mixed disulfides. Thus, the conformational events that occur in the polypeptide chain and lead to the formation of a given S-S bond are faster than the oxidation of the corresponding cysteines.

The latter conclusion indicates that, if some secondary or tertiary structure elements were *stable* in the absence of some S-S bonds they would have ample time to get formed before the S-S bonds and should therefore be detected by a far-UV CD change *before* the formation of these bonds. Yet, no detectable secondary structure could be observed before the second disulfide bond was made, indicating that no stable secondary structure was formed before at least two, presumably native, S-S bonds were present. This strongly suggested that the formation of at least two native S-S bonds might be the rate limiting step in the formation of a nucleation center around which, once it is present, the polypeptide chain would fold in less than a second following the pathways proposed by Dobson et al. (1994). Indeed, the model of Dobson et al. relies on experiments performed with HEWL in which the disulfide bonds were kept intact in the unfolded state. Hence, the putative nucleation center was present from the onset of the refolding process.

Such a model, in which the formation of a limited number of native disulfide bonds would be followed by the quasi-instantaneous appearance of the secondary structure (Chaffotte et al., 1992) and by the very rapid completion of the folding (Dobson et al., 1994) is compatible with an initially "random" search for the native pairing of cysteines, kinetically driven not by the conformational properties, but rather by the spacing of the cysteines along the polypeptide chain as proposed by Camacho and Thirumalai (1995). When enough of the disulfides required for the formation of a nucleation center would be formed, the resulting fast folding of the polypeptide around it would shield these native disulfide bonds from the solvent, thus preventing the reducing component of the interchange catalyst from driving back the molecule upstream the folding pathway. That the rapid refolding of denatured lysozyme with its disulfides intact efficiently protects its S-S bonds against reduction was indeed observed in previously reported stopped-flow experiments showing that the presence of the redox catalyst did not modify the kinetics of appearance of the far-UV ellipticity (Roux et al., 1997). On the contrary, it is expected that species with wrong disulfides would be unable to fold further, thus leaving their disulfides exposed for reduction or intramolecular exchange. This would predict that, as already observed with BPTI (Weissman & Kim, 1991), HEWL folding intermediates with nonnative disulfide bonds should not be observed in significant amounts. Testing this prediction, as well as determining whether some disulfides play a more important role than others, will require to find out which disulfides are present at each stage the refolding. Experiments aimed at answering these questions are in progress in our laboratories.

Materials and methods

Proteins and chemicals

Hen egg white lysozyme (HEWL) and oxidized glutathione (GSSG) were purchased from Boehringer Mannheim GmbH (Mannheim, Germany), reduced dithiothreitol (DTT) and *Micrococcus lysodeikticus* from Sigma (St. Louis, Missouri), guanidine hydrochloride (GdnHCl) and urea from ICN Biomedicals (Costa Mesa, California), and iodoacetamide from Fluka Biochemica (Buchs, Switzerland).

PD-10 columns were purchased from Pharmacia (Uppsala, Sweden).

Preparation of the reduced/denatured lysozyme

Reduced/denatured lysozyme was prepared as described by Goldberg et al. (1991).

Refolding of reduced denatured lysozyme

The lyophilized reduced/denatured lysozyme was dissolved in 6 M GdnHCl, 0.1 M acetic acid, pH 2.5. Refolding was initiated upon a 100-fold dilution, under strong vortex agitation, in renaturation buffer (0.5 M GdnHCl, 0.1 M Tris HCl, pH 8.2, 1 mM EDTA, 20 μM reduced DTT, 60 μM GSSG). The samples were then incubated at 25 °C.

Enzyme assays

Lysozyme activity was measured as described earlier (Roux et al., 1997). The protein concentration in the assay was 2 μg/mL.

Carboxymethylation of the refolding lysozyme

Aliquots of reduced and denatured HEWL were diluted into renaturation buffer to provide 1 mL samples at 0.1 mg/mL. After incubation at 28 °C for the desired refolding time, 800 μL of these samples were mixed by rapid pipetting with 800 μL of a 2 M solution of iodoacetamide (dissolved in 0.5 M GdnHCL, 0.1 M TrisHCl, pH 8.2, 1 mM EDTA) in a low-binding tube wrapped in aluminum foil. The iodoacetamide solution was obtained according to Gray (1993) by dissolving the iodoacetamide powder in the buffer at 65 °C for not more than 5 min. The solution was cooled to room temperature before use. The alkylating reaction was carried out for 20 s at room temperature in the dark. The samples were

then acidified to pH 3 by addition of 120 μL of pure acetic acid and immediately loaded on a PD-10 column equilibrated and eluted using 0.1 M acetic acid. One milliliter fractions were collected and their absorbance at 280 nm was measured using as a reference the acetic acid solution. The fractions containing the protein were then lyophilized and kept at $-20\,^{\circ}$C. Samples of native and denatured/reduced lysozyme were incubated for 15 s in renaturation buffer and treated as above, except that IAM was omitted from the labeling solution. The protein concentrations in the samples loaded on the column and in the eluted fractions were determined by the method of Bradford or by absorbance at 280 nm, respectively. The recovery for both species was the same and was always between 80 and 100%, showing that no species was selectively retained on the PD-10 column.

Double-labeling experiments

To identify the species that were incompletely labeled (odd number of carboxyamidomethylations), samples labeled with iodoacetamide and lyophilized were split into two fractions. One fraction was left as such. The other one was denatured and reduced in the presence of 10 M urea, 10 mM DTT in 10 mM sodium phosphate buffer at pH 10, or in the presence of 6 M GdnHCl, 10 mM DTT in 10 mM phosphate buffer at pH 10. After incubation at 25 °C for 2 h, the pH of the buffer was lowered to pH 7, and the sample was diluted 1:1 with a 0.1 M phosphate buffer, containing 0.2 M N-ethyl maleimide and the same denaturant concentration. After a 10 min incubation at 25 °C, the sample was loaded on a PD-10 column equilibrated in 0.1 M acetic acid. The protein was eluted as indicated in the previous section and lyophilized. The fractions after single- and double-labeling were both analyzed by mass spectrometry.

Mass spectrometry analysis

ESMS analysis were carried out using a BIO-Q triple quadrupole mass spectrometer equipped with an electrospray ion source (Micromass, Beverly, Massachusetts). The protein samples were dissolved in H_2O containing 2% CH_3COOH and diluted 1/1 with CH_3CN; 10 μL (10–20 pmol/μL) were introduced into the ion source via loop injection at a flow rate of 300 μL/h. Spectra were recorded by scanning the quadrupole at 10 s/scan. Data were acquired and elaborated by the MassLynx software. Each population of intermediates was accurately quantified by measuring the total ion current produced by each species provided that the different components are endowed of comparable ionization capabilities. Mass-scale calibration was performed by means of multiply charged ions from a separate injection of horse heart myoglobin (average molecular mass 16,951.5 Da) or of HEWL (average molecular mass 14,305.1 Da). The errors between replicates on the molecular masses were as indicated in Table 1.

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