Protein Science (1994), *3*:883–887. Cambridge University Press. Printed in the USA.

Native disulfide bonds greatly accelerate secondary structure formation in the folding of lysozyme

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(RECEIVED December 16, 1993; ACCEPTED March 18, 1994)

Abstract

To assess the respective roles of local and long-range interactions during protein folding, the influence of the native disulfide bonds on the early formation of secondary structure was investigated using continuous-flow circular dichroism. Within the first 4 ms of folding, lysozyme with intact disulfide bonds already had a far-UV CD spectrum reflecting large amounts of secondary structure. Conversely, reduced lysozyme remained essentially unfolded at this early folding time. Thus, native disulfide bonds not only stabilize the final conformation of lysozyme but also provide, in early folding intermediates, the necessary stabilization that favors the formation of secondary structure.

Keywords: CD; disulfide bonds; folding intermediates; lysozyme

In a recent study on the refolding of hen egg white lysozyme (HEWL) we observed that an intermediate with a far-UV circular dichroism spectrum compatible with native-like secondary structure was formed in less than 4 ms (Chaffotte et al., 1992). At this early stage of the folding process, the polypeptide chain has no defined tertiary structure as judged from the lack of near-UV CD in the aromatic and disulfide bond regions. Moreover, pulsed proton exchange followed by NMR identification of the protected protons showed that, at this stage, no proton involved in tertiary contacts and a very small fraction only of the backbone amide protons were protected against exchange with the solvent (Radford et al., 1992). Thus, a "molten globule" with native-like secondary structure is formed within the first 4 ms of folding of HEWL. Are disulfide bonds involved in this rapid folding step? We consider 2 alternative models for the effect of correct disulfide bonds on the formation of early folding intermediates of hen lysozyme, when its disulfide bonds are left intact during unfolding:

Model 1: Early intermediates do not involve correct tertiary contacts. The stability, and also the rate of formation of these intermediates, is the same whether or not correct disulfide bonds are present.

Model 2: Although the probes used thus far to characterize early intermediates in a variety of proteins have detected only secondary structure at such early stages of the folding process, nevertheless correct tertiary contacts are required for their stability. Therefore, the presence of correct disulfide bonds contributes to their stability and increases the rate of their formation.

A decision could be made between these 2 models by comparing the rate of formation of early folding intermediates when correct disulfide bonds are either present or absent. A study of early intermediates during the renaturation of reduced HEWL was therefore undertaken.

After reduction of its disulfides, HEWL can refold and reoxidize into native HEWL (Epstein & Goldberger, 1963; Wetlaufer et al., 1974). But at the slightly alkaline pH values needed for efficient refolding and oxidation of reduced HEWL and at the protein concentrations used for stopped-flow CD studies (about 0.1 mg/mL), reduced HEWL forms significant amounts of aggregates, which hinders its renaturation (Goldberg et al., 1991). This apparently precluded any reliable CD measurements on reduced HEWL. We describe here experiments in which this technical difficulty was overcome by measuring the far-UV CD signal of early folding intermediates before any significant aggregation occurred.

Results

Aggregation was avoided and the 4-ms intermediate was observed as follows (see Fig. 1). Because the committed step in aggregate formation occurs in the 0.5–5-s range during the folding of reduced HEWL (Goldberg et al., 1991), reduced unfolded HEWL was mixed under continuous flow in a 1/70 ratio with renaturation buffer over a 500-ms period in a stopped-flow apparatus (see Materials and methods). This resulted in an 8-mL/s

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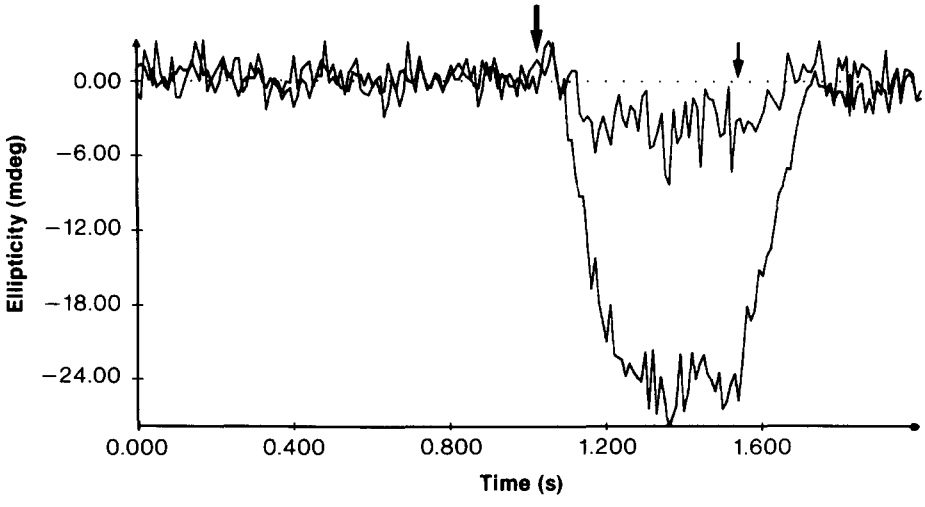

Fig. 1. Continuous-flow determination of the ellipticity of the 4-ms intermediate during folding of reduced and nonreduced HEWL. The ellipticity at 222 nm of HEWL after 4 ms of folding was recorded during a continuous-flow experiment conducted as described in Materials and methods. The tracings shown (upper for reduced HEWL and lower for nonreduced HEWL) are the average of 48 successive recordings. The large arrow indicates the start of mixing, and the small arrow indicates the start of rinsing.

continuous flow, through the observation cell, of reduced HEWL. With the SFM3 stopped-flow configuration and the observation cell used, this flow rate resulted in an aging time of 4 ms for the refolding process. This first injection phase was followed, without interruption of the flow, by a phase of washing with buffer only, thus preventing HEWL from remaining in the lines, mixer, and observation cell of the stopped-flow apparatus for more than a few milliseconds. Aggregates therefore had no time to form inside the stopped flow and could not clog the instrument. Neither could they seriously impair the far-UV CD signal of the 4-ms intermediate monitored in the observation cell. The result of such an experiment is shown in Figure 1, compared with that obtained under identical conditions with nonreduced HEWL. By repeating similar experiments at several wavelengths in the 213–245-nm range, the far-UV CD spectrum of the 4-ms intermediate was constructed for both reduced and nonreduced HEWL. These spectra are shown in Figure 2. That obtained with nonreduced HEWL confirmed our previous observation of the rapid regain of a large far-UV CD signal in the 222-nm region. Conversely, the spectrum of the 4-ms intermediate for reduced HEWL was smooth and did not show the negative ellipticity peak around 222 nm, typical of α-helices. This indicated an essentially random conformation.

Discussion

The spectra in Figure 2 show a dramatic difference in behavior between reduced and nonreduced HEWL. Qualitatively, they suggest that the 4-ms intermediate has a large amount of secondary structure (essentially α-helices) for nonreduced HEWL, whereas it has a random conformation for reduced HEWL. A more precise examination of these spectra shows, however, that they deviate from the expected spectra. Indeed, the spectrum of the reduced HEWL intermediate shows a slight rise in ellipticity with decreasing wavelengths, which deviates significantly from the spectrum obtained at equilibrium for the unfolded protein (Fig. 2). This might originate from traces of aggregates (correction of the far-UV CD spectrum for aggregates requires absorption flattening and quantitative light scattering measurements, which could unfortunately not be made in the stopped flow in the 4-ms time range), from the presence of small amounts of β-structure, or from an experimental artifact related to the impossibility of properly adjusting the photomultiplier high voltage (see Materials and methods). We very much favor the latter interpretation, mainly because the control spectrum obtained with the nonreduced intermediate in these series of experiments shows a similar deviation when compared to that obtained previously (Chaffotte et al., 1992) with the appropriate high voltage adjustment. Efforts are currently under way to develop a procedure that will avoid such artifacts.

Because of this artifact of a possible contribution from the very small amounts of aggregates that perhaps appear in 4 ms, and because the wavelength range investigated was too limited, no attempt was made to estimate the secondary-structure content of the 4-ms intermediate of reduced HEWL from its CD

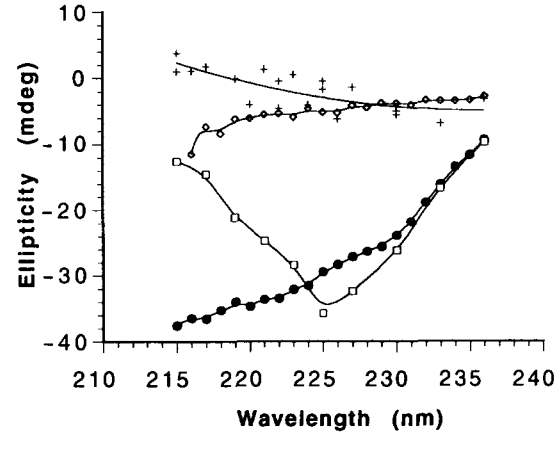

Fig. 2. Continuous-flow CD spectra of the 4-ms folding intermediates of reduced and nonreduced HEWL. Experiments identical to that described in Figure 1 were repeated at different wavelengths, as indicated on the abscissa. At each wavelength, the ellipticity of the 4-ms intermediate was determined as the difference between the plateau during the HEWL injection phase and the plateau before the injection and was plotted as a function of the wavelength for reduced (+) and nonreduced (□) HEWL. For comparison, the spectra recorded at equilibrium for reduced HEWL unfolded in 6 M guanidinium chloride, pH 3.0 (◇), and native HEWL (●) are also shown.

spectrum. The observed spectrum, however, clearly indicates the absence of detectable native-like secondary structure after 4 ms of folding of reduced HEWL. This result demonstrates that the tertiary topological constraints imposed by the 4 native disulfide bonds on denatured nonreduced HEWL greatly accelerate the formation of native-like secondary structures.

That nonreduced HEWL gets renatured much more rapidly than reduced HEWL had been known since the early studies of Epstein and Goldberger (1963). Even after optimizing the reoxidation conditions, a renaturation half time of several minutes was found for reduced HEWL (Wetlaufer et al., 1974) as compared to a few seconds only for the nonreduced protein (Kuwajima et al., 1985). Moreover, reduction of disulfide bonds in HEWL (Privalov et al., 1989; Radford et al., 1991), as in other proteins (Creighton, 1975, 1988; Kuroki et al., 1992), is known to decrease the stability of the native protein. The results described in the present report might therefore appear, at first sight, as trivial. This is not the case. Indeed, previous studies dealt with the effects of S-S bonds on the stability and kinetics of formation of the *final native tertiary structure* of HEWL. On the contrary, our study deals with an *early molten globule intermediate* that, up to now, was thought to have only native-like secondary structure but no defined tertiary structure. Because the folding of HEWL is clearly not a 1-step event, there was a priori no reason to expect that the influence of disulfide bonds on the kinetic and stability properties of an intermediate formed in less than 4 ms would be the same as their influence on the rate of formation (which takes about 3 orders of magnitude longer) and stability of native HEWL.

To our knowledge, this is the first report on the secondary structure of early intermediates during the effective refolding of a fully reduced, unmodified, disulfide-containing protein. It was reported previously that several reduced proteins in nondenaturing buffer lack detectable secondary structure even at equilibrium, thus suggesting that intermediates with stable secondary structure should not exist. For instance, fully reduced bovine pancreatic trypsin inhibitor (BPTI) has a far-UV CD spectrum characteristic of a random coil (Kosen et al., 1981). The same was reported for bovine ribonuclease (Galat et al., 1981) or lysozyme (Privalov et al., 1989; Radford et al., 1991) with their disulfide bonds disrupted. In these experiments, however, the cysteine residues of the unfolded reduced proteins had to be carboxymethylated to avoid their reoxidation during studies at equilibrium. This prevented folding of the protein into its native state, and it seems quite likely that the bulky carboxymethyl groups introduced in the "interior" of the modified protein could also destabilize the nascent secondary structure of the polypeptide chains. In the experiments we describe, no chemical modification of the cysteines was introduced. The observations reported here are therefore novel and more convincingly reflect the properties of early folding intermediates in the natural folding process of HEWL.

Our observations on reduced HEWL obviously diverge from those reported for α-lactalbumin, a protein yet very similar in sequence and conformation to HEWL. For α-lactalbumin, it was recently shown that reduction of 1 of the 4 disulfide bonds resulted in a significant decrease in the stability of the native protein but had only a marginal effect on the far-UV CD spectrum of an early intermediate observed by stopped flow (Ikeguchi et al., 1992). Furthermore, the reduced protein exhibited far-UV CD spectra compatible with large amounts of native-like secondary structure (Ikeguchi & Sugai, 1989). These observations on α-lactalbumin supported the commonly accepted view that disulfides are formed at late stages of the folding process, and that "they have a stabilizing effect on the native state without determining either the folding pathway or the final three-dimensional structure of the protein" (Jaenicke & Buchner, 1993:7). Our results show, on the contrary that, at least during the refolding of nonreduced HEWL, disulfide bonds modify the folding process at a very early stage by drastically increasing the rate of formation and/or the stability of folding intermediates. From the 2 models that were proposed in the introductory section, this clearly rules out model 1 and supports model 2, according to which tertiary contacts (the native disulfide bonds) are required for the rapid formation and the stability of secondary structure in HEWL early folding intermediates.

This conclusion establishes that long-range interactions are responsible for early steps on the folding pathway of nonreduced HEWL. Thus, folding of the nonreduced protein does not appear to be a hierarchical process that would begin by making local sequence interactions independently of long-range interactions and end with tertiary interactions between residues that are distant in sequence. Rather, it appears as a process in which tertiary interactions, fixed by the S-S bonds, are important from the beginning because the stability of local secondary structures depends on the presence of these long-range interactions. This comes as a support to the hypothesis of Karplus and Weaver (1976) that protein folding must involve a "selective" process in which long-range interactions contribute to the emergence and stabilization of the intermediate states that play a crucial role in dramatically reducing the conformational entropy of a polypeptide chain at early stages of its folding.

Whether or not disulfide bonds also play a role at early stages of the folding of reduced HEWL is, however, still an open question on which we shall now speculate. Under the pH and temperature conditions used in this study (Wetlaufer et al., 1974; Goldberg et al., 1991), the chemistry of disulfide bond formation is rapid (Creighton, 1975, 1988), and the formation of native disulfides should be controlled essentially by the rate of appearance of intermediate conformations that bring together the proper thiol groups (Creighton & Goldenberg, 1984). What limits the rate of renaturation of reduced HEWL must therefore be the rate of appearance and the stability of intermediates with secondary and supersecondary structures that bring about the proper pairing of cysteinyl residues (Creighton, 1988). In the absence of preformed disulfide bonds, such intermediates are not present in detectable amounts after 4 ms of folding, which is likely to explain the slow rate of renaturation of reduced HEWL.

Several mechanisms could account for the failure of reduced HEWL to form significant amounts of structured intermediates early during its folding. One would originate from the propensity of reduced HEWL to aggregate. Whereas intermediates "committed" to aggregate are formed only in a time range of about a second (Goldberg et al., 1991), "softer" aggregates might already be formed within the first 4 ms of folding and prevent the formation of secondary structure. This soft aggregation would, however, not be irreversible because reduced HEWL can ultimately fold and reoxidize into its native conformation. Moreover, if such soft aggregates, clearly corresponding to off-pathway intermediates, were indeed shown to form with reduced HEWL and not with the nonreduced protein, this would be an

additional, direct proof that the presence of native disulfide bonds modify not only the folding kinetics but also the folding pathway of HEWL. However, the presence of transient aggregates cannot be detected with current methods. Similarly, it has not been possible to find experimental conditions where reduced HEWL can fold without aggregate formation, and therefore to test whether soft aggregation is responsible for the slow appearance of secondary structure in HEWL. Though this hypothesis seems to us unlikely (because even irreversibly aggregated proteins exhibit large amounts of secondary structure), testing it certainly remains an interesting challenge.

A second possible mechanism accounting for the effect of S-S bonds on the secondary structure of the 4-ms intermediates would be to assume that secondary structure formation results from a general compaction of the polypeptide chain, that hydrophobic interactions would not suffice to maintain in the case of reduced HEWL. In nonreduced HEWL, the presence of disulfide bonds would greatly contribute to the compaction, thus indirectly helping secondary structure formation. A possible way of testing this hypothesis of a "nonspecific collapse" would be to investigate the secondary structure of 4-ms intermediates of HEWL with non-native disulfide bonds. It also would be of interest, using site-directed mutagenesis, to find out whether the 4 native disulfide bonds of HEWL play equivalent roles or if some only contribute to secondary structure formation.

The third possible mechanism deals with an intrinsically low thermodynamic stability of HEWL secondary structure in the absence of specific tertiary interactions and particularly of native disulfide bonds. Thus, the development of secondary structure should be coupled to the formation of S-S bonds. This coupling is usually considered as resulting from the formation of disulfide bonds at the end of the folding pathway. Alternatively, such a coupling might also occur at *early* stages of the folding process. Indeed, the role of S-S bonds in stabilizing local secondary structure was already demonstrated by Staley and Kim (1990) through studies at equilibrium of model peptides corresponding to disulfide-bonded, structured regions of native BPTI. To account for such a coupling, one can assume that secondary structure formation precedes the formation of disulfide bonds. Thus, a small, undetectable fraction of reduced HEWL molecules would rapidly fold into a form (or forms) having a native-like secondary structure, and this form(s) would ultimately favor the formation of native disulfide bonds. Disulfide bond formation would then pull the equilibrium toward the native-like secondary structure. This hypothesis deviates from the classical view only in that the disulfide bonds would form early on the folding pathway, i.e., at the stage of the molten globule, rather than near the end of the folding process. An alternative model, equally plausible though more heterodox, is that disulfide bonds should form first to induce native-like secondary structure formation. That native rather than random disulfide bonds are detected in significant amounts during the folding/oxidation of reduced proteins (Weissman & Kim, 1991) in the presence of disulfide interchange catalysts would then be explained by assuming that only *native* disulfide bonds would lead to the rapid formation of secondary and supersecondary structures that can protect the S-S bonds against the action of disulfide exchange catalysts. Thus, whereas non-native S-S bonds would be rapidly reduced and exchanged, native ones would be maintained and lead to the completion of the folding process. Whether the secondary structure forms first and induces the proper pairing of the cysteines, or S-S bonds form first and, if native, help in secondary-structure formation, is an open and challenging question.

Finally, the striking difference reported here between the folding properties of reduced and nonreduced HEWL emphasizes that results from studies on the renaturation of nonreduced disulfide containing polypeptide chains should not be extrapolated without great care to the biologically significant problem of protein folding, which deals with initially reduced polypeptide chains.

Materials and methods

HEWL was purchased from Boehringer and used without further purification. Reduced HEWL was prepared as described earlier (Goldberg et al., 1991). Reduced or nonreduced HEWL were dissolved in a solution of 6 M guanidinium chloride in water at pH 3.0. The HEWL concentration was adjusted to 7 mg/mL as previously described (Chaffotte et al., 1992) and introduced into the small syringe of an SFM3 stopped-flow apparatus (Bio-Logic, Claix, France) adapted as an option to a CD6 spectrodichrograph (Jobin-Yvon, Longjumeau, France). The 2 large syringes contained 10 mM potassium phosphate buffer at pH 7.8. The temperature was maintained at 20 °C. The observation cell (0.5-cm optical path) was initially filled with buffer. Recording of the ellipticity at 222 nm (with a 10-ms sampling interval and a 10-ms filtering time constant) was triggered at time 0 and continued for 2 s. One second after triggering, 57 μL of unfolded HEWL from the small syringe and 1,972 μL of buffer from each large syringe were continuously injected over 500 ms. This results in a constant flow (8 mL/s) of HEWL molecules (0.1 mg/mL) through the observation cell. Then, without interruption of the flow (i.e., 1.5 s after triggering), 1 mL of buffer from each large syringe was injected continuously over 250 ms, at which time the flow was interrupted. At each wavelength, the standard procedure of the stopped-flow option software was used to adjust the photomultiplier high voltage. This is done at the beginning of each series of runs, i.e., with the buffer present in the observation cell. This is likely to create a minor artifact in the ellipticity measured for the protein, mainly at low wavelengths, because of an insufficient signal due to light absorption by the protein. Because of the aggregation problem, it was unfortunately not possible to have the reduced HEWL in the cell during high-voltage adjustment.

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