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The oxidative refolding of hen lysozyme and its catalysis by protein disulfide isomerase

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The oxidative refolding of hen lysozyme has been studied by a variety of time-resolved biophysical methods in conjunction with analysis of folding intermediates using reverse-phase HPLC. In order to achieve this, refolding conditions were designed to reduce aggregation during the early stages of the folding reaction. A complex ensemble of relatively unstructured intermediates with on average two disulfide bonds is formed rapidly from the fully reduced protein after initiation of folding. Following structural collapse, the majority of molecules slowly form the four-disulfide-containing fully native protein via rearrangement of a highly native-like, kinetically trapped intermediate, des-[76–94], although a significant population (~30%) appears to fold more quickly via other three-disulfide intermediates. The folding catalyst PDI increases dramatically both yields and rates of lysozyme refolding, largely by facilitating the conversion of des-[76–94] to the native state. This suggests that acceleration of the folding rate may be an important factor in avoiding aggregation in the intracellular environment.

Keywords: disulfide bond/hen lysozyme/oxidative refolding/protein disulfide isomerase/refolding intermediate

Introduction

The sequence of events by which an unfolded protein achieves its native structure is one of the most complex problems in structural biology. Understanding how the information defining the protein fold is contained within the amino acid sequence is not only a major intellectual challenge but has important practical consequences, for example in contributing to the efforts to predict protein structures from amino acid sequences. Such an objective has particular importance in view of the extensive sequence data emerging from the various genome projects (Miklos and Rubin, 1996). Folding of proteins is also recognized to be linked with a variety of biological processes, including the control of metabolic processes and regulation of the cell cycle (King *et al*., 1996). Moreover, misfolding of proteins is now known to be associated with a wide range of diseases (Thomas *et al*., 1995).

At present, the vast majority of information concerning folding has been derived from studies of small, single domain proteins either lacking disulfide bonds or containing the native disulfide bonds throughout the folding reaction (Kim and Baldwin, 1990; Radford *et al*., 1992; Sosnick *et al*., 1992). Relatively little is known about the folding of disulfide-containing proteins since the oxidative folding of only a small number of proteins has been investigated in any detail, most notably bovine pancreatic trypsin inhibitor (BPTI) (Creighton, 1974; Weissman and Kim, 1991), ribonuclease T_1 (Ruoppolo and Freedman, 1994, 1995; Frech and Schmidt, 1995), α-lactalbumin (Creighton and Ewbank, 1994; Peng *et al*., 1995; Wu and Kim, 1998) and ribonuclease A (Rothwarf and Scheraga, 1993; Xu and Scheraga, 1996). The relative lack of oxidative refolding data is in contrast to the fact that cysteine residues comprise ~3.5% of the amino acid residues of extracellular eukaryotic proteins (Nakashima and Nishikawa, 1994). Therefore, disulfide bond formation is likely to play an important role in the folding of a considerable number of eukaryotic proteins. The formation of such bonds occurs concomitantly with folding, usually within the endoplasmic reticulum, and is catalysed by millimolar concentrations of reduced and oxidized glutathione (GSH/GSSG) (Hwang *et al*., 1992). Oxidative refolding studies are therefore of considerable importance from a physiological point of view because of their relevance to the folding of disulfide-containing proteins *in vivo*.

In the intracellular environment, a whole array of molecular chaperones helps to prevent the association of unfolded protein molecules that leads to aggregation, and facilitates correct folding (Gething and Sambrook, 1992; Hartl, 1996). In addition, proteins that catalyse disulfide bond formation are present (Freedman, 1995), one of the most extensively studied of which is protein disulfide isomerase (PDI) (Lyles and Gilbert, 1991; Weissman and Kim, 1993; Puig and Gilbert, 1994; Hawkins and Freedman, 1995; Walker and Gilbert, 1995; Darby *et al*., 1998), a 55 kDa protein that is present in near millimolar concentrations in the endoplasmic reticulum (Gilbert, 1998). It is thought to be the major catalyst of native disulfide bond formation in proteins (Gilbert, 1997), although the exact mechanism by which this occurs remains to be elucidated. PDI is also thought to display molecular chaperone activity under certain conditions (Gilbert, 1998).

Lysozyme (Figure 1) is an important model system for understanding protein folding, since its folding in the presence of its four native disulfide bonds has been studied in great detail (Chaffotte *et al*., 1992; Radford *et al*., 1992; Dobson *et al*., 1994; Eyles *et al*., 1994; Hooke *et al*.,

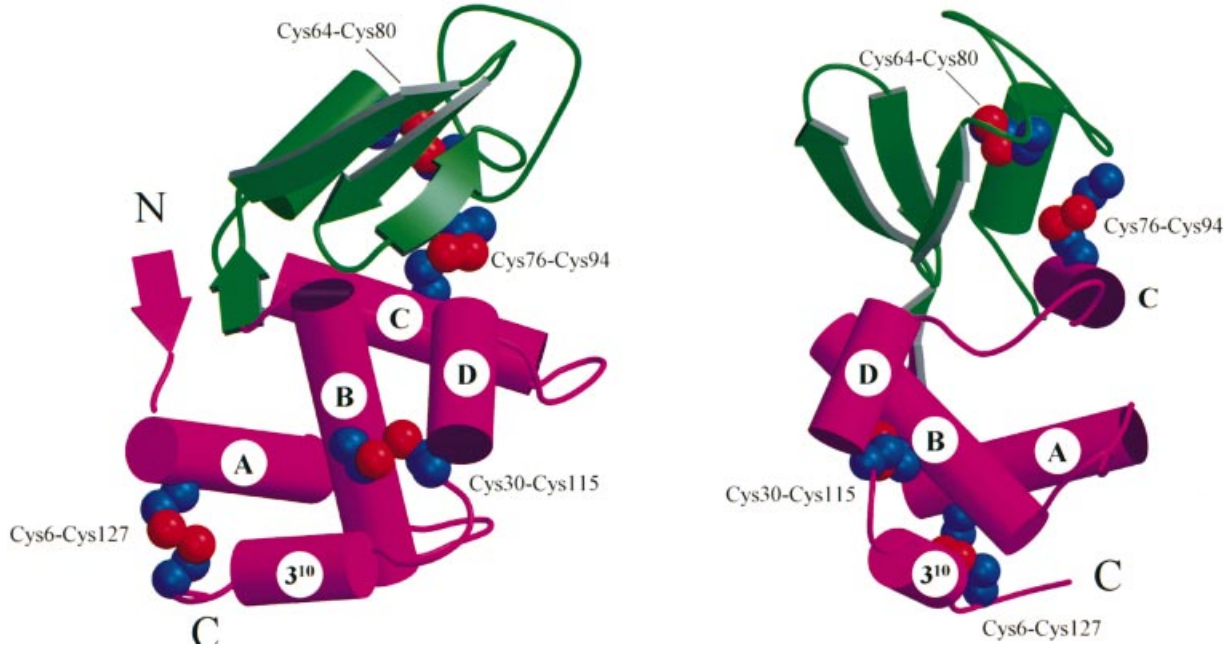

Fig. 1. MOLSCRIPT representations of native hen lysozyme showing the α-domain (residues 1–40 and 90–129) with the α-helices A–D and a C-terminal 3_{10} helix in purple. The β-domain, containing an antiparallel three-stranded β-sheet and a long loop, is shown in green. The helices are represented as cylinders. The C_α and C_β atoms of the cysteine residues of the four native disulfide bonds are drawn as CPK models in blue, with their S_γ atoms shown in red. Two disulfide bonds are present within the α-domain (Cys6–Cys127 and Cys30–Cys115). The remaining disulfide bonds are Cys64–Cys80, located within the β-domain, and Cys76–Cys94, linking the two domains.

1994; Matagne *et al*., 1997; Wildegger and Kiefhaber, 1997; Mizuguchi *et al*., 1998). Although its oxidative folding from the fully reduced state was first investigated more than 20 years ago (Anderson and Wetlaufer, 1976), only recently have methods that allow efficient refolding of reduced denatured lysozyme at higher concentrations been devised (Hevehan and De Bernadez-Clark, 1997; Roux *et al*., 1997). This allows the use of many biophysical techniques to probe the development of different aspects of the native structure during oxidative folding in a time-resolved mode, which previously was only possible for its non-oxidative folding process. The present work describes a detailed study of the oxidative folding of lysozyme designed to give insight into the underlying determinants of the folding reaction including the role of the disulfide bonds, under conditions more relevant to the intracellular environment.

Results and discussion

Oxidative refolding monitored by optical spectroscopy and NMR

Figure 2 shows the oxidative renaturation of lysozyme followed by a variety of types of optical spectroscopy; the values of the time constants obtained from each are summarized in Table I. Figure 2A, in which the kinetics of disulfide bond formation are shown, indicates clearly that formation of disulfide bonds occurs in distinct phases, the kinetics of which can be described to a good approximation by a double exponential curve. During the initial fast phase (τ_1 ~200 s), on average two disulfide bonds are formed within the ensemble of intermediates. During a second, much slower phase (τ_2 ~1700 s), the formation of the disulfide bonds is completed.

The intrinsic and ANS fluorescence changes during refolding can also be described well by double exponential kinetics, with similar time constants to those for the formation of the disulfide bonds (Figure 2B and C). The fast component (τ_1 ~200–300 s) of the kinetic profiles suggests that burial of tryptophan residues and a degree of hydrophobic collapse occur rapidly after initiation of folding during the phase when on average two disulfide bonds are formed. However, the magnitude of the ANS fluorescence change is relatively small and, compared with the large increase observed in 'molten globule' states of proteins (Semisotnov *et al*., 1991), indicates that the population of hydrophobically collapsed states during the initial stages of refolding is not large. The slow component of the kinetic profiles (τ_2 ~1700 s) shows that later in folding structural rearrangements occur with significant changes in the environment of the tryptophan residues, as indicated by the intrinsic fluorescence, accompanied by the formation of a tightly packed structure, as suggested by the concomitant decrease in ANS fluorescence.

The far-UV circular dichroism (CD) signal at 225 nm (Figure 2D) follows apparently single exponential kinetics with a time constant (τ ~1000 s) intermediate between those observed for disulfide bond formation and the recovery of the native fluorescence. By contrast, the development of the near-UV CD signal, reflecting the establishment of stable and persistent tertiary structure, shows an initial fast event with a time constant (τ_1 ~200 s) similar to those of the fast phases observed for the formation of the disulfide bonds, intrinsic fluorescence and ANS binding (Figure 2E). This suggests that the intermediates formed at this stage of folding are characterized by a lack of stable tertiary interactions; the negative amplitude of this phase is likely to reflect the formation of disulfide bonds (Rauk, 1984; Chaffotte *et al*., 1992). The subsequent slow phase of the near-UV CD signal has

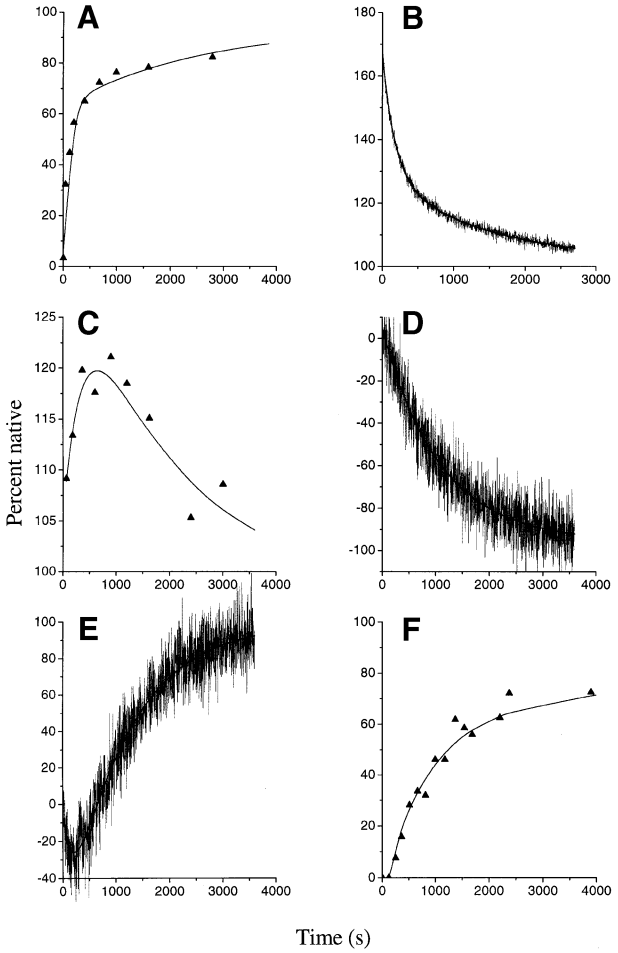

Fig. 2. Renaturation of 25 μM lysozyme in the presence of 1.0/0.2 mM GSH/GSSG at pH 8.5 monitored by optical spectroscopy. (**A**) Formation of disulfide bonds. (**B**) Intrinsic fluorescence. (**C**) ANS binding. (**D**) Far-UV CD. (**E**) Near-UV CD. (**F**) Recovery of enzymatic activity. Formation of disulfide bonds, ANS binding and recovery of enzymatic activity were monitored for 24 h; only the initial parts of their kinetics are shown to facilitate comparison with the other spectroscopical data. For details see Materials and methods.

Table I. Refolding of lysozyme at pH 8.5 as monitored by activity measurements, optical spectroscopy and 1D ^{1}H NMR spectroscopy

Technique	τ_1	τ_2	τ_3
S–S bond formation	200 ± 50	1700 ± 200	–
Intrinsic fluorescence	200 ± 20	1600 ± 100	–
ANS binding	340 ± 150	1700 ± 300	–
Far-UV CD	–	1020 ± 150	–
Near-UV CD	200 ± 30	1100 ± 100	–
Enzymatic activity	–[a]	900 ± 150	15 000 ± 2000
NMR α-domain	–	925 ± 115	–
NMR β-domain	–	1425 ± 140	–

Time constants (in s) are averages of at least two or three experiments (the fast component of the various kinetics as fitted to a double exponential corresponds to τ_1, the slow component to τ_2). Lysozyme concentrations were 25 μM for the optical studies and 35 μM for NMR. GSH/GSSG concentrations were 1.0/0.2 mM.

[a]Due to the difficulties of fitting the lag-phase during the recovery of enzymatic activity, no value could be obtained for τ_1. The remaining part of the enzymatic activity data has been fitted to a double exponential with time constants τ_2 and τ_3.

a positive amplitude and reflects formation of stable tertiary structure. The time constant of this phase is slightly longer (τ_2 ~1100 s) than the single time constant observed for the far-UV CD signal. Although the qualitative difference in the nature of the development of the far- and near-UV CD signals makes a direct comparison of their kinetics inappropriate, it is clear that the overall development of the near-UV CD signal is significantly slower than that of the far-UV CD signal; after 1500 s only ~50% of the native near-UV CD signal is present, whereas this is ~70% for the far-UV CD signal (Figure 2D and E).

Disulfide bonds can also contribute to the observed ellipticity changes at 225 nm (Chaffotte *et al*., 1992). However, the fact that the development of the far-UV CD signal does not show an initial fast phase as seen in the kinetics of the near-UV CD signal suggests that the contribution of disulfide bonds to the CD at 225 nm is small. Since the contributions of aromatic side chains to the CD in this wavelength region are also known to be small (Chaffotte *et al*., 1992), the observed changes in ellipticity at 225 nm are likely to reflect predominantly formation of secondary structure, which is therefore slow relative to the fast initial events observed in the formation of disulfide bonds and fluorescence (Table I and Figure 2).

The recovery of enzymatic activity (Figure 2F) shows a lag-phase of ~2–3 min which, as has been described previously (Roux *et al*., 1997), is very difficult to fit to a simple exponential model. However, its existence is consistent with the notion from the near-UV CD kinetics that the early intermediates do not yet possess stable tertiary structure. Following the lag phase, the data fit reasonably well to a double exponential, the first phase of which has a time constant comparable to that of the far-UV CD signal (τ ~900 s), whereas the second phase is very slow (τ ~15 000 s). This very slow phase is only apparent in the enzymatic activity measurements and suggests that the intermediates occurring during the later stages of the folding process have very native-like structures but substantially lower enzymatic activities than the native protein.

The changes observed in the optical properties of the protein described above reflect the global properties of the folding molecules. More localized information was obtained by following the NMR signals of individual residues in both the α- and β-domains during folding (Figure 3). The average time constant of the development of the native chemical shifts of the resolved resonance of one of the methyl groups of Leu17 and of a composite resonance from methyl groups of Leu8/Leu17/Ile98/Met105, all of which are located in the α-domain, is 925 ± 115 s. By contrast, the time constant of the development of the native chemical shift of the overlapping resonances from the β-domain residues T51/L56 is 1425 ± 140 s. The kinetics of the appearance of the resonances of α-domain residues at their native chemical shifts can be seen to be closely similar to the kinetics of development of the far-UV CD signal. The kinetics of the resonances of the β-domain residues, however, are significantly slower and more similar to the kinetics of development of the near-UV CD signal (Figure 3 and Table I). This suggests that in the intermediates that are formed, the α-domain is on average more native-like than the β-domain. Throughout the NMR spectra, only

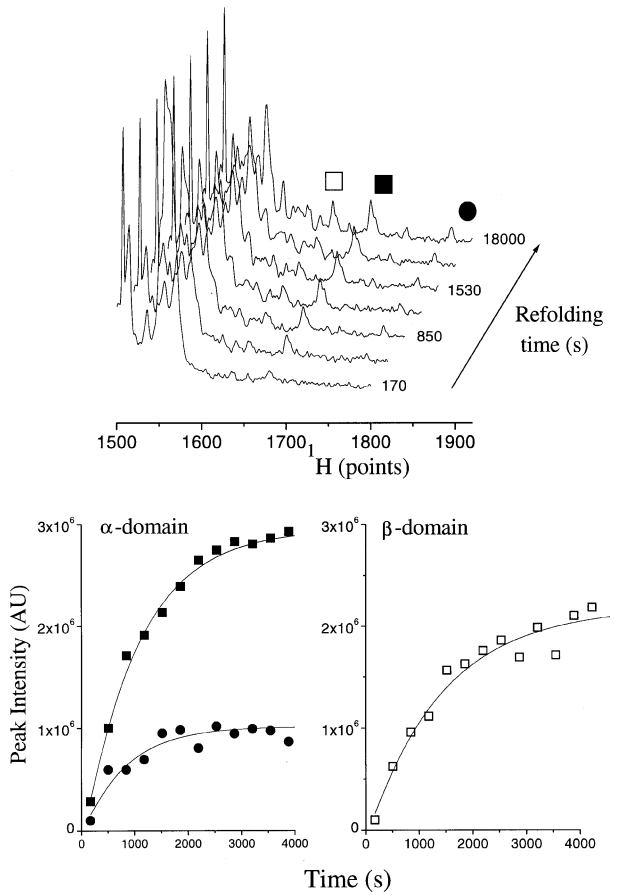

Fig. 3. Oxidative refolding of 35 μM lysozyme followed by 1D ^{1}H NMR spectroscopy at pH 8.5, in the presence of 1.0/0.2 mM GSH/GSSG. The top panel shows the high-field methyl region of several 1D spectra of the refolding protein. The resonances for which the kinetics are shown in the bottom panels are indicated. Left panel: resonances of L8δ_1/L17δ_2/M105ε/I98δ (■), all of which are located in the α-domain, and L17δ_1 (●). Right panel: resonances of T51γ/L56δ_1 (□), which are located in the β-domain. Since the dead time of these NMR experiments is ~4 min, no lag phase is observed in the kinetic measurements and the data fit well to single exponential curves.

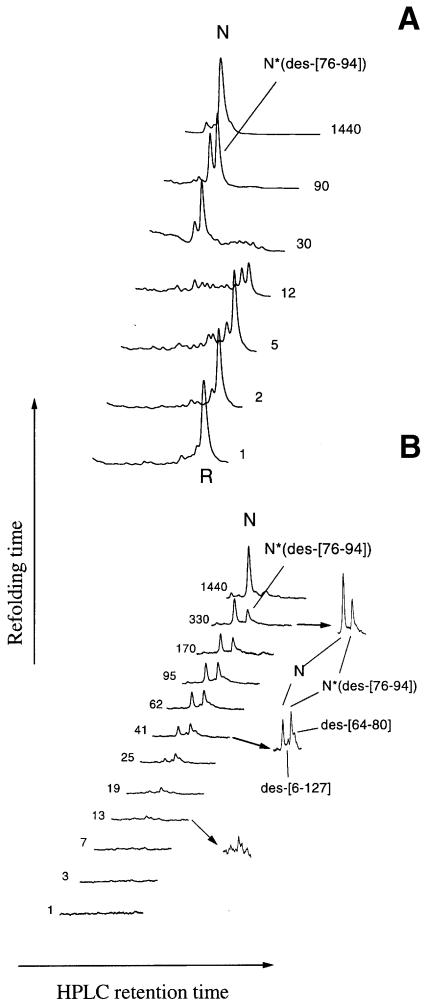

Fig. 4. (**A**) Refolding of 25 μM lysozyme in 2 M urea at pH 8.5 followed by reversed-phase HPLC. The elution positions of native lysozyme (N), fully reduced lysozyme (R) and the three-disulfide intermediates des-[6–127], des-[64–80] and des-[76–94] are indicated in the chromatograms. (**B**) Partial elution profiles focusing on the three-disulfide region. The concentrations of GSH/GSSG were 1.0/0.2 mM.

relatively sharp resonance lines are observed, resulting from either largely unstructured species early in folding or highly native-like species formed later in the folding process. No significant line broadening or resonance intensity loss is observed, indicating that compact, highly dynamic states akin to the molten globular species seen in non-oxidative refolding (Dobson *et al*., 1994) are not substantially populated in the ensembles of oxidative folding intermediates, which is consistent with the results of the ANS binding experiments.

Refolding kinetics of three-disulfide intermediates

An acid-quench methodology in combination with reverse-phase HPLC at pH 2 (Weissman and Kim, 1991) has been described recently to separate lysozyme refolding intermediates at neutral pH and to identify three native-like three-disulfide intermediates that occur later during folding (van den Berg *et al*., 1999). The same approach was used to separate the intermediates formed during the oxidative refolding of lysozyme at pH 8.5 (Figure 4A). Although folding at pH 8.5 is approximately a factor of 10 faster than folding at pH 7.4, the elution profiles are very similar under both conditions, and the same native-like three-disulfide species occur during the later stages of the process (Figure 4B). The most abundant of these has been identified as des-[76–94], a highly native-like species which has three of the four native disulfide bonds but which lacks the Cys76–Cys94 disulfide bond. The two minor species that elute close to the major intermediate des-[76–94] are des-[6–127] and des-[64–80], which are also three-disulfide species with native-like structures (van den Berg *et al*., 1999).

The kinetic profiles of the disappearance of the fully reduced protein (R) and the appearance of the three-disulfide intermediates and the native protein (N) are shown in Figure 5. The results confirm that des-[76–94] is the most abundant folding intermediate, accounting for up to 40% of the total protein at some stages of the folding reaction. Late in folding, the native protein and des-[76–94] are the only species populated at significant levels. This provides an explanation for the observation of the final very slow kinetic phase in the recovery of enzymatic activity, since, although des-[76–94] has a

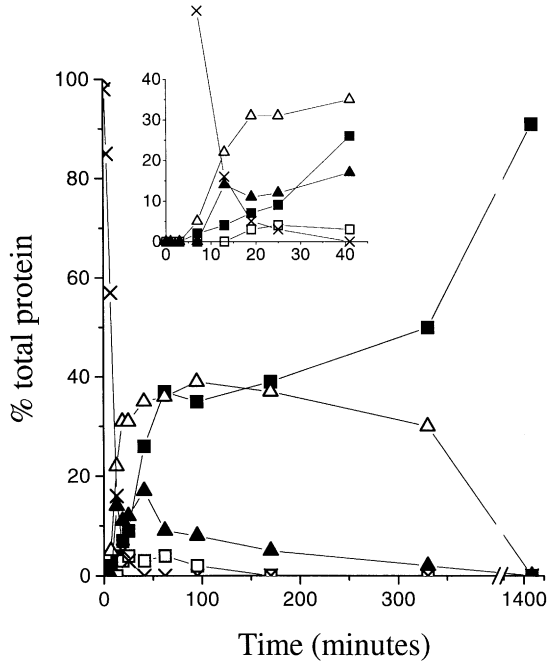

Fig. 5. Refolding kinetics of reduced lysozyme (×), native lysozyme (■) and the three-disulfide intermediates des-[6–127] (□), des-[64–80] (▲) and des-[76–94] (△) as determined by reverse-phase HPLC. The inset shows the early kinetics more clearly. Lysozyme (25 μM) was refolded at pH 8.5 in the presence of 2 M urea and 1.0/0.2 mM GSH/GSSG.

highly native-like structure with essentially native optical properties, its enzymatic activity is substantially lower (50 ± 5%) than that of the native protein (van den Berg *et al*., 1999). As a consequence, this phase is only evident in the recovery of enzymatic activity but not in the CD and fluorescence data.

Of the other native three-disulfide species, des-[64–80] accumulates to significantly lower levels (10–15%) under these conditions and appears to be oxidized more quickly than des-[76–94]. Finally, des-[6–127] is only present at low levels (<5%) during the folding process. The fact that the later stages of the reaction are dominated by a single intermediate, des-[76–94], suggests that the conversion of this species into the native protein is likely to be the rate-limiting step in the folding of the majority of lysozyme molecules. The kinetic profile of des-[76–94], however, shows that its conversion to the native protein is a very slow process with a time constant of several hours, which is consistent with the slow phase in the recovery of enzymatic activity (Figure 2F, Table I). This is clearly too long to account for the presence of ~35% native protein after 60 min of refolding under these conditions (Figure 5), and indicates that a considerable fraction (~25–30%) of lysozyme molecules do not form the native state via des-[76–94]. These molecules could fold via the minor intermediates des-[6–127] and des-[64–80], which are likely to convert more rapidly to the native protein, and we speculate that they reach the native state more quickly than those folding through des-[76–94]. This heterogeneity in the folding population is analogous to the 'fast' and 'slow' tracks described for the folding process of lysozyme containing its native disulfide bonds (Matagne *et al*., 1997; Wildegger, 1997; Kulkarni *et al*., 1999).

Effects of urea and PDI on folding

The refolding of reduced lysozyme is complicated by the fact that after initiation of folding a rapid partitioning occurs between species committed to productive folding and species committed to aggregation (Goldberg *et al*., 1991). Although in an intracellular environment molecular chaperones help to prevent aggregation of folding polypeptide chains (Hartl, 1996), the efficient refolding of lysozyme described here shows that low concentrations of urea are also effective in preventing initial aggregation of the refolding molecules. In this regard, they mimic the action of molecular chaperones. Even so, at lysozyme concentrations above ~35 μM in the presence of 2.0 M of urea, refolding yields drop rapidly. Since it is not unreasonable to assume that any process that speeds up folding also reduces the likelihood of aggregation, the influence of the folding catalyst PDI was investigated. PDI is very suitable for this purpose, since it is by far the most abundant folding catalyst present in the lumen of the endoplasmic reticulum. Moreover, PDI is also known to act as a molecular chaperone under certain conditions (Puig and Gilbert, 1994; Gilbert, 1998). As shown in Figure 6A, low concentrations of PDI already have a dramatic effect on lysozyme refolding even in the presence of 1 M urea. Under the experimental conditions where PDI still has ~60% of its isomerase activity (Hawkins and Freedman, 1995), the recovery of enzymatic activity is ~15 times faster in the presence than in the absence of PDI. In addition, the observed yields of active protein, which are rather low (~40%) in the absence of PDI, are increased by a factor of two in the presence of PDI. Since the contribution of any chaperone activity of PDI is unlikely to be dominant under these conditions, the data suggest that the increased rate of folding could serve as a mechanism to prevent protein aggregation, which is generally relatively slow. The large influence of PDI on the lysozyme folding kinetics is illustrated clearly by the intrinsic fluorescence data (Figure 6B).

These findings differ significantly from those of earlier studies, where PDI present at substoichiometric concentrations relative to lysozyme exhibited anti-chaperone activity and actively reduced the yield of fully refolded protein (Puig and Gilbert, 1994). The difference between the present and previous findings may be due to the reduced tendency of unfolded lysozyme to aggregate under the conditions used here as a consequence of the addition of 1.0 M of urea to the refolding buffer. Remarkably, even when PDI is added after the disappearance of the fully reduced protein and most of the early intermediates, refolding yields still improve dramatically (Figure 6C and D). This shows that competition exists between productive folding and aggregation (Goldberg *et al*., 1991) not only early during folding, but also at the later stages of the folding reaction when more structured intermediates are populated.

In the initial phase of folding, PDI is likely to assist the rapid interconversion of intermediates with one or two disulfide bonds by accelerating the rates of disulfide bond breakage and reformation. The rate of acceleration by PDI of the later stages in lysozyme folding is likely to be due to an effect on the three-disulfide species that accumulate, most notably des-[76–94] (Figure 6C and D). Compared with the native protein, des-[76–94] accumulates to a

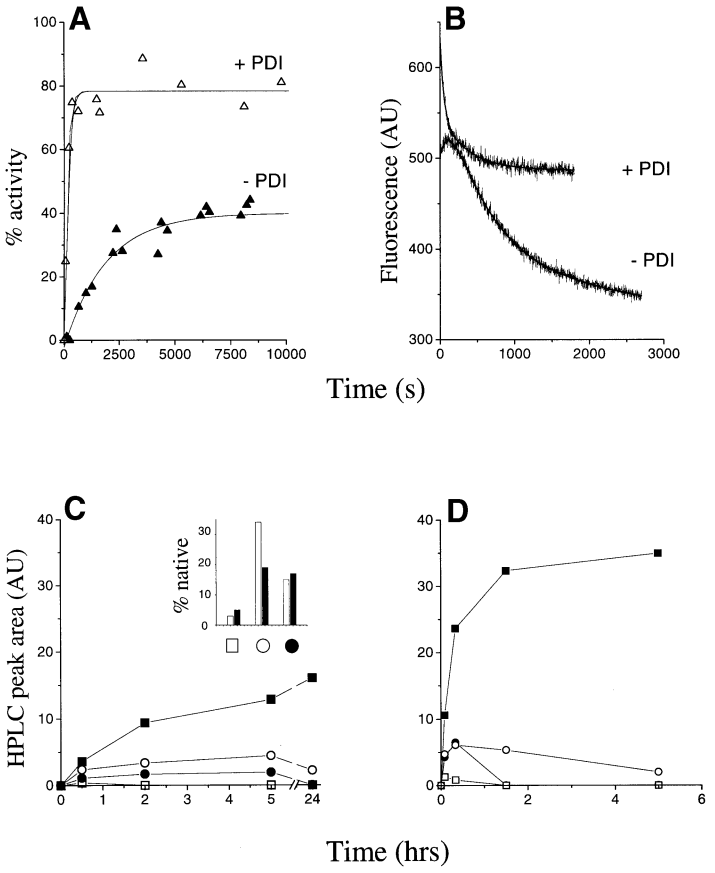

Fig. 6. Refolding of lysozyme (6 μM) in 1 M urea in the absence and presence of 2 μM PDI at pH 7.4 as determined by (**A**) recovery of enzymatic activity and (**B**) intrinsic fluorescence. Refolding kinetics of native lysozyme (■), des-[6–127] (□), des-[64–80] (●) and des-[76–94] (○) obtained by refolding lysozyme in the absence (**C**) and presence (**D**) of PDI at pH 7.4 and in the presence of 1 M urea and 2.0/0.4 mM GSH/GSSG. Refolding was initiated with 30 μM lysozyme in the presence of 2 M urea; after 1 h of refolding, samples were diluted with equal volumes of refolding buffer without urea and in the absence (C) or presence (D) of 3 μM PDI, and folding was allowed to proceed (see Materials and methods). The inset in (C) shows the observed maximum levels of the three-disulfide intermediates (expressed as a percentage of native protein) in the absence (white bars) and presence of PDI (black bars).

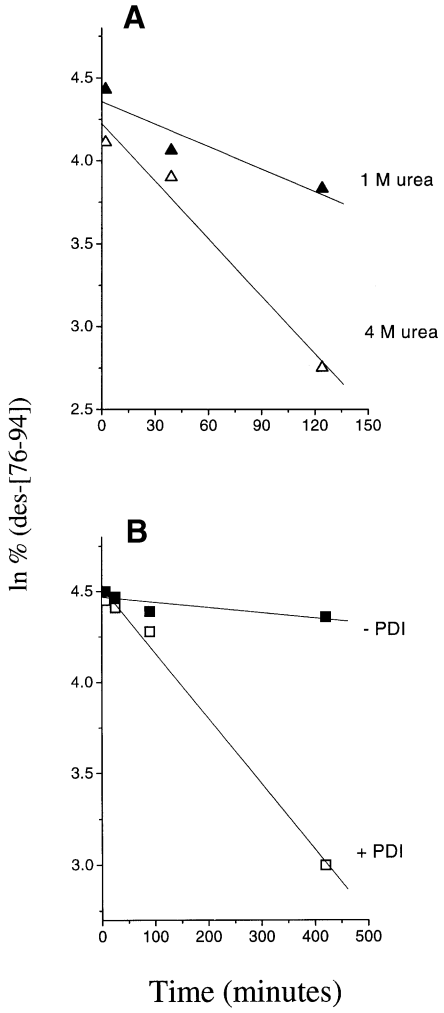

Fig. 7. (**A**) Rearrangement of 55 μM des-[76–94] at pH 8.5 in the presence of 1 and 4 M urea and in the absence of GSH/GSSG. (**B**) Effect of PDI (5 μM) on the reoxidation of des-[76–94] (55 μM) at pH 7.4, in the presence of 1 M urea and 2.0/0.4 mM GSH/GSSG.

relatively lesser extent in the presence of PDI than in its absence. This results in the observation of similar levels of des-[64–80] and des-[76–94] during folding in the presence of PDI (Figure 6C). The quantity of des-[6–127], in which the free thiols are likely to be solvent accessible (Radford *et al*., 1991) is not significantly affected by PDI and supports the previous conclusion from studies of BPTI that PDI has relatively smaller effects on intermediates that are readily oxidized in its absence (Weissman and Kim, 1993). PDI can be seen to catalyse particularly efficiently the conversion of des-[76–94] to the native protein, which accounts for at least part of the observed acceleration of folding in the presence of PDI. This notion is supported by the fact that a slow phase is absent from the kinetics of the recovery of enzymatic activity when lysozyme is refolded in the presence of PDI (Figure 6A).

Des-[76–94] acts as a kinetic trap

Figure 7A shows that the conversion of purified des-[76–94] to the native protein is significantly faster (by almost a factor of 3) in the presence of 4 M urea than in 1 M urea. This suggests that des-[76–94] has to unfold in order to form the Cys76–Cys94 disulfide bond and generate the fully native protein. By monitoring the refolding of des-[76–94], it is clear that under these conditions its rate is ~8 times faster in the presence than in the absence of PDI (Figure 7B). This situation is similar to that found in the folding of BPTI, where two highly native-like intermediates have been shown to retard folding (Creighton, 1974; Weissman and Kim, 1991). As found here with lysozyme, high concentrations of urea were found to increase the rate of rearrangement of these intermediates (Weissman and Kim, 1991), whereas PDI was shown to catalyse specifically and efficiently their disulfide bond rearrangements. These results led to the suggestion that PDI could function by promoting both local unfolding and disulfide bond rearrangements in structured intermediates (Weissman and Kim, 1993).

The similar effects of urea and PDI on the rearrangement of des-[76–94] suggest that PDI facilitates the unfolding of native-like structure in this intermediate. Data obtained for mutant human lysozymes suggest that the thiol group of Cys76 in des-[76–94] will be solvent accessible,

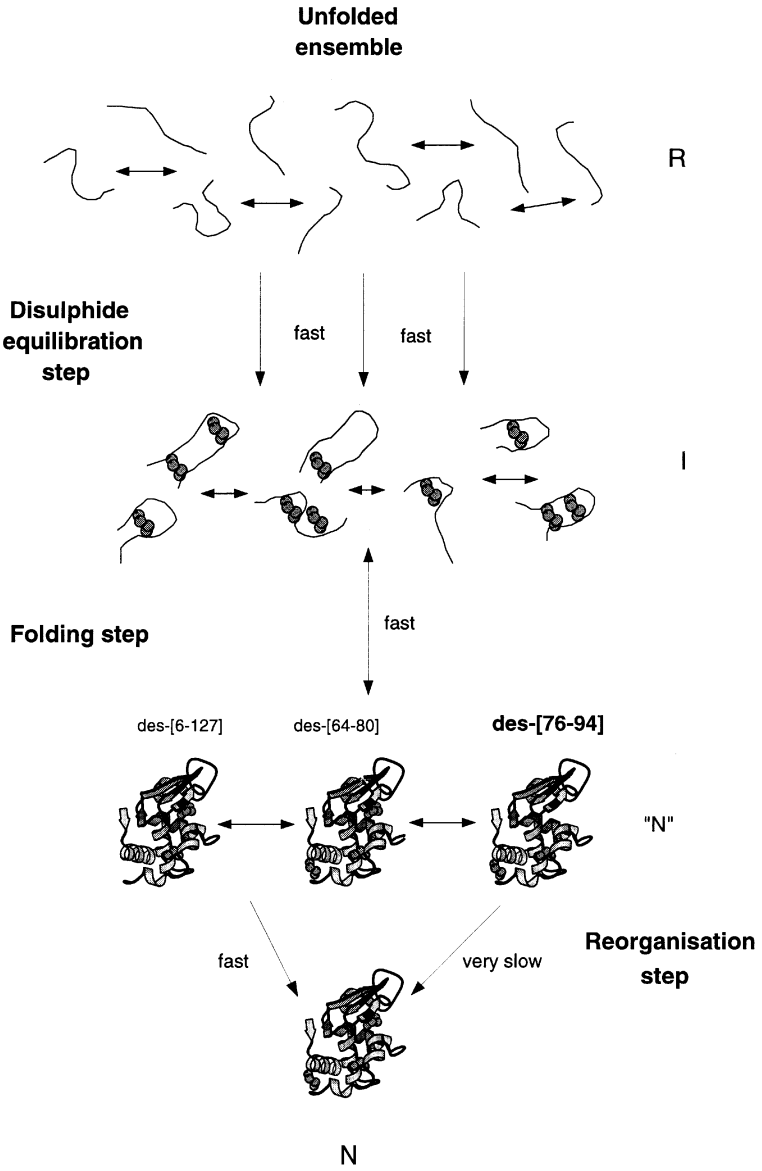

Fig. 8. Schematic representation of the oxidative refolding of lysozyme, illustrating the observed kinetics and the statistical nature of protein folding. The fully reduced protein is indicated by R, the ensemble of early unstructured intermediates by I, the native-like three-disulfide intermediates by 'N' and the fully native protein by N. The details of the final reorganization steps are not yet known (see text).

whereas that of Cys94 is buried and not accessible to GSSG or a protein disulfide (Kikuchi *et al*., 1990; Taniyama *et al*., 1991). This is the likely origin of the slow conversion of des-[76–94] to the fully native state in the absence of PDI and high concentrations of urea (van den Berg *et al*., 1999). The details of this conversion are, however, not yet known. Formation of the fully native protein could occur via direct oxidation of des-[76–94] but, as in the native-like BPTI intermediates [14–38, 30–51] and [14–38, 5–55], a more random rearrangement process involving substantial loss of structure could occur (Weissman and Kim, 1991). One interesting possibility for the rearrangement process is that reduction of a disulfide bond in des-[76–94] by GSH or PDI could take place, resulting in a largely unfolded two-disulfide intermediate. This species could then reoxidize to form the native protein via either des-[64–80] or des-[6–127]. Support for the viability of such a mechanism is provided by the fact that des-[64–80] accumulates to low levels during the conversion of des-[76–94] to the fully native protein.

Distributions of conformers during folding

Identification of oxidative folding intermediates provides information on the conformational distributions at different stages in folding (Creighton, 1974). For a protein with four disulfide bonds, there are 764 distinct chemical entities possible (28 single-disulfide, 210 two-disulfide, 420 three-disulfide and 105 four-disulfide species). When mixed protein–GSH disulfide bonds are considered, the total number of possible intermediates increases to over 7000. The experimental data show that during the later stages of folding only a very small fraction of the possible three- and four-disulfide intermediates accumulate to significant levels. Indeed, only three three-disulfide species, all with native disulfide bonds, have been identified, and only a single four-disulfide species, the native protein

(Figure 4B). By contrast, during the early stages of folding, the number of detectable intermediates is relatively large (Figure 4A). These observations imply that there is a rapid and substantial reduction of accessible conformational space even at the early stages of the folding process.

Although single sequential pathways have been suggested for the oxidative folding of lysozyme (Roux *et al*., 1997), the experimental data obtained under the conditions of the present work are better explained by assuming the existence of many parallel events during the earlier stages of folding which converge to much smaller numbers during the later stages. A striking observation from the present work is that although the oxidative refolding process of lysozyme is highly complex, the data can be described well by relatively simple kinetic schemes (Figure 2). This demonstrates a very important aspect of the new view of protein folding, which is the existence of 'hidden' complexities in protein folding processes as a result of the fact that there are a multiplicity of ways of reaching the native state (Sali *et al*., 1994; Baldwin, 1995; Dill and Chan, 1997; Dobson *et al*., 1998).

A schematic description of the oxidative folding of hen lysozyme derived from the variety of spectroscopic methods used here is shown in Figure 8. The fully reduced denatured protein (R) is present in solution as an ensemble of conformers with considerable local and global heterogeneity (Schwalbe *et al*., 1997). Depending on the conformation of a given molecule at the initiation of folding, structural interactions can be formed in different orders. This results in multiple parallel events during which a rapid formation of a relatively large number of highly unstructured intermediates with one or two disulfide bonds occurs (Figures 2 and 4). These initial intermediates are not substantially collapsed and do not possess significant levels of native-like secondary structure. Therefore, they do not resemble the molten globule states of proteins observed in the early stages of non-oxidative folding in the presence of the native disulfide bonds (Radford *et al.*, 1992; Dobson *et al*., 1994). The relatively unstructured character of these intermediates is likely to facilitate the search of conformational space by allowing rapid interconversions between them.

This 'disulfide equilibration' phase is followed by authentic folding events in which the formation of a third disulfide bond is associated with the stabilization of collapsed intermediates with extensive native-like tertiary structure. This step in the folding reaction is the key one for the formation of the overall fold, which for most molecules occurs via a slow route involving des-[76–94]. A minor population of molecules is able to form the native state much faster, via routes that could involve the three-disulfide intermediates des-[6–127] and des-[64–80]. The fact that at the stage of folding in which species with three disulfide bonds are dominant only intermediates with native disulfide bonds accumulate strongly suggests that native disulfide bonds are dominant in the species formed earlier in folding. Some clues concerning the identity of the early intermediates arise from studies of the structurally homologous protein α-lactalbumin (Peng *et al*., 1995; Wu and Kim, 1998). Here, local structural preferences greatly favour the formation of the Cys28–Cys111 disulfide bond. This has led to the suggestion that the core region surrounding the Cys28–Cys111 disulfide bond plays an important role in α-lactalbumin folding by stabilizing native-like structure (Peng *et al*., 1995; Wu and Kim, 1998). It is tempting to speculate that the analogous disulfide bond Cys30–Cys115 is formed early in the folding of lysozyme, and that it is present in at least a high proportion of intermediates prior to the process of structural collapse. Support for the notion that this disulfide bond is important in the early stages of lysozyme folding comes from the fact that des-[30–115] is the only native three-disulfide species that has not been detected during the refolding reaction.

Although the data suggest that molten globule-like intermediates are not populated to a high extent, such species are likely to exist in rapid equilibrium with the more extended structures even in the fully reduced protein (Schwalbe *et al*., 1997). It is probable that a transient population of native-like collapsed structures is the origin of the preferential formation of native disulfide bonds even in the absence of high populations of collapsed states. They may therefore play a key role in the development of the overall native-like fold of the protein. Indeed, such processes are observed clearly in the non-oxidative folding of both native and des-[6–127] proteins from denaturing conditions (Radford *et al*., 1992; Eyles *et al*., 1994).

An overview of lysozyme folding

The present study illustrates that the heterogeneity and complexity characteristic of the non-oxidative folding process of lysozyme (Radford *et al*., 1992; Matagne *et al*., 1997; Wildegger and Kiefhaber, 1997) are also characteristic of its oxidative folding. During non-oxidative folding under certain conditions, most molecules fold via a slow track involving an intermediate that has a native-like α-domain, but which lacks persistent structure in the β-domain (Radford *et al*., 1992; Dobson *et al*., 1994; Matagne *et al*., 1997). The remaining population of molecules folds fast, via a near-native intermediate which has stable structure in both domains but in which the active site is not yet completely formed. Recent results suggest that even the molecules of the major slow folding track fold via this near-native intermediate (Matagne *et al*., 1997; Kulkarni *et al*., 1999). During oxidative folding, there are also distinct populations of molecules that form the native state with greatly different rates. Moreover, the identities of the native-like three-disulfide species that accumulate during the later stages of the folding process, in particular des-[76–94], show that most molecules attain a highly native-like structure in both the α- and β-domains prior to formation of the fully native state. The kinetic NMR data, which indicate that fully native structure is formed in the α-domain before the β-domain, are compatible with these results and mainly reflect the subtle structural differences within the β-domain and the α/β domain interface of des-[76–94] compared with the native protein (van den Berg *et al*., 1999). The observation for lysozyme that folding can occur independently in both domains, and that folding is completed by a coalescence of the domains to form the fully native structure, may well be an important feature in the folding of all but the simplest proteins (Panchenko *et al*., 1996).

Taken together, the results from non-oxidative and oxidative folding studies of lysozyme, along with the characterization of denatured states of the protein

(Schwalbe *et al*., 1997), enable the beginnings of a unified picture of its folding process to be described. Initial states in the folding process are very heterogeneous, but evidence indicates that these include small populations of molecules that are compact and have native-like features (Schwalbe *et al*., 1997). This will result in native-like disulfide bonds being generally more stable than non-native ones. When a disulfide bond forms, the large relative decrease in the entropy of the more unfolded state will lead to the relative stabilization of the more collapsed species. At a critical point in folding, which for lysozyme under the conditions used here is on average between the formation of the second and third disulfide bond, the protein undergoes a major conversion from a largely unfolded state to a largely folded state. The latter has a highly native-like character and the final reorganization step in folding for the majority of molecules involves a search for the lowest energy state in an environment where enthalpic barriers exist as a consequence of the compact nature of the protein structure (Dobson *et al*., 1998).

The results described here illustrate many essential features of the current thinking about the mechanisms of protein folding (Sali *et al*., 1994; Baldwin, 1995; Dill and Chan, 1997; Dobson *et al*., 1998), including the intrinsic heterogeneity of the initial stages and the dominance of native-like interactions resulting in the funnelling of conformations towards the native state. In addition, the results are complementary to those obtained previously from studies of its non-oxidative folding which focus on the crucial collapse process of unstructured intermediates (Dobson *et al*., 1994), and suggest that the underlying determinants of folding are similar in both the oxidative and non-oxidative processes. The low concentrations of urea necessary for efficient folding may act in a manner similar to molecular chaperones in reducing aggregation of the highly unfolded states. The effectiveness of PDI reflects its known importance in physiological refolding (Freedman, 1995; Gilbert, 1997, 1998), and supports earlier findings that it has a role in enhancing reorganization of highly structured intermediates (Weissman and Kim, 1993). Moreover, the acceleration of folding resulting from the effects of PDI may well represent a general strategy to reduce aggregation of unfolded and partially folded states. The present study of the oxidative folding of lysozyme, therefore, represents a first step to understanding how the folding of this protein might occur in its intracellular environment.

Materials and methods

Reduction of lysozyme, renaturation and assay of enzymatic activity

Lysozyme reduction and denaturation were carried out as described previously (van den Berg *et al*., 1999). To achieve renaturation at pH 8.5, the reduced denatured protein was dissolved initially in buffer 1 (8 M urea/100 mM Tris–HCl/100 mM NaCl/1 mM EDTA, pH 8.5); renaturation was initiated by rapid 4-fold dilution into buffer 2 (100 mM Tris–HCl/100 mM NaCl/1 mM EDTA/1.33 mM GSH/0.27 mM GSSG, pH 8.5). Under these conditions, final refolding yields are 75–80%. Folding reactions, all carried out at 20°C, were quenched by addition of 1/25 vol. of 2.5 M HCl to pH 2. GSH/GSSG solutions were freshly made before use. Refolding buffers were degassed with helium prior to use. Lysozyme activities were assayed at 20°C by following the decrease in absorbance at 450 nm of a *Micrococcus lysodeikticus* cell wall suspension in 20 mM sodium phosphate pH 7.0.

Reverse-phase HPLC chromatography

Native and reduced lysozyme and intermediates were separated by reverse-phase HPLC at pH 2 using an Analytical Vydac column (218TP54; 250×4.6 mm) or a preparative Dynamax-300 A C8 column (83-323-C5; 250×21.4 mm), with linear gradients consisting of solvent A (H_2O/0.1% TFA) and B (90% CH_3CN/10% H_2O/0.1% TFA). Details have been published elsewhere (van den Berg *et al*., 1999).

Real-time optical spectroscopy and 1D NMR

Experiments were carried out with 25 μM lysozyme under standard refolding conditions at pH 8.5. For determination of the kinetics of disulfide bond formation, lysozyme refolding aliquots were taken after certain times, desalted using Pharmacia NAP-25 columns in formic acid pH 2, and lyophilized. Samples subsequently were dissolved in buffer containing 8 M urea (without GSH/GSSG), immediately diluted to 2 M urea, and the number of free thiols was determined directly by absorbance of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) at 412 nm. The number of assayed thiol groups in fully reduced lysozyme was 7.7 (96%). Intrinsic tryptophan fluorescence was monitored in real-time using a Perkin Elmer LS 50B fluorimeter (excitation/emission at 280/350 nm; slit widths 2.5 nm). ANS binding experiments (excitation/emission 394/478 nm; slit widths 5.0 nm) were performed by addition of ANS (200 μM) after various times to refolding aliquots of lysozyme, since refolding in the presence of ANS yielded strange kinetics, presumably due to ANS remaining bound to the refolding protein. Kinetic CD experiments were done at 225 (far-UV CD) and 289 nm (near-UV CD) using a Jasco J720 spectropolarimeter with cells having path lengths from 1 to 5 mm. In all cases, rapid mixing of solutions to initiate refolding was performed manually, giving a typical dead time in these experiments of ~10 s. CD ellipticities and fluorescence values of reduced lysozyme in renaturation buffer without GSH/GSSG were quite close to extrapolated values at –10 s, indicating that no significant kinetic events occur in the dead time of the experiments. Control experiments were performed where the reduced protein was replaced by native lysozyme. All kinetic data were fitted to single and double exponential curves with ORIGIN (MicroCal software). Reported time constants τ are defined as half-times of the kinetic events, and are average values of at least two or three experiments.

Real-time kinetic 1D ^1H NMR experiments were carried out in D_2O at 20°C with 35 μM lysozyme at pH 8.5 (uncorrected meter reading). All buffer components except GSH and GSSG (1.0 and 0.2 mM, respectively) were deuterated. Mixing was done manually, giving a total dead time in these experiments of ~4 min (including locking/shimming). Sequential 1D scans were recorded at 600 MHz using a 62.4° pulse in combination with a relaxation delay of 0.55 s. In this way, 512 transients of 2 K points could be recorded for each 1D spectrum in 338 s. A total of 200 1D experiments was recorded.

Refolding of lysozyme in the absence and presence of PDI

For direct observation of recovery of enzymatic activity and intrinsic fluorescence changes in the presence and absence of PDI, lysozyme (6 μM) was refolded in the presence or absence of 2 μM PDI at pH 7.4, in buffer containing 1.0 M urea and 2.0/0.4 mM GSH/GSSG. Since PDI has only ~10% activity in 2 M urea (Hawkins and Freedman, 1995), a different strategy had to be employed in order to be able to monitor the kinetics of the three-disulfide intermediates in the presence of PDI by reverse-phase HPLC and at the same time to avoid aggregation of the reduced protein and early folding intermediates (Figure 6C and D). In order to achieve this, lysozyme was refolded initially at high concentration (30 μM) in 2.0 M urea. After 1 h of refolding, the solutions were diluted into equal volumes of refolding buffer without urea and in the presence and absence of 3 μM PDI, to give a final refolding buffer with 15 μM lysozyme and 1.0 M urea/100 mM Tris/100 mM NaCl/1 mM EDTA/2.0 mM GSH/0.4 mM GSSG at pH 7.4. Folding was then allowed to proceed.

Rearrangement of des-[76–94]

Rearrangement of purified acid-quenched des-[76–94] (55 μM) was carried out at pH 8.5 in refolding buffers containing 1 or 4 M urea in the absence of PDI and GSH/GSSG. For experiments involving PDI, des-[76–94] (55 μM) was allowed to refold in 1.0 M urea at pH 7.4 in the presence and absence of 5 μM PDI (2.0/0.4 mM GSH/GSSG). Peak areas in the HPLC chromatograms were determined using the GILSON Unipoint analysis software program.

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