A kinetic explanation for the rearrangement pathway of BPTI folding

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Bovine pancreatic trypsin inhibitor (BPTI) does not fold by simple sequential formation of its native disulphide bonds. Instead, an initially formed intermediate, termed N', first rearranges to a more stable species in a slow process that requires substantial unfolding. We find that direct oxidation of N' is also inhibited by native structure which slows both the intermolecular step in oxidation—formation of a mixed disulphide bond with the oxidizing agent GSSG—as well as the subsequent intramolecular step. Folding does not occur appreciably by direct oxidation because the high GSSG concentrations required for efficient mixed disulphide formation cause N' to accumulate as a nonproductive, double-mixed disulphide species. The need to unfold previously acquired native structure, observed in the folding of BPTI, may be a common feature of disulphide-linked folding reactions.

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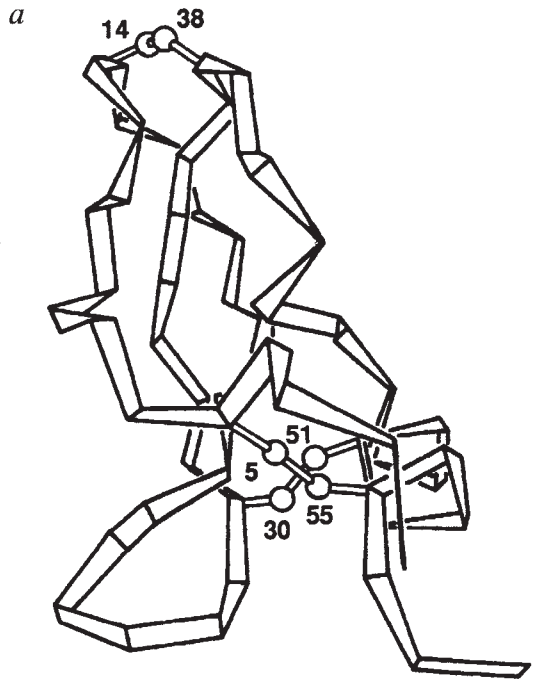
Folding of proteins that contain disulphide bonds presents an unusual problem[1]: on the one hand, it is native structure that stabilizes one set of disulphide bonds over the numerous alternate possibilities; yet on the other hand, native structure can inhibit formation of native disulphide bonds by burying and constraining the free thiols of a protein. As a consequence, native-like structure in a folding intermediate can, paradoxically, inhibit the rate at which the final native state is reached. These difficulties are illustrated clearly in the oxidative folding pathway of bovine pancreatic trypsin inhibitor (BPTI)[2–6]. BPTI, therefore, provides an excellent model system for examining this phenomenon, as well as for studying how rapid disulphide formation is facilitated in the cell[7–9].

BPTI is a small (58 residues) protein that has a melting temperature at neutral pH in excess of 100 °C (refs 10,11). The native structure of BPTI is stabilized by three disulphide bonds between residues 30–51, 5–55, and 14–38 (Fig. 1*a*). On reduction of these disulphide bonds, BPTI unfolds spontaneously. Thus, protein folding is linked thermodynamically to disulphide bond formation. The folding pathway of BPTI has been described (Fig. 1*b*) in terms of the disulphide-bonded intermediates that accumulate during the oxidative folding of the protein[2–6]. These intermediates are denoted in brackets by the Cys residues involved in disulphide bonds; native BPTI (N) is thus [30–51; 5–55; 14–38].

Formation of a disulphide bond in a protein by oxidized glutathione (GSSG) is a redox reaction that proceeds in two steps (Fig. 2*a*). First, in an intermolecular step, a free thiol in the protein attacks the disulphide bond of GSSG, releasing a molecule of reduced glutathione (GSH). This step yields a species with a disulphide bond between a protein Cys residue and a glutathione molecule, referred to as a 'mixed' disulphide. Second, in an intramolecular step, a different protein thiol attacks the protein-glutathione mixed-disulphide bond, resulting in the formation of a disulphide bond between two Cys residues in the protein and the release of a second molecule of GSH. Intramolecular rearrangement of existing disulphide bonds in a protein occurs by a mechanism that is similar to the second step in the oxidation reaction, but the protein thiol attacks a protein–protein disulphide bond instead of a protein–glutathione disulphide bond (Fig. 2*b*). The rates of intramolecular rearrangements are therefore independent of glutathione concentration.

When BPTI folds in strongly oxidizing conditions (Fig. 1*b*), only half of the molecules form native BPTI (N). The remaining molecules become kinetically trapped in the form of a very stable, native-like intermediate, [5–55; 14–38], which has been designated N* (refs 3,4,12). Although N* lacks the 30–51 disulphide, it has a structure that is essentially identical to that of native BPTI, as judged by nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography[12,13]. This native structure in N* buries Cys 30 and Cys 51 (Fig. 1*a*), rendering them inaccessible to oxidizing agents[3,4] and thereby preventing formation of a mixed disulphide between the protein and GSSG. In the conditions of these experiments, N* is a dead-end intermediate that can persist for weeks[3,8].

For the fraction of molecules that do form native BPTI, folding does not proceed by a simple sequential acquisition of native disulphide bonds[2,4]. Instead, at neutral pH, folding proceeds as follows (Fig. 1*b*). (i) The native two-disulphide intermediate, [30–51; 14–38], accumulates readily to high levels. This intermedi-



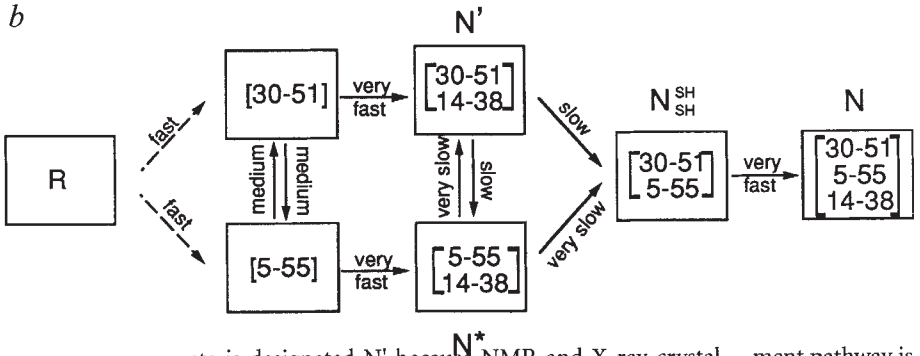


Fig. 1 Structure and folding pathway of BPTI. *a*, Schematic representation of the crystal structure for BPTI[22,23]. The residues involved in disulphide bonds are labelled. The 14–38 disulphide bond is accessible to solvent, exposing ~50% of its total surface area, whereas the 30–51 and 5–55 disulphide bonds are inaccessible, exposing 0% of their total surface area[21]. *b*, Schematic representation of the kinetically preferred pathway[4] for the folding of BPTI at pH 7.3, 25 °C. R denotes the reduced protein. All of the well-populated intermediates contain only native disulphide bonds. Qualitative descriptions of the relative rates of the intramolecular transitions associated with each step are indicated (see Table 1 of ref. 7). N* is a kinetically trapped intermediate that is stable for weeks under these conditions. The dotted arrows indicate that R is oxidized initially to several species, which then rearrange rapidly to [30–51] and [5–55]. Recent experiments indicate that a substantial fraction of the one-disulphide species rearrange through the [14–38] intermediate[6].

ate is designated N' because NMR and X-ray crystallography studies indicate that it folds to a stable structure that is very similar to native BPTI, despite the absence of the 5–55 disulphide bond[4,14,15]. Under the conditions used in these studies, formation of N' occurs predominantly by direct oxidation of the 14–38 disulphide bond in the native single disulphide species [30–51] (refs 4,7). In more alkaline conditions, where half of the cysteine sidechains are expected to be negatively charged (pH 8.7), a fraction of [30–51] first forms either the 5–14 or 5–38 disulphide bond[16], followed by a rapid intramolecular disulphide rearrangment to produce N' (refs 4,5). (ii) N' does not form the final disulphide bond directly, but instead rearranges to a more stable native two-disulphide intermediate, [30–51; 5–55], denoted N^{SH}_{SH}, which is also known to be folded into a structure essentially identical to that of native BPTI[17]. The rearrangement of N' to N^{SH}_{SH} is the rate-determining transition in the productive folding of BPTI[2,4]. (iii) Finally, the 14–38 disulphide bond in N^{SH}_{SH} is oxidized readily to form native BPTI.

The following observations led us to conclude previously[4] that the rearrangement of N' to N^{SH}_{SH} is a random process that requires the loss of native structure. First, addition of high concentrations of chemical denaturant (6 M urea) increases substantially the rate of productive rearrangement of N' (ref. 4). Second, as discussed above, N' folds to a structure that is very similar to that of native BPTI. This structure is expected to bury Cys 5 and Cys 55 since the 5–55 disulphide bond is inaccessible to solvent in native BPTI. Finally, rearrangement of N' shows no preference for formation of the productive intermediate N^{SH}_{SH} over the dead-end species N* (refs 3,4), even though these two rearrangements must proceed through a different set of non-native species. This conclusion was extended by experiments indicating that two non-native species observed during folding, [30–51; 5–14] and [30–51; 5–38], do not act to enhance the rate at which N' rearranges to N^{SH}_{SH} (ref. 5).

The conclusion[5] that the major transition state for the rearrangement pathway is extensively unfolded is also supported strongly by a recent examination of the effect of mutagenesis on the rate of folding and unfolding of BPTI by Goldenberg and coworkers[18]. These studies found a direct relationship between the extent that a mutation

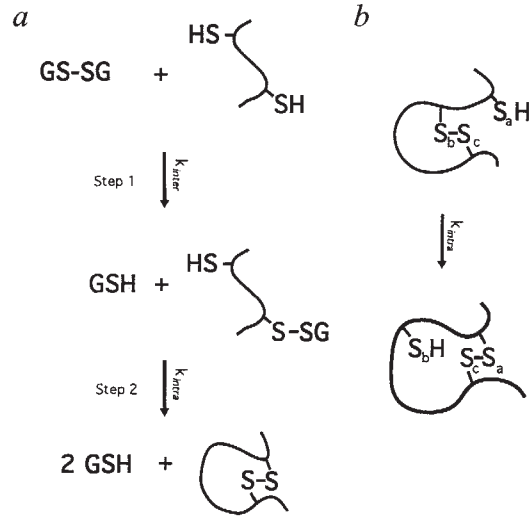


Fig. 2 Diagram of steps in the formation and rearrangement of disulphide bonds. *a*, Formation of a protein disulphide bond by GSSG. Oxidation occurs in two steps. Step 1 is the intermolecular attack of a protein thiol on the glutathione-glutathione disulphide bond. Step 2 is the intramolecular attack of a second protein thiol on the protein-glutathione mixed disulphide. *b*, Intramolecular thiol-disulphide exchange between a protein thiol and an existing protein disulphide bond.

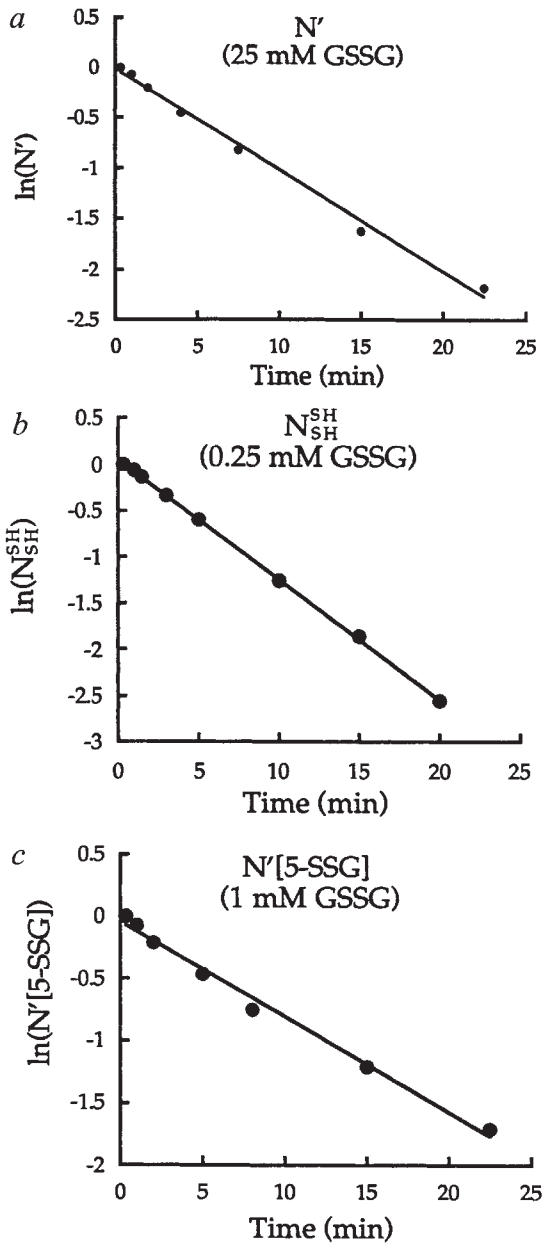


Fig. 3 The free thiols in N' react slowly with GSSG. *a*, Acid-quenched, HPLC-purified N' was dissolved in pH 7.3 buffer containing 25 mM GSSG at 25 °C. At the indicated times, a portion of the sample was removed, quenched with acid and analyzed by HPLC. The concentration of N' remaining as a function of reaction time is plotted. *b*, Time course of the reaction of N^{SH}_{SH} with 0.25 mM GSSG. *c*, Time course of the reaction of N'[5–SSG] with 1 mM GSSG.

destabilizes the native structure of BPTI and the extent to which the rate of rearrangement is accelerated for a number of mutations located throughout the tertiary structure of BPTI.

A central question raised by the BPTI folding studies is why the rearrangement of N' is preferred kinetically over direct oxidation. The preference for the rearrangement pathway is particularly puzzling given that the rearrangement of N' is slow and requires essentially complete unfolding. To address this issue, we have taken advantage of the ability to produce purified, reversibly trapped intermediates[4] to measure directly the rate for each of the two steps in the direct oxidation of N'. Our results indicate that structure in the N' intermediate inhibits formation of a mixed-disulphide in N'. As a consequence, at the modest GSSG concentrations generally used in previous BPTI folding studies, formation of a mixed-disulphide derivative of N' is slower than the intramolecular rearrangement of N' to N^{SH}_{SH}. Mixed-disulphide derivatives could be formed at high GSSG concentrations but, under these conditions, productive folding will not result because the single mixed-disulphide derivatives of N' react readily with GSSG to yield a non-productive, double mixed-disulphide derivative. Our results also suggest that, as in the rearrangement pathway, direct oxidation requires substantial, if not complete loss of structure in the N' intermediate.

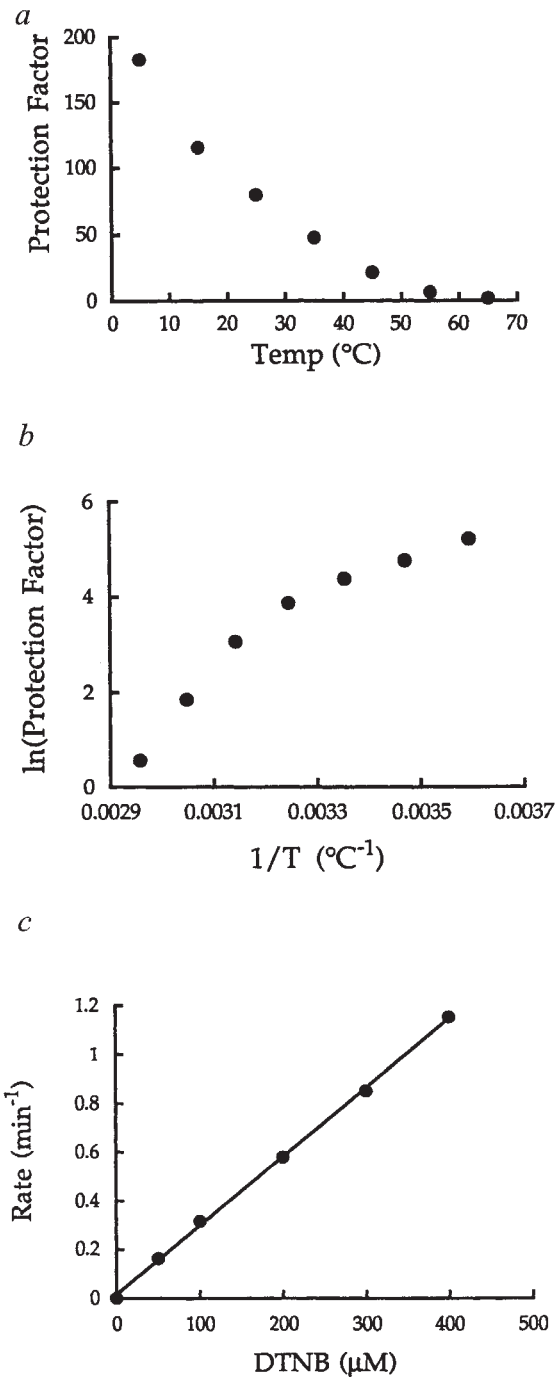
Rate of the first step in N' oxidation

Because the thiolate anion is the reactive species in thiol-disulphide exchange, it is possible to quench folding rapidly by lowering the pH. Protonation is reversible, however, so acid-quenched intermediates must be separated rapidly and at low pH to avoid further oxidation or rearrangement. Previous control experiments established the validity of using acid-quenching and reversed-phase high performance liquid chromatography (HPLC) at pH 2 for studies of BPTI folding[4]. A critical advantage of acid-quenching is that it is reversible. As a result, it is possible to purify an acid-quenched intermediate to chromatographic homogeneity and subsequently to allow further disulphide bond formation or rearrangement[4].

Using reversibly trapped N', we measured directly the rate of the first step of oxidation; reaction of N' with the external oxidizing agent, GSSG. This step yields the single mixed-disulphide derivatives of N' in which either Cys 5 or Cys 55 is involved in a disulphide bond with glutathione (termed N'[5–SSG] and N'[55–SSG], respectively). The rate at which N' reacts with GSSG was determined as follows: (i) the reaction was initiated with the addition, to N', of pH 7.3 buffer containing GSSG; (ii) at various times, the reaction was quenched with the addition of acid; (iii) for each time point, the fraction of N' that had not reacted with GSSG was determined by reversed-phase HPLC.

We find that the rate of reaction of N' with GSSG is low (Fig. 3*a*). For comparison, we also measured the rate of mixed-disulphide formation in a different two-disulphide intermediate, N^{SH}_{SH}. Compared to N', the N^{SH}_{SH} intermediate reacts with GSSG 150-fold more rapidly at pH 7.3 and 25 °C (Fig. 3*b*). Under more alkaline conditions (pH 8.7), similar to those used in earlier BPTI folding studies[2–4], the free thiols in N' are still protected substantially, reacting with GSSG ~70-fold less rapidly than does N^{SH}_{SH} (data not shown). Thus, at both pH 7.3 and pH 8.7, the low reactivity of the free thiols in N' contributes substantially to the failure of BPTI to fold by direct oxidation.

Fig. 4 Temperature dependence of the reactivity of the free thiols in N'. The reaction of the free thiols in the N' and N^{SH}_{SH} intermediates with DTNB was determined by monitoring absorbance at 412 nm. *a*, Plot of the thiol protection factor (that is, the ratio of the reactivity of N^{SH}_{SH} to N') as a function of temperature. *b*, A van't Hoff plot of protection factors. *c*, Rate of reaction of N' as a function of DTNB concentration at 25 °C.



N' is reactive at high temperatures

NMR studies[4,14,15] and X-ray crystallography studies (A.A. Kossiakoff, *personal communication*) have shown that the N' intermediate is folded into a structure that is very similar to that of native BPTI. At pH 2.0, the midpoint of the thermal unfolding transition (T_m) of N', as measured by circular dichroism at 222 nm, is ~40 °C. It is not possible to measure directly the stability of the N' intermediate at neutral pH since, on unfolding, N' rapidly undergoes intramolecular disulphide bond rearrangements. However, a recombinant model[15,19] of the N' intermediate, in which Cys 5 was replaced by valine and Cys 55 by alanine, termed $N'_{V5,A55}$, which has comparable stability to N' at pH 2, was found to have a T_m of ~60 °C and a free energy of unfolding of 4.3 kcal mol^{-1} at 5 °C and neutral pH (data not shown).

To determine if the low rate of mixed-disulphide formation in N' is caused by native structure in this intermediate, we investigated the temperature dependence of the reactivity of N' (Fig. 4). For these studies, the reactivity of the free thiols with 5'-dithio-bis-2-nitrobenzoic acid (DTNB) was determined because the course of this reaction can be monitored directly by the change in absorbance at 412 nm. Because the reaction of N^{SH}_{SH} with DTNB was too rapid to measure readily at pH 7.3, these studies were carried out at pH 6.0. The results (Fig. 4) show that the protection factor for the free thiols in N' decreases sharply with increasing temperature. Moreover, near the T_m of $N'_{V5,A55}$ (~60 °C) the free thiols in N' are essentially normally reactive. We conclude, therefore, that the low reactivity of the thiols in N' results primarily from the native-like structure of this intermediate.

Rate of second step in N' oxidation

The second step in the direct oxidation of N' is the intramolecular transition of the single mixed-disulphide derivatives of N' (N'[5–SSG] and N'[55–SSG]) to native BPTI. In order to measure these rates directly, it was necessary to produce purified, reversibly trapped N'[5–SSG] and N'[55–SSG]. Production of these species, however, is non-trivial as they do not accumulate significantly during a folding reaction. We therefore developed a two-step strategy for producing N'[5-SSG] and N'[55–SSG] (Fig. 5). In the first step, N' is reacted with DTNB to yield a species, termed N'[5–TNB, 55–TNB], that contains the 30–51 and 14–38 disulphide bonds and mixed-disulphide bonds with 5-thio-2-nitrobenzoic acid (TNB) on thiols 5 and 55. In the second step, N'[5–TNB, 55–TNB] is reacted with a 1:1 mixture of dithiothreitol (DTT) and GSH, yielding an approximately equimolar mixture of N', N'[5–SSG], N'[55–SSG] and N'[5–SSG; 55–SSG] (Fig. 5). The rationale for this strategy is described in Methods.

To measure the rate of the intramolecular step in the oxidation of N', purified N'[5–SSG] or N'[55–SSG] was allowed to rearrange in the absence of glutathione. N'[5–SSG] formed native BPTI spontaneously with a half time of ~4 h (Fig. 6*a*). There were no well-populated intermediates observed during this rearrangement, suggesting that the transition of N'[5–SSG] to N occurs by direct attack of the Cys 55 thiol on the Cys 5-glutathione mixed-disulphide bond. By contrast, the N'[55–SSG] intermediate did not form native BPTI directly. Instead, N'[55–SSG] first rearranged relatively rapidly (~30 minutes) to form predominantly N'[5–

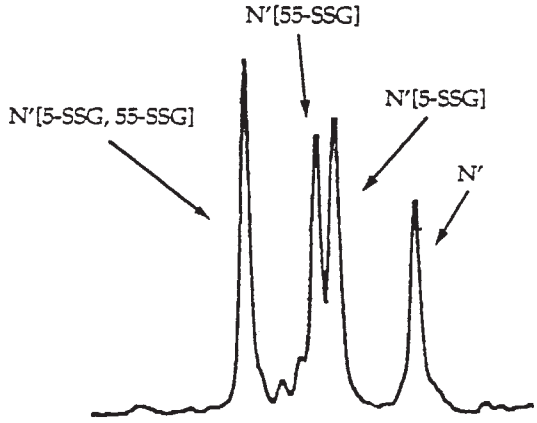


Fig. 5 HPLC chromatogram of the reaction yielding reversibly trapped N'[5–SSG] and N'[55–SSG] (see Methods).

SSG] (Fig. 6*b*). Because N'[55–SSG] forms N'[5–SSG] rapidly, the overall rates for formation of N from N'[5–SSG] and N'[55–SSG] are similar.

Rapid formation of second mixed disulphide

In the presence of GSSG, the intramolecular step for direct oxidation of N' competes with formation of a double mixed-disulphide derivative of N', termed N'[5–SSG; 55–SSG], in which both Cys 5 and Cys 55 are involved in disulphide bonds with glutathione (Fig. 7). In contrast to N', we find that the mixed-disulphide species, N'[5–SSG], reacts readily with GSSG (Fig. 3*c*). Thus, the formation of a mixed disulphide on residue 5 appears to alter substantially the local environment of residue 55 in the N' intermediate. Because N'[5–SSG; 55–SSG] contains no free thiols, this species is a dead-end for folding in the absence of a reducing reagent.

N' structure inhibits first oxidation step

In the studies presented here, we investigated why the N' intermediate does not fold by direct formation of the final native disulphide bond. Disulphide bond formation requires two steps: the intermolecular reaction of a protein thiol with GSSG to yield a protein-glutathione mixed-disulphide species, and the intramolecular attack of the protein-glutathione mixed disulphide by a second thiol in the protein to yield a protein–protein disulphide bond (Fig. 2*a*). The failure of N' to oxidize directly could be due to the low rate of the first or second step, or both. Our results indicate that native structure retards greatly the rate of both steps.

The rate of the first step in the oxidation of N', reaction of the free thiols with the external oxidizing agent GSSG to form the single mixed-disulphide derivatives, N'[5–SSG] and N'[55–SSG], is slowed by ~150-fold compared to the rate observed for N^{SH}_{SH}. Although differences in the intrinsic reactivity of the thiols in BPTI have been observed (M. Dadlez & P.S.K., unpublished results), the observation that N' and N^{SH}_{SH} have similar reactivity at elevated temperature and the observation that N'[5–SSG] reacts readily with GSSG indicate that the low reactivity of the thiols in N' is primarily the result of structure in this intermediate.

In order to react with an external disulphide reagent, denoted RSSR, the free thiols in N' must be exposed, at

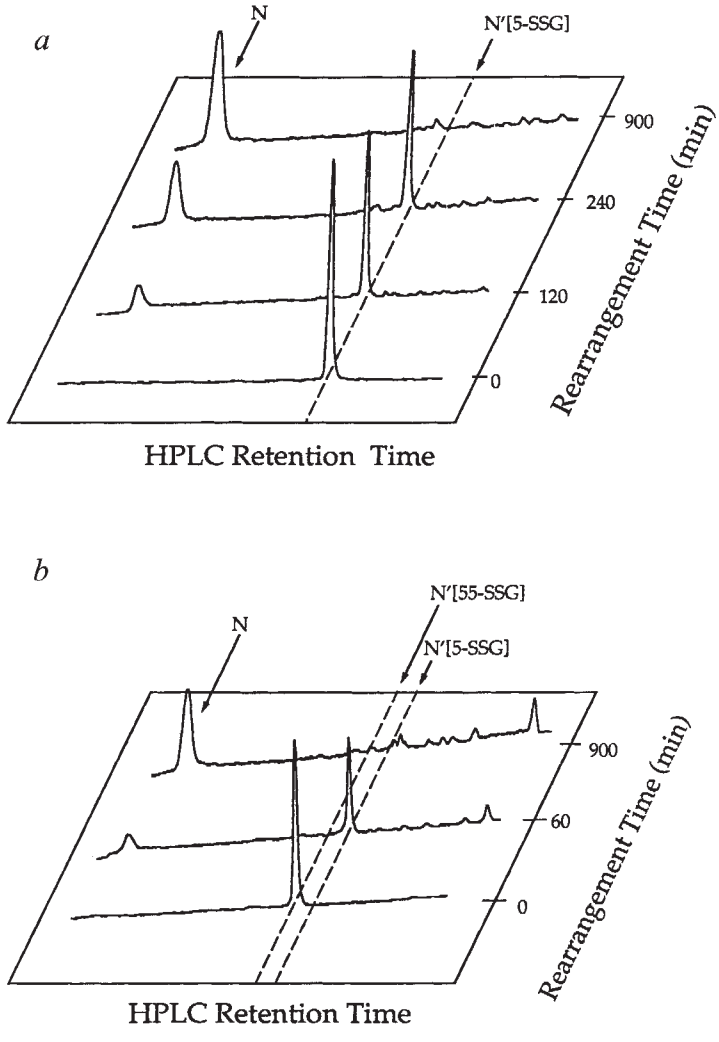


Fig. 6 Disulphide bond formation in the single mixed-disulphide derivatives of N'. *a*, Rearrangement of N'[5–SSG]. Acid-quenched, HPLC-purified N'[5–SSG] was allowed to rearrange by raising the pH to 7.3 in the absence of redox reagents. At the indicated times, a portion of the sample was removed, quenched with acid and analysed by HPLC. *b*, Rearrangement of N'[55–SSG].

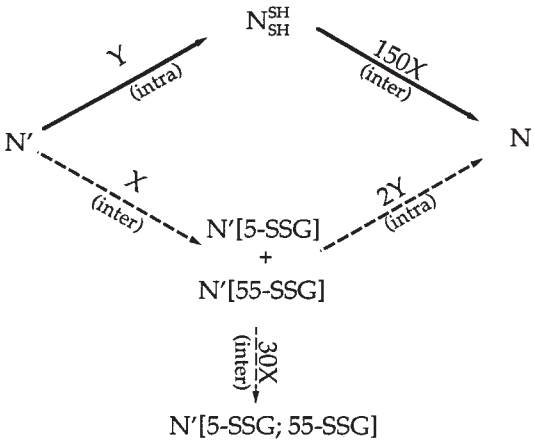


Fig. 7 Relative rates of the steps in the direct oxidation and rearrangement of N'. Steps involved in the rearrangement pathway are indicated by solid lines. The rearrangement of N' to the dead-end intermediate N* is not included but is substantial[3,4]. Steps in the direct oxidation pathway are indicated by dashed lines. '(intra)' denotes that the indicated step involves only intramolecular transitions. '(inter)' denotes that the indicated step is a bimolecular reaction with a rate that is proportional to the concentration of the external oxidizing agent, GSSG. Because the rate of N'[55–SSG]→N'[5–SSG] is rapid compared to the rate of N'[5–SSG]→N, the overall rates of N'[55–SSG]→N and N'[5–SSG]→N are similar (see Fig. 6). At pH 7.3, 25 °C, Y (the rate of formation of the first mixed-disulphide bond in the N' intermediate) is 3 min^{-1} M^{-1}, and X (the rate of the intramolecular rearrangement of N' to N^{SH}_{SH}) is 1.4×10^{-3} min^{-1}. At low GSSG concentrations, the rate of mixed-disulphide formation in N' is slower than the rate of rearrangement of N' to N^{SH}_{SH}. Formation of N'[5-SSG] and N'[55-SSG] is favoured at high GSSG concentrations (>500 μM). Under these conditions, however, the single mixed-disulphide derivatives rapidly react with GSSG to form a second mixed disulphide, yielding the dead-end intermediate N'[5–SSG, 55–SSG].

least transiently, to solvent. Thus, the reaction of N' with RSSR can be depicted as:

$$N' \underset{k_2}{\overset{k_1}{\rightleftharpoons}} X \xrightarrow{k_3[RSSR]} N'[5/55\text{-}SSR]$$

where X is the ensemble of conformations of N' in which the free cysteines are accessible to RSSR, N'[5/55–SSR] represents a species in which either Cys 5 or Cys 55 has formed a mixed disulphide with the disulphide reagent, and k_3[RSSR] is the rate of mixed-disulphide formation for solvent-accessible protein thiols (*cf.* ref. 20).

When $k_3[RSSR] << k_1+k_2$, the apparent first-order rate constant (k_{app}) is approximated by:

$$k_{app} \simeq \frac{k_1}{k_2}(k_3[RSSR]) \qquad (1)$$

The measured value of k_{app} is related linearly to the concentration of DTNB (Fig. 4*c*), indicating that equation 1 is valid for N' under these conditions. The value of k_3[RSSR] is approximated by the rate of mixed-disulphide formation in N^{SH}_{SH} since, unlike N', one of the thiols in N^{SH}_{SH} (Cys 14) is accessible to solvent even in the fully folded protein[21–23]. Thus, the thiol protection factor (that is, the ratio of the reactivity of the thiols in N^{SH}_{SH} divided by the reactivity of the thiols in N') provides an estimate of the equilibrium constant (k_2/k_1) between folded N' and the conformations of N' which are able to react with disulphide reagents.

Comparison of the protection factor of the thiols in N' with the free energy for global unfolding of this species indicates that, at low temperatures, reaction of the free thiols in N' proceeds through a partially structured intermediate. For example, the protection factor of the free thiols in N' at 5 °C is 170. By contrast, the free energy of unfolding of $N'_{V5,A55}$ is 4.3 kcal mol^{-1}, indicating that the equilibrium constant between folded and unfolded N' at this temperature is ~2000. This observation is in contrast to that for reduction of the constant fragment (C_L) of the immunoglobulin light chain, where the kinetics of reduction by dithiothreitol (DTT) are consistent with reduction proceeding through a globally unfolded species[24].

The proposal that mixed disulphide formation in N' at low temperature does not require complete unfolding is also supported by the finding that a subdomain of BPTI consisting of the C-terminal α-helix and the central β-sheet is able to form a stable, autonomously folding unit[25]. It may be, therefore, that N' is able to react with external disulphide reagents without unfolding completely if the N-terminal 3_{10}-helix becomes disordered, thereby exposing Cys 5, while the subdomain remains folded (see also ref. 15). At temperatures above the T_m of the subdomain, 30–40 °C (refs 25–27), formation of the mixed disulphide would be predicted to occur by global unfolding. Consistent with this expectation, the nonlinearity of the van't Hoff plot observed above 35 °C (Fig. 4*b*) could be due to a change in the mechanism of mixed disulphide formation from local to global unfolding.

N' structure inhibits second oxidation step

Starting with the two single mixed-disulphide derivatives, N'[5–SSG] and N'[55–SSG], it was possible to measure directly the rate of the intramolecular step in the direct oxidation of N'. We find that both N'[5–SSG] and N'[55–SSG] form native BPTI (N) at a low rate. For example, the intramolecular step in the oxidation of N' is ~400-fold slower than the average rate of the intramolecular step for the formation of the first disulphide bond in reduced, unfolded BPTI[7].

Although the rate of the intramolecular step in the direct oxidation of N' is low, this step is nonetheless approximately two-fold faster than the rate-limiting transition in the rearrangement pathway, rearrangement of N' to N^{SH}_{SH} (Fig. 7). The rearrangement of N' involves substantial, if not complete loss of structure in N' (refs 4,5,18). The similarity in the rates of the intramolecular steps in the direct oxidation and the rearrangement of N' suggests strongly that direct oxidation also requires extensive loss of structure.

No direct oxidation

A striking feature of the BPTI folding pathway is that folding proceeds through intramolecular disulphide bond rearrangements. Our measurements provide an explanation for why folding does not occur efficiently by direct oxidation of N' (Fig. 7). The thiols in N' are found to be buried and inaccessible to oxidizing agents. As a consequence, as with rearrangement to N^{SH}_{SH}, formation

of a mixed-disulphide bond in N' requires loss of strucure. Thus, at low or moderate GSSG concentrations, the intramolecular rearrangement of N' (to N^{SH}_{SH}) occurs more rapidly than the first step of direct oxidation (reaction of N' with GSSG to yield a single mixed-disulphide derivative) since the effective concentration[28–30] of the disulphide bonds in N' is higher than the concentration of GSSG. At high GSSG concentrations, mixed disulphide formation can be favoured. However, productive folding does not result because the single mixed-disulphide derivatives react readily with GSSG to yield the double mixed-disulphide derivative N'[5–SSG; 55–SSG]. Thus, rather than leading to an increase in the rate of productive folding of N', raising the concentration of GSSG causes the N' intermediate to accumulate as the nonproductive, double mixed-disulphide derivative N'[5–SSG; 55–SSG]. Consequently, in the absence of external reducing agents, there is no GSSG concentration that will allow folding to proceed efficiently by direct oxidation.

It is possible, however, that a significant fraction of BPTI molecules would fold by direct oxidation of N' in the presence of high concentrations of both oxidized and reduced glutathione (see ref. 31). Interestingly, by providing a solvent-accessible thiol with a relatively high effective concentration, the cysteine-containing pro-region of BPTI allows folding of BPTI to proceed by direct oxidation of a third disulphide bond[7]. In addition, destabilization of the folding intermediates by increasing temperature allows folding to proceed readily by the direct oxidation pathway[32].

Implications for disulphide-linked folding

In conclusion, as was observed with the N* intermediate, native structure in N' inhibits formation of the final disulphide bond. This structure buries and constrains the remaining free thiols (Fig. 1*a*). As a consequence, N' is a kinetically trapped intermediate in which both the intramolecular rearrangement of existing disulphide bonds and the formation of a new disulphide bond requires substantial loss of structure.

The premature 'locking in' of structure by the presence of native disulphide bonds has also been found to inhibit folding of other proteins. Both ribonuclease T1[33] and the constant fragment of the immunoglobulin light chain[34] fold even in the absence of any disulphide bonds. In the case of ribonuclease T1, formation of the two native disulphide bonds retards the rate at which the protein folds when it is diluted from denaturant, even though the disulphide bonds increase the stability of the native state substantially[35]. In the case of the immunoglobulin light chain, the rate-limiting step in both the oxidation and reduction of the protein appears to require complete unfolding of the native structure[24].

It is possible that the rate-limiting transition in the oxidative folding of proteins that contain disulphide bonds will often require the loss of previously acquired native structure, as most disulphide bonds in proteins are inaccessible to solvent[36]. A number of proteins, such as DsbA[37] and protein disulphide isomerase (PDI), that catalyze the oxidative folding of proteins in vivo have been identified. DsbA acts primarily by promoting formation of disulphide bonds[37,38] in solvent accessible cysteine residues. It has little effect, however, on the rate of productive folding of the highly structured, kinetically trapped BPTI disulphide intermediates (ref. 39; J.S.W. & P.S.K., unpublished results). By contrast, PDI was found to catalyze dramatically the rate of disulphide rearrangements in the kinetically trapped N' and N* intermediates, while having only a modest effect on the rate of disulphide formation in intermediates that do not contain buried cysteine residues[8]. The mechanism by which PDI gains access to buried thiol residues and catalyzes disulphide rearrangements in highly stable folding intermediates is poorly understood, but is likely to require unfolding of such species.

Methods

Production of disulphide intermediates. Reversibly trapped N' was produced as described previously[4]. $N'_{V5,A55}$ was produced by overexpression in *Escherichia coli* as described elsewhere[15]. Because the N'[5–SSG] and N'[55–SSG] intermediates are poorly populated during folding, it was not possible to purify these species directly from a folding reaction. Instead, reversibly trapped N'[5–SSG] and N'[55–SSG] were produced using the following strategy (Fig. 5). (i) Purified N' was dissolved in a solution containing 5 mM 5, 5'-dithio-*bis*-2-nitrobenzoic acid (DTNB, ref. 40), 30 mM Tris, pH 8.7, 6 M guanidine hydrochloride. DTNB reacts with the free thiols in N', yielding a species termed N'[5–TNB, 55–TNB], that contains the 30–51 and 14–38 disulphide bonds, and mixed-disulphide bonds with 5-thio-2-nitrobenzoic acid (TNB) on thiols 5 and 55. (ii) After 5 min, N'[5–TNB; 55–TNB] was desalted in 5% acetic acid (PD-10, Pharmacia) and dried to completion. (iii) Lyophilized N'[5–TNB; 55–TNB] was dissolved in a solution containing 4.5 M urea, 100 mM sodium formate, pH 4.0, 50 mM reduced glutathione (GSH), 50 mM reduced dithiothreitol (DTT). Because TNB is a much better leaving group than a cysteine or glutathione thiol, reaction of a protein–TNB disulphide bond with GSH results in the rapid release of TNB and formation of a protein–glutathione mixed-disulphide bond. Similarly, reaction of a protein–TNB disulphide bond with reduced DTT yields a protein–DTT mixed-disulphide bond. DTT is a circular disulphide reagent, however, so the second free thiol in DTT rapidly attacks the protein–DTT mixed-disulphide bond, yielding a free thiol on the protein. Thus, the reaction of N'[5–TNB, 55–TNB] with a 1:1 mixture of GSH and DTT for a period long enough to remove all of the TNB mixed-disulphide bonds, while not so long as to reduce protein disulphide bonds, yields an approximately equimolar mixture of N', N'[5–SSG], N'[55–SSG] and N'[5–SSG; 55–SSG]. (iv) After 7 min, the reaction was quenched with 1/10th volume of 6 M HCl. N'[5–SSG] and N'[55–SSG] were purified from the mixture by reversed-phased HPLC on a Vydac analytical C-18 column (Fig. 5). The identities of the N'[5–SSG] and N'[55–SSG] species were determined with a fluorescent labelling method as described previously[4]. The molecular weights of N'[55–SSG] (observed 6810 AMU, calculated 6819 AMU) and N'[5–SSG] (observed 6820 AMU, calculated 6819 AMU) were confirmed by laser desorption mass spectrometry (Finnigan MAT, Lasermat).

Measurement of thiol reactivity. The rates of reaction with GSSG for N', N'[5–SSG] and N^{SH}_{SH} were determined by dissolving purified intermediates in degassed pH 7.3 folding buffer (150 mM NaCl, 100 mM sodium phosphate, pH 7.3, 1 mM (ethylenedinitrilo) tetraacetic acid (EDTA)) or pH 8.7 folding buffer (200 mM KCl, 100 mM Tris, pH 8.7, 1 mM EDTA) containing different concentrations of GSSG.

These experiments were carried out in a circulating water bath at 25 °C. Folding reactions were quenched with 1/10th volume of 6 M HCl and analyzed by reversed-phase HPLC. HPLC separations were performed on a Vydac C-18 analytical column heated to 37 °C. The concentrations of intermediates present in the HPLC chromatograms were determined by absorbance at 229 nm. The rate constants for the reactions were determined using a nonlinear least squares-fitting program (Kaleidagraph, Abelbeck Software).

The rates of reaction of the thiols in N' and N^{SH}_{SH} with DTNB were determined as follows: (i) 1900 µl of pH 6.0 buffer containing DTNB (final concentration 10–100 µM DTNB, 100 mM sodium citrate, pH 6.0, 150 mM NaCl, 1 mM EDTA) was equilibrated to the appropriate temperature in a stirred quartz cuvette. (ii) The reaction was initiated by the addition of 100 µl of purified N' or N^{SH}_{SH} (final concentration, 1–5 µM) in 10 mM HCl, pre-equilibrated to the appropriate temperature. (iii) The time course of the reaction was determined by monitoring the increase in absorbance at 412 nm using an Aviv UV/VIS spectrophotometer (model 118DS) equipped with a thermoelectric temperature controller. The rate constants were determined as above.

Rearrangement of purified intermediates. Rearrangement reactions were initiated by the addition of pH 7.3 folding buffer to lyophilized intermediates in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI). Folding buffer was degassed by stirring in the anaerobic chamber for several days prior to the experiment. The rearrangement reactions were carried out in a circulating water bath at 25 °C. Aliquots were removed from the rearrangement mix at the appropriate time, quenched with 1/10th volume of 6 M HCl and analyzed by HPLC[4,5] with a Vydac C-18 analytical column heated to 37 °C. Samples were stored at 4 °C prior to HPLC analysis. Buffer A is 0.1% TFA in water, and buffer B is 0.1% TFA, 90% acetonitrile, 10% water. In the experiments depicted in Fig. 6, a gradient with linear segments was used: 0 min, 90% A; 5 min, 83% A; 25 min, 74% A; 85 min, 62% A.

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