Oxidative refolding of recombinant prochymosin

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The disulphide-coupled refolding of recombinant prochymosin from *Escherichia coli* inclusion bodies was investigated. Prochymosin solubilized from inclusion bodies is endowed with free thiol groups and disulphide bonds. This partially reduced form undergoes renaturation more efficiently than the fully reduced form, suggesting that some native structural elements existing in inclusion bodies and remaining after denaturation function as nuclei to initiate correct refolding. This assumption is supported by the finding that in the solubilized prochymosin molecule the cysteine residues located in the N-terminal domain of the protein are not incorrectly paired with the other cysteines in the C-terminal domain. Addition of GSH/GSSG into the refolding system facilitates disulphide rearrangement and thus enhances renaturation, especially for the fully reduced prochymosin. Based on the results described in this and previous papers [Tang, Zhang and Yang (1994) Biochem. J. **301**, 17–20], a model to depict the refolding process of prochymosin is proposed. Briefly, the refolding process of prochymosin consists of two stages: the formation and rearrangement of disulphide bonds occurs at the first stage in a pH 11 buffer, whereas the formation and adjustment of tertiary structure leading to the native conformation takes place at the second stage at pH 8. The pH 11 conditions help polypeptides to refold in such a way as to favour the formation of native disulphide bonds. Disulphide rearrangement, the rate-limiting step during refolding, can be achieved by thiol/disulphide exchange initiated by free thiol groups present in the prochymosin polypeptide, GSH/GSSG or protein disulphide isomerase.

Key words: disulphide rearrangement, inclusion bodies, oxidative state.

INTRODUCTION

Generation of correct disulphide bonds is required for formation and stabilization of the native conformation of a disulphide-containing protein, and the kinetics and thermodynamics of disulphide formation can dominate the rate and pathway of protein folding and in turn determine the refolding efficiency. Therefore, studies on the mechanism of disulphide formation during protein refolding will facilitate not only the understanding of protein refolding but also the recovery of functional forms of recombinant proteins from inclusion bodies. Since some disulphide-containing proteins such as RNase A [1] refold with high efficiency into their native structures, whereas others such as insulin-like growth factor I [2] do not, it is necessary to investigate the oxidative refolding of each target protein individually. Prochymosin contains three disulphide bonds linking Cys-45 to Cys-50, Cys-206 to Cys-210 and Cys-250 to Cys-283 (pepsin numbering). As the zymogen of a commercially important enzyme for the production of cheese, chymosin has been the subject of the numerous studies on recombinant proteins since the early 1980s. In 1984 Marston et al. [3] first devised a protocol to produce chymosin from prochymosin-containing inclusion bodies: the inclusion bodies were solubilized and denatured in urea and then renatured by a process involving alkaline treatment followed by neutralization; it was found that the alkaline treatment was critical to high renaturation. Later, Sugrue et al. [4] rationalized this empirical procedure by an argument that the dilution of urea-solubilized prochymosin into a high-pH buffer converts prochymosin irreversibly denatured by urea into a form of the protein that more readily refolds to the native conformation. It is worthwhile to note that this argument was based on denaturation studies on prochymosin with intact native disulphide bonds, and the disulphide-bond formation was not taken into consideration. In fact, renaturation of prochymosin from inclusion bodies includes both the regeneration of native non-covalent interactions and the formation of natural disulphide bonds. To further elucidate the mechanism of prochymosin refolding, the role of disulphide-bond formation in the refolding of recombinant prochymosin was investigated in our laboratory. By using protein disulphide isomerase (PDI) as a probe to monitor the process of recombinant prochymosin refolding, we have demonstrated that formation of native disulphide bonds plays a critical role in correct refolding of prochymosin [5]. Under optimal conditions in the presence of PDI, renaturation efficiency of up to 90 % can be achieved (B. Tang, Y. Zhang, and K. Yang, unpublished work). Site-directed mutagenesis studies indicated that among the three disulphide bonds Cys-250–Cys-283 is indispensable to the correct refolding of prochymosin [6], whereas Cys-45–Cys-50 and Cys-206–Cys-210 are dispensable ([7] and H. Chen, G. Zhang, and K. Yang, unpublished work). It has also been demonstrated that the renaturation efficiency of recombinant prochymosin depends not only on the renaturation conditions but also the solubilization (denaturation) conditions. Compared with pH 8, solubilization of prochymosin-containing inclusion bodies at pH 11 leaves prochymosin molecules in a more reduced and more unfolded state which undergoes renaturation more readily [8]. Based on these findings, the effect of the oxidation states of the unfolded polypeptides and the redox conditions on the renaturation of recombinant prochymosin was further investigated. A model to depict the oxidative refolding of prochymosin is thus proposed.

MATERIALS AND METHODS

Solubilization of inclusion bodies and renaturation

Inclusion bodies prepared as described previously [5] were solubilized in buffer A (50 mM KH_2PO_4/50 mM NaCl/1 mM EDTA, pH 11) containing 8 M urea. After 2 h at 30 °C the

Abbreviations used: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; PDI, protein disulphide isomerase; PVDF, polyvinylidene difluoride.

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solution was centrifuged at 20000 ***g*** for 10 min. The supernatant was recovered as inclusion-body solution. The renaturation process consisted of two steps: first, the inclusion-body solution (2 mg/ml of protein) was diluted with 11.5 vol. of buffer A and left at 15 °C for 1–24 h; secondly, the pH 11 refolding solution was adjusted to pH 8 with 1 M HCl, maintained at 15 °C for 1 h and dialysed against buffer B (20 mM Tris/HCl/50 mM NaCl/1 mM EDTA, pH 8) overnight at 4 °C.

Activation and milk-clotting activity assay

The renatured prochymosin was acidified to pH 2, incubated for 2 h at 15–20 °C and then adjusted to pH 6.3 [9]. After incubation at 4 °C for 1 h the milk-clotting activity was measured by the method of Emtage et al. [10]. Authentic chymosin was used as a standard. One unit of activity is equivalent to the clotting activity of 1 μg of authentic chymosin. The renaturation efficiency was expressed as a percentage of active chymosin detected in relation to the prochymosin input in the refolding solution.

Titration of thiol group

The free thiol content of prochymosin was estimated by 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) titration as described by Ellman [11].

Disulphide analysis

For disulphide analysis the method developed by Anderson and Wetlaufer [12] was followed.

Analysis of disulphide-bonding pattern

To determine the disulphide-bonding pattern of the solubilized prochymosin, a peptide-mapping method was established. The procedure (C. Wei, H. Chen, Y. Zhang and K. Yang, unpublished work) is described briefly as follows. The freshly prepared inclusion-body solution (2 mg/ml of protein) was treated with 2% (v/v) 4-vinyl pyridine at pH 8 and room temperature for 1 h to block any free thiols present in the unfolded prochymosin. The chemically modified prochymosin was purified by acetone precipitation and preparative SDS/PAGE. The purified prochymosin was digested with CNBr ($\sim$ 60–100-fold excess over methionine) in 70% formic acid at room temperature for 24 h. The digest was diluted approx. 10-fold with distilled water and lyophilized. The lyophilized preparation was dissolved in loading buffer (50 mM Tris/HCl, pH 6.8/2% SDS/10% glycerol) and subjected to one-dimensional SDS-urea/PAGE according to the method of Swank and Munkres [13]. The gel containing the separated polypeptides was excised and soaked in the loading buffer with 5% 2-mercaptoethanol for 30 min and then subjected to SDS-urea/PAGE in the second dimension at right angles to the first. The peptide fragments were transferred from the gel to a PVDF membrane and identified by N-terminal sequencing on a FEADI 491-A protein sequencer (ABI Company).

Estimation of molecular mass of peptide fragments

The molecular masses of CNBr fragments were estimated by SDS-urea/PAGE [13].

Purification of prochymosin

The refolded prochymosin was purified by DEAE-Sepharose CL 6B column chromatography as described previously [7].

Reduction of prochymosin

The prochymosin solubilized from inclusion bodies and the purified prochymosin were reduced with 0.15 mM and 1 mM dithiothreitol (DTT) respectively in the presence of 8 M urea at 30 °C, pH 11 for 2 h. Full reduction was confirmed by thiol titration.

Protein determination

The amount of protein was determined by the method of Lowry et al. [14].

CD spectroscopy

CD spectra were measured with a Jasco J-720 spectropolarimeter using 1 mm path-length cuvettes. The protein concentration of the sample was 0.2 mg/ml. The purified prochymosin, after denaturation and reduction followed by gel filtration, was subjected to renaturation in the presence of GSSG/GSH. The refolding process was monitored by measuring the CD ellipticity at 220 nm and CD spectra.

Fluorescence measurement

Fluorescence changes during the refolding of the reduced and purified prochymosin were measured with a Hitachi F4010 spectrofluorimeter using 0.5 cm path-length cuvettes. The protein concentration of the sample was 0.2 mg/ml. The fluorescence was excited at 281 nm and the intensity of the emission at 335 nm and fluorescence spectra were recorded as a function of time.

RESULTS

Oxidation state of the unfolded prochymosin solubilized from inclusion bodies

The prochymosin used in this study was derived from inclusion bodies. We have demonstrated [8] that solubilization of inclusion bodies with 8 M urea at pH 11 leaves the prochymosin molecule in a more reduced and unfolded state, which undergoes renaturation with one-fold higher efficiency than its counterpart solubilized at pH 8 (8 M urea). This result indicates that the unfolded state of prochymosin has great influence on its renaturation. To further characterize the prochymosin unfolded at pH 11 with 8 M urea, the freshly solubilized prochymosin was treated immediately with 4-vinylpyridine to block its free thiol groups and was purified by acetone precipitation and preparative SDS/PAGE, and its oxidation state was then analysed. The Ellman assay revealed that 3.0 ± 0.3 free thiols per solubilized prochymosin molecule on average were detected. One-dimensional electrophoresis (Figure 1A) and diagonal electrophoresis (Figure 1B) of the CNBr cleavage products of the solubilized prochymosin indicate that only peptide fragment b can be reduced into two peptides (b_1, b_2), corresponding to fragments d and e. N-terminal sequencing and molecular mass determination (Table 1) demonstrated that the larger polypeptide (b_1) of the two fragments lying off the diagonal contains Cys-206, Cys-210 and Cys-250, whereas the smaller one (b_2) harbours Cys-283; fragment c lying on the diagonal was identified as the N-terminal polypeptide in which Cys-45 and Cys-50 reside. The fact that fragments d and e in the unreduced sample are separated after first-dimension electrophoresis (Figure 1A, lane 1) and retained on the diagonal after second-dimension electrophoresis (Figure 1B) reveals that they are not linked by a disulphide bridge, indicating that Cys-283 in part of the solubilized prochymosin is in a free form. As indicated in Table 1, the fragments c, d and e contain 2 (Cys-45, Cys-50), 3 (Cys-206, Cys-210, Cys-250) and 1

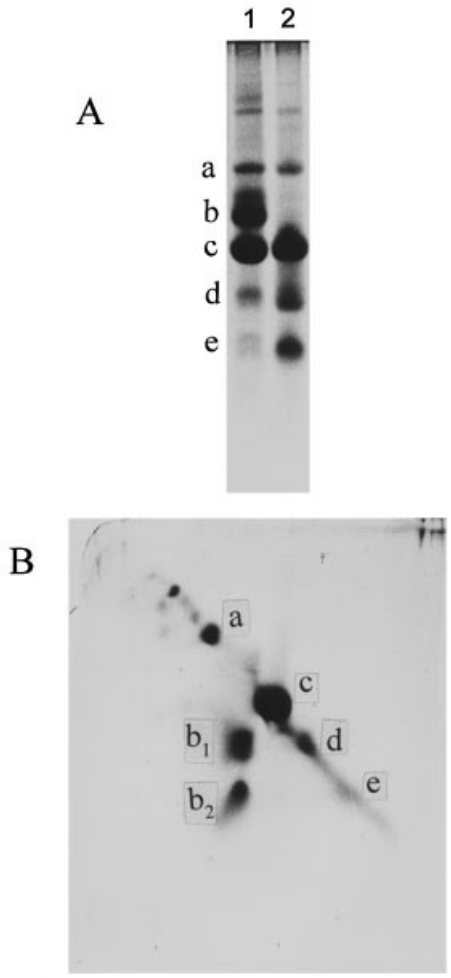


Figure 1 Electrophoresis map of the CNBr-cleavage products of the prochymosin solubilized from inclusion bodies with 8 M urea at pH 11

(**A**) One-dimensional electrophoresis map. CNBr cleavage products, unreduced (lane 1) and reduced (lane 2), were subjected to SDS-urea/PAGE. (**B**) Diagonal electrophoresis map. After one-dimensional electrophoresis of the unreduced sample, the gel slice containing separated peptides was soaked in the loading buffer with 2-mercaptoethanol for 30 min and then subjected to SDS-urea/PAGE in the second dimension (for details, see the Materials and methods section).

(Cys-283) cysteine residue(s) respectively. Cys-283 must be in a free thiol form, while the others remain to be determined. To address this, fragments c and d were eluted from the first-dimension gel and subjected to UV absorbance measurement and disulphide analysis. UV absorbance scanning indicated that a peak with a maximum at 257 nm was observed for these two

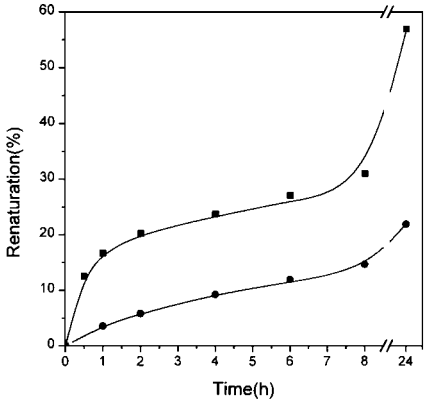


Figure 2 Renaturation progress of partially and fully reduced prochymosin following the time course of the first stage of refolding in air

An 80 μl inclusion-body solution (160 μg of protein), unreduced or reduced with 0.15 mM DTT, was diluted with 920 μl of buffer A and incubated at 15 °C for various times as indicated. The refolding mixture was then adjusted to pH 8 with 1 M HCl, incubated at 15 °C for 1 h and dialysed against buffer B overnight at 4 °C. Samples were acidified and assayed for milk-clotting activity as described in the Materials and methods section. ■, Partially reduced prochymosin; ●, fully reduced prochymosin.

fragments (results not shown). This is consistent with the property of the pyridylethyl group with a characteristic absorption around 254–260 nm [15,16], revealing that some free thiol(s) present in these fragments were alkylated by vinylpyridine during the SH-blockage step. According to disulphide analysis [12], disulphide bridges have also been detected in these fragments (results not shown). These results demonstrated that fragments c and d contain both thiol group(s) and disulphide bond(s), in accordance with the fact that they were derived from solubilized prochymosin and consequently endowed with heterogeneity with respect to oxidation state. Taking all the results described above into consideration, it is concluded that prochymosin solubilized from inclusion bodies with 8 M urea at pH 11 contains both free thiols and disulphide bonds. Most importantly, Cys-45 and Cys-50, whether in a reduced form or in an oxidized form, are not incorrectly paired with the other four cysteine residues. This state is designated as a partially reduced form. For comparison, the partially reduced form was converted into the fully reduced form by complete reduction with DTT to examine the influence of oxidation states on refolding.

Oxidative refolding of prochymosin with different oxidation states in air

As mentioned in the Materials and methods section, renaturation of prochymosin consists of two stages: dilution at pH 11 and dialysis at pH 8. It has been proved that renaturation of

Table 1 Identification of CNBr peptide fragments containing cysteine residues

* Determined by SDS-urea/PAGE

Peptide fragment	N-terminal sequence		Mass (Da)		Position of cysteine residues
	Expected	Sequenced	Expected	Determined*	
b_1 (d)	L-T-L-G-A	L-T-L-G-A	9657.7	9500	206, 210, 250
b_2 (e)	Y-P-L-T-P	Y-P-L-T-P	6377.8	6000	283
c	A-E-I-T-R	A-E-I-T-R	13968.2	14000	45, 50

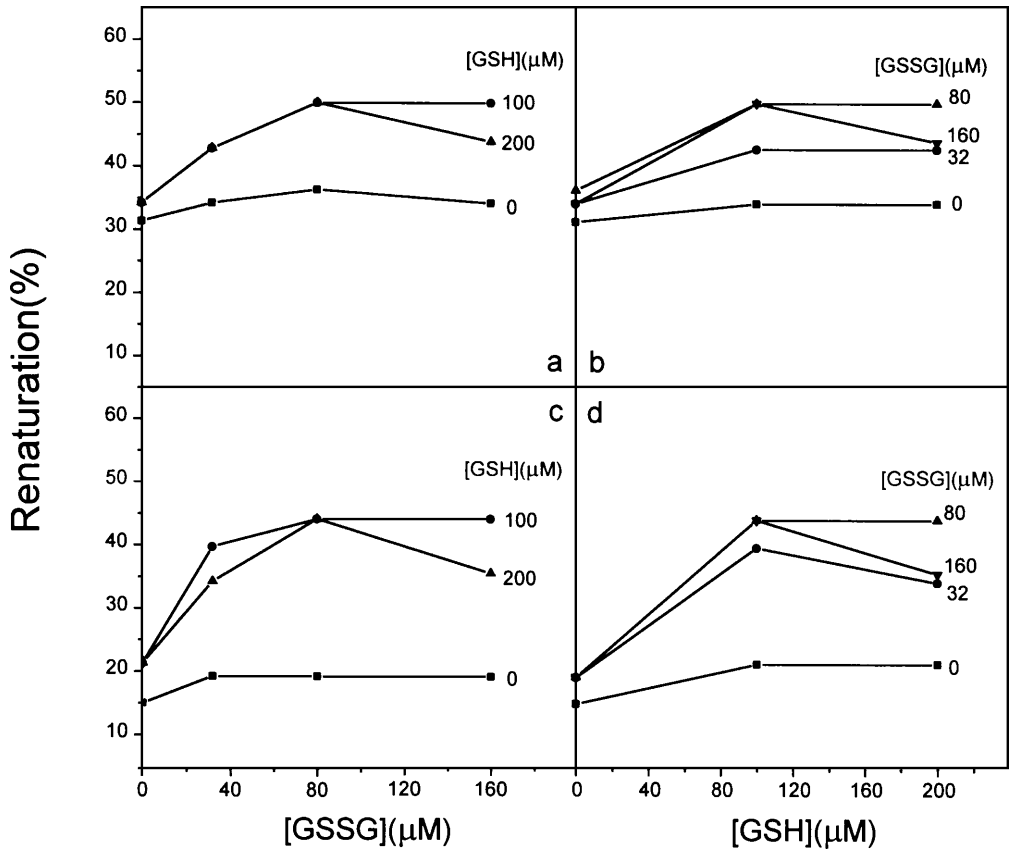


Figure 3 Effect of GSH and/or GSSG concentration on the renaturation of partially and fully reduced prochymosin

The procedure described in the legend to Figure 2 was followed, except that the refolding mixture containing different concentrations of GSH and/or GSSG, as indicated, was left at pH 11 and 15 °C for 8 h. (**a**) and (**b**) Partially reduced prochymosin; (**c**) and (**d**) fully reduced prochymosin.

prochymosin is highly pH-dependent at the first stage: higher pH is critical to higher renaturation [3,5]. Based on this finding the effect of the dilution pH on the renaturation of prochymosin with different oxidation states was investigated. It was demonstrated that both states exhibit similar pH-renaturation profiles with identical pH optimum around pH 11. However, their renaturation efficiencies are quite different. The active enzyme obtained from the fully reduced prochymosin is much lower than that from the partially reduced form. This holds true when the incubation time is prolonged to 24 h (Figure 2), indicating that partially reduced prochymosin undergoes renaturation more efficiently than the fully reduced form. These results may be rationalized by consideration of their different oxidation states. As mentioned above, for partially reduced prochymosin, Cys-45 and Cys-50 are not incorrectly paired with the other four cysteine residues. Most likely, they are restricted to a specific region of the molecule. This restriction may prevent them from forming non-native disulphide bonds on refolding. In other words, for partially reduced prochymosin, disulphide formation and rearrangement mainly occurs among four cysteine residues. On the other hand, for fully reduced prochymosin all six cysteine residues are in a free form, therefore, disulphide formation and rearrangement must occur among six cysteine residues. According to Anfinsen and Scheraga [17], the number of the possible combinations of $2n$ SH groups to form disulphide bonds in proteins increases rapidly with the increase in n. It is therefore reasonable to assume that after oxidation there will be 15 isomers of the fully reduced prochymosin and three isomers of the partially reduced one. Since only one isomer with native disulphide bonds is active and the others are inactive, the lower renaturation of fully reduced prochymosin may be attributed to more scrambled isomers being formed during reoxidation in air.

Oxidative refolding of prochymosin with different oxidation states in the presence of GSH/GSSG

From the results described above we speculated that the GSH couple GSH/GSSG may enhance renaturation of prochymosin via thiol/disulphide interchange to reduce molecules with scrambled disulphide bonds. Experiments began with the determination of the optimal ratio of GSH to GSSG for prochymosin renaturation. Figure 3 reveals that GSH or GSSG alone can only promote renaturation of both forms of prochymosin slightly. A remarkable effect is observed when GSH and GSSG are present simultaneously. The optimal ratio of GSH to GSSG is 100 μM/80–160 μM. It is important to note that under the optimal redox conditions the boost to renaturation efficiency of fully reduced prochymosin is larger than that of partially reduced prochymosin (193% compared with 59%). This difference also suggests that more scrambled molecules are derived from fully reduced prochymosin. Scrambled molecules can only be converted into molecules with native disulphide bonds via thiol/disulphide interchange initiated by GSH/GSSG at the later stages of refolding when all free thiols of the

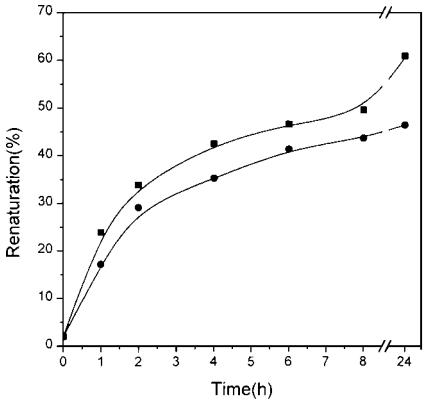


Figure 4 Renaturation progress of partially and fully reduced prochymosin following the time course of the first stage of refolding in the presence of GSH/GSSG

The procedure described in the legend to Figure 2 was followed, except that the refolding solution containing 100 μM GSH and 80 μM GSSG was left at pH 11 and 15 °C for various times as indicated. ■, Partially reduced prochymosin; ●, fully reduced prochymosin.

Table 2 Effect of GSH/GSSG, added at different stages of the refolding process, on the renaturation of prochymosin

The renaturation procedure described in the legend to Figure 2 was followed, except that 100 μM GSH and 80 μM GSSG were added to the renaturation solution on dilution at pH 11 (the first stage) or on neutralization at pH 8 (the second stage) respectively. Milk-clotting activities are given as the means of triplicate assays ± S.D.

	Milk-clotting activity (units/ml)	
[GSH]/[GSSG] (100 μM/80 μM)	Partially reduced prochymosin	Fully reduced prochymosin
No GSH/GSSG	10.6 ± 0.2	1.3 ± 0.4
Added at the first stage	15.4 ± 0.3	10.6 ± 0.2
Added at the second stage	11.7 ± 0.2	1.7 ± 0.4

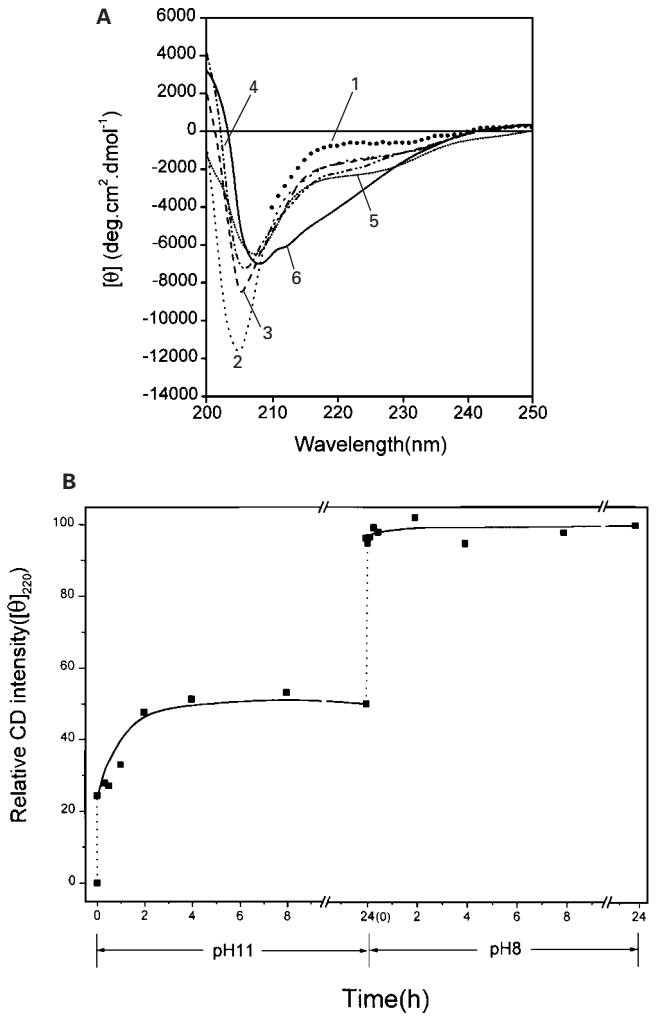


Figure 5 Changes in far-UV CD spectra and ellipticity at 220 nm during two-stage renaturation of purified prochymosin in the presence of GSH/GSSG

Purified prochymosin, after denaturation and reduction followed by gel filtration, was subjected to refolding at a concentration of 0.2 mg/ml in the presence of GSH/GSSG. (**A**) Changes in the CD spectra were measured at different time intervals as indicated during refolding. Curves: 1, unfolded and reduced prochymosin (0.2 mg/ml in 8 M urea pH 11 buffer); 2, 0 min in pH 11 buffer (immediately after dilution); 3, 30 min in pH 11 buffer; 4, 6 h in pH 11 buffer; 5, 24 h in pH 11 buffer; 6, 24 h in pH 8 buffer. (**B**) Changes in CD ellipticity at 220 nm, $[\theta]_{220}$, were measured during refolding and are expressed as relative values. The initial $[\theta]_{220}$ in the presence of 8 M urea and the final $[\theta]_{220}$ are -799 deg·cm^2·dmol^{-1} and -4163 deg·cm^2·dmol^{-1} respectively. Dotted lines, CD change occurred within the mixing time of the experiment.

polypeptide are paired. Therefore, in the presence of GSH/GSSG the larger enhancement is observed for fully reduced prochymosin.

This postulation is supported by the results presented in Figure 4. In the presence of GSH/GSSG the fully reduced prochymosin proceeds to refold in a manner similar to that of partially reduced prochymosin. Compared with the refolding in the absence of GSH/GSSG (Figure 2) the differences in renaturation efficiency between them at every test point during refolding are markedly reduced, suggesting that the conversion of unproductive product into productive product mediated by GSH/GSSG must be related to the conversion of the non-native disulphide bonds into native disulphide bonds.

We have demonstrated that the thiol/disulphide exchange catalysed by PDI occurred mainly at the first stage (pH 11) of the prochymosin refolding process rather than the second stage (pH 8), and proposed that pH 11 is favourable for such refolding of polypeptide as at this pH two relevant cysteine residues are brought into proximity for the formation of correct disulphide bonds [5]. According to this point of view, we assumed that GSH/GSSG may also exert their function at the first stage. Indeed, a minor effect on prochymosin renaturation is observed when GSH/GSSG is added at the second stage; inclusion of GSH/GSSG into the refolding system at the first stage results in a marked enhancement of renaturation (Table 2). In this situation we prefer to conclude that for all the disulphide rearrangement reactions of prochymosin, spontaneous or catalysed by PDI or GSH/GSSG, proceeding at pH 11 favours the formation of native disulphide bonds.

Conformational change of prochymosin during the two-stage renaturation

To get insights into the changes in the secondary and tertiary structures of prochymosin during refolding, the purified active prochymosin was fully reduced and denatured and then subjected to renaturation in the presence of GSH/GSSG; at different time

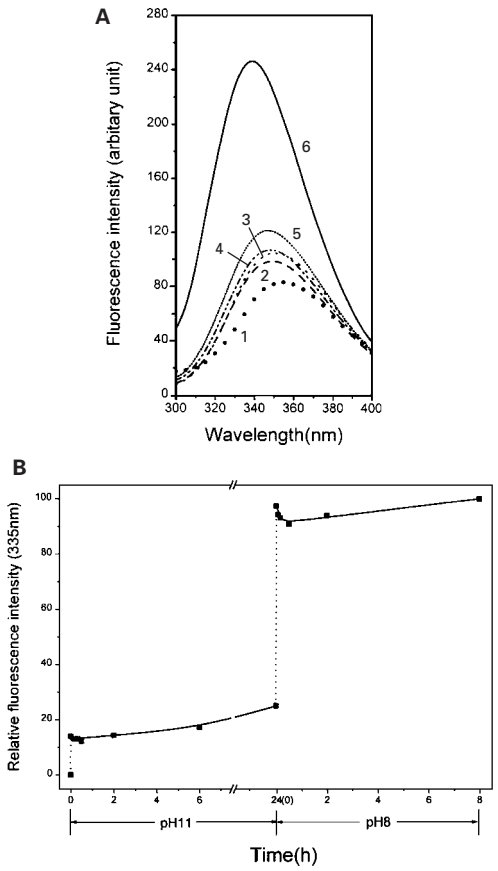


Figure 6 Changes in the intrinsic fluorescence spectra and fluorescence intensity at 335 nm during two-stage renaturation of the purified prochymosin in the presence of GSH/GSSG

The procedure described in the legend to Figure 5 was followed, except that the fluorescence was measured. (**A**) Changes in the fluorescence spectra were measured at different time intervals as indicated during refolding. Curves: 1, unfolded and reduced prochymosin (0.2 mg/ml in 8 M urea pH 11 buffer; 2, 0 min in pH 11 buffer (immediately after dilution); 3, 30 min in pH 11 buffer; 4, 6 h in pH 11 buffer; 5, 24 h in pH 11 buffer; 6, 8 h in pH buffer. (**B**) Changes in fluorescence intensity at 335 nm. Dotted line, fluorescence change occurred within the mixing time of the experiment.

intervals aliquots were withdrawn and analysed by far-UV CD and intrinsic fluorescence measurements. As shown in Figure 5, with the prolongation of the incubation time the intensity of CD at 220 nm increases, gaining 50 % of ellipticity at the pH 11 stage and the other half at the pH 8 stage (Figure 5B). Meanwhile, the far-UV CD spectra undergo changes from the type characteristic of a random coil to the type characteristic of an ordered secondary structure with a predominance of β-sheet, as expected from the known structure of prochymosin (Figure 5A). It is worthwhile to mention that although 50 % of the ellipticity at 220 nm has been gained after the first stage renaturation, the CD spectrum of the protein at this stage is still different from that of the final product. Figure 6 demonstrates that the intrinsic fluorescence at 335 nm is increased by only 25 % after the pH 11 stage, while the other 75 % fluorescence is recovered after the pH 8 stage; the blue shift of fluorescence emission maxima from 354 nm (unfolded state) to 346 nm (after the first stage) to 338 nm (after the second stage) is also evident. From all these results it is reasonable to conclude that during the first stage of renaturation prochymosin proceeds to refold, forming intermediates that assume some ordered but still more open structures, and that the final native conformation can only be formed after the second stage of renaturation.

DISCUSSION

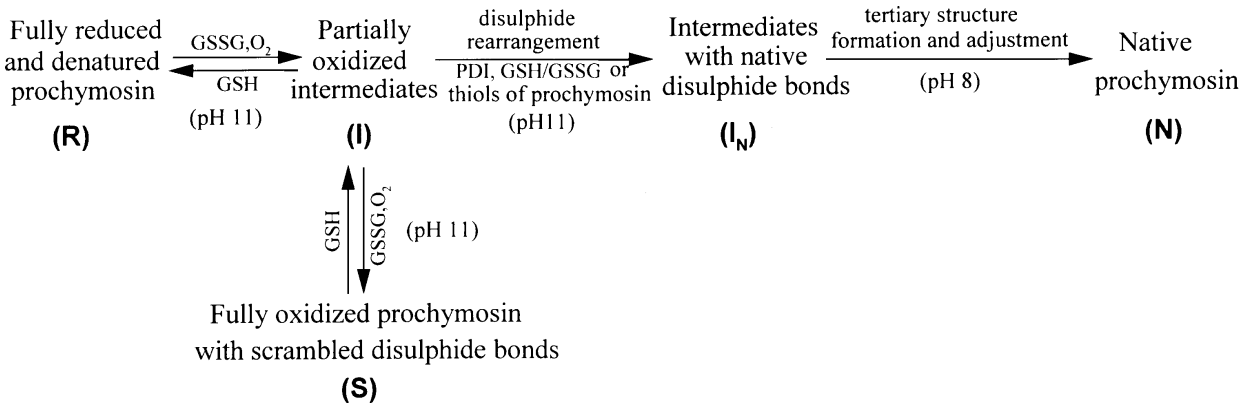
Partially reduced prochymosin solubilized from inclusion bodies undergoes renaturation more readily than the fully reduced prochymosin

From Figures 2–4, it is evident that a higher degree of renaturation is achieved from partially reduced prochymosin than from the fully reduced protein both in air and in the GSH/GSSG system, suggesting that the former contains some native structural elements to initiate correct refolding. Oberg et al. [18] and Przybycien et al. [19] determined the structures of interleukin-1β and β-lactamase inclusion bodies expressed in *Escherichia coli* by attenuated total reflectance FTIR and Raman spectroscopy respectively, and found that both of them have high contents of secondary structures. Meanwhile, more and more lines of experimental data have shown that some proteins under denaturing conditions still possess some ordered structures rather than totally random coil, although they possess no tertiary structure at all [20]. Similarly, we found that Cys-45 and Cys-50 were not incorrectly paired with other cysteine residues in prochymosin molecules solubilized from inclusion bodies by urea at pH 11, suggesting that some ordered structures exist in partially reduced prochymosin. Prochymosin consists of two domains: Cys-45 and Cys-50 are located in the N-terminal domain, while the other cysteine residues are found in the C-terminal domain. The fact that Cys-45 and Cys-50 are not involved in non-native disulphide bridges implies that they are restricted in some native secondary structures. This may result from co-translational folding during the synthesis of nascent polypeptide. The native structural elements may act as nuclei to initiate correct refolding, leading to higher yield of active product. When the partially reduced prochymosin is converted into the fully reduced form with DTT, it may be considered as a random coil with no native secondary structures. Random coil polypeptide is more liable to form incorrect disulphide bonds, resulting in lower renaturation efficiency.

Model of the oxidative refolding process of prochymosin

Based on the results described above and in [5], a model to depict the refolding process of prochymosin is proposed (Scheme 1). The main points of this model are as follows. (1) The refolding process consists of two stages: the formation and rearrangement of disulphide bonds occur at pH 11, whereas the formation and adjustment of tertiary structure leading to the native conformation mainly take place at pH 8. (2) The unfolded polypeptides begin to refold as soon as the urea concentration is diluted below 0.8 M with pH 11 buffer. The high pH helps polypeptides to refold in such a way as to favour the formation of the native disulphide bonds. (3) The rearrangement of disulphide bonds is a rate-limiting step during refolding; without an intermediate with native disulphide bonds (I_N) there would be no active product (N). Disulphide rearrangement can be initiated by free thiol groups of prochymosin itself, GSH/GSSG or PDI.

Creighton et al. [21] have indicated that for a reduced and very unfolded protein, formation of the initial disulphide bonds is essentially random and depends primarily on the proximity of the cysteine residues in the covalent structure; further disulphide-bond formation is also a statistical event and is increasingly unfavourable energetically, until the protein adopts non-random conformations that either favour or disfavour particular disulphide bonds. This point of view can be used to explain the



Scheme 1 Model of the oxidative refolding process of prochymosin

proposed model. It is assumed that after dilution in a pH 11 buffer the fully reduced and denatured prochymosin proceeds to refold, forming partly refolded species, including partially oxidized intermediates (I), scrambled prochymosin (S) and intermediates with native disulphide bonds (I_N) (see Scheme 1). The formation of these species can be considered as the earlier events during refolding before the formation of the non-random conformation. This speculation is supported by the observation that during the pH 11 stage the increases in ellipticity at 220 nm and the fluorescence intensity at 335 nm never exceed 50% and 25% respectively and the red-shift of the fluorescence emission maxima is evident, suggesting that all the species formed assume a solvent-exposure conformation. It is likely that the more open structure of these intermediates renders the free cysteine residues and disulphide bonds accessible for further thiol/disulphide interchange, leading to the production of more I_N molecules, which eventually adopt the active conformation after the native tertiary interactions are completed at pH 8. Obviously, this model can be used to explain why partially reduced prochymosin from inclusion bodies and the pH 11 stage are favourable for renaturation. It should be emphasized that for most disulphide-containing proteins reported so far the acquisition of native conformation and the reformation of correct disulphide bonds are simultaneously accomplished during a single refolding process. In contrast, prochymosin is characterized for its two-stage oxidative refolding. Most interestingly, its homologous protein, pepsinogen, also follows a one-stage renaturation process and pH values above 8 are unfavourable for its renaturation [22], although they share similar conformation. The mechanism underlying this difference remains to be elucidated.

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