

Assisted refolding of recombinant prochymosin with the aid of protein disulphide isomerase

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Protein disulphide isomerase (PDI) was shown to be able to accelerate the refolding of unfolded recombinant prochymosin and to enhance the overall yield of active protein. Unlike previous reports in this study PDI was found to be active at pH values as high as 11. The coincidence of the similar apparent optimum pH values of uncatalysed and PDI-catalysed reactions suggests that

conditions favourable to spontaneous refolding of proteins may help PDI to catalyse thiol/disulphide interchange. Under the conditions described here no exogenously added dithiothreitol was required for PDI-catalysed renaturation, implying that the disulphide form of PDI was reduced to its active form by the free thiol groups in prochymosin molecules.

INTRODUCTION

With the wide application of genetic engineering to the production of active proteins of commercial importance the demand for developing feasible downstream procedures has increased. Recombinant proteins accumulating to high levels in *Escherichia coli* usually exist in the form of inclusion bodies. Solubilization and renaturation are essential steps to obtain active molecules [1]. In addition, for disulphide-containing proteins correct pairing of cysteine residues must be taken into consideration. Several lines of evidence have indicated that protein disulphide isomerase (PDI) (EC 5.3.4.1) plays an important role in the formation of the native disulphide bonds of proteins both *in vivo* and *in vitro* due to its ability to catalyse thiol/disulphide interchange [2]. Therefore we are interested in studying its possible ability to facilitate the renaturation of recombinant proteins from inclusion bodies. Okumura et al. [3] first reported that PDI was capable of accelerating the refolding of recombinant pro-urokinase but failed to increase its overall yield. Here, we provide evidence to demonstrate that PDI is able to enhance the renaturation efficiency of recombinant prochymosin, in addition to increasing its refolding rate.

MATERIALS AND METHODS

Preparation of inclusion bodies

E. coli JM105 cells harbouring an expression plasmid of a cDNA clone for bovine prochymosin, pTaAC5 [4], were grown in Luria–Bertani medium containing ampicillin (50 µg/ml) at 37 °C. After induction with isopropylthiogalactoside (IPTG) for 4.5 h at 42 °C cells were collected and suspended in STET buffer [8% (w/v) sucrose, 50 mM Tris/HCl, (pH 8.0), 20 mM EDTA, 5% (v/v) Triton X-100] at 4 °C overnight. The cell suspension was sonicated and centrifuged. The pellets were recovered as inclusion bodies.

Solubilization and renaturation

The procedure developed by Marston et al. [5] was followed basically except that PDI was included in the refolding system. In brief, inclusion bodies were dissolved in buffer A [50 mM Tris/HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA] containing 8 M

urea. For the inclusion bodies from every 0.1 g wet wt. of cells 1 ml of buffer was used. After 2 h at room temperature the solution was centrifuged at 10000 g for 10 min and the supernatant was diluted with 11.5 vol. of phosphate buffer [50 mM KH₂PO₄ (pH 11), 1 mM EDTA, 50 mM NaCl] supplemented with 25 µg of PDI for every 290 µg of inclusion body protein and left at room temperature for 60 min. Finally, the solution was adjusted to pH 8.0 with 0.1 M HCl, maintained at room temperature for 60 min and dialysed against buffer B [20 mM Tris/HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA] overnight at 4 °C. The dialysate was acidified to pH 2, incubated for 2 h at room temperature and then adjusted to pH 6.3. After incubation for 1 h the milk-clotting activity was measured by the method of Emtage et al. [6]. 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 in relation to prochymosin present in inclusion bodies, as judged by gel scanning.

Isolation of PDI

PDI was purified to homogeneity, as judged by SDS/PAGE, from bovine liver according to the method of Hillson et al. [7]. The activity of PDI was assayed by measuring the re-activation of sulphonated RNAase [8]. Briefly, an appropriate amount of PDI was incubated with sulphonated RNAase [pretreated with dithiothreitol (DTT)] in 100 µl of 50 mM Tris/5 mM EDTA buffer, pH 7.5, at 30 °C for 3 min. The activity of re-activated RNAase in a 50 µl aliquot was determined spectrophotometrically in 1 ml of 50 mM Tris/25 mM KCl/5 mM MgCl₂ buffer, pH 7.5, containing 500 µg of RNA at 37 °C for 3 min. One unit of PDI activity was defined as the amount of enzyme catalysing re-activation of sulphonated RNAase at a rate of 1 RNAase unit per min. One RNAase unit is defined as the amount of the enzyme degrading RNA and leading to the increase of one unit of A₂₆₀/min. The specific activity of purified PDI was 2.2 × 10⁵ units/g under these assay conditions.

Protein determination

The amount of protein in inclusion bodies or in PDI was determined by the method of Lowry et al. [9].

RESULTS AND DISCUSSION

pH dependence of renaturation

It has been proved that the renaturation of prochymosin polypeptide is highly pH-dependent [5]. The alkaline pH is critical for facilitating the conversion of the urea-unfolded state into the alkaline-unfolded state, which refolds readily leading to the increase of active products [10]. On the other hand, PDI was generally used to catalyse thiol/disulphide interchange at pH 7.8 [2]. To optimize the conditions for refolding the effect of pH on the renaturation of recombinant prochymosin in the presence of

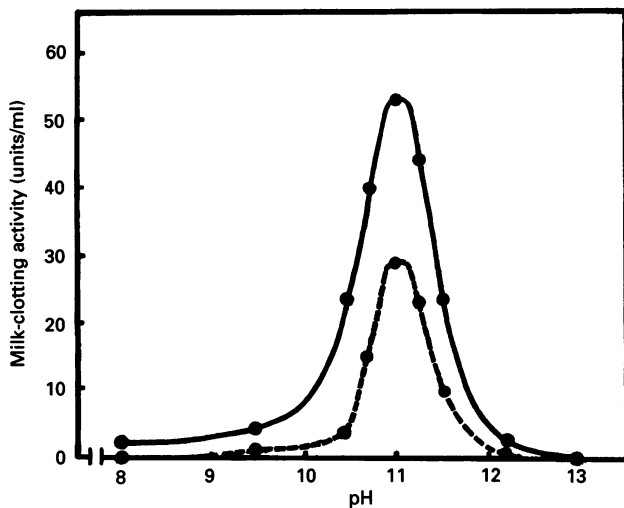


Figure 1 Effect of pH on the renaturation of prochymosin

Solubilized inclusion-body solution (80 μ l; 290 μ g of protein) was diluted with 920 μ l of phosphate buffer (50 mM KH_2PO_4 , 1 mM EDTA, 50 mM NaCl adjusted to different pHs as indicated) containing 25 μ g of PDI and incubated at room temperature for 1 h. At the end of the incubation period the pH dropped by 0.2–0.3 unit, the refolding mixture was then adjusted to pH 8, incubated at room temperature for 1 h and dialysed against buffer B overnight at 4 $^\circ\text{C}$. Samples were acidified and assayed for milk-clotting activity as described in the Materials and methods section. Activities were expressed as units/ml of refolding mixture. (—), PDI present; (---), PDI absent.

Table 1 Stability of oxidized PDI at pH 11

Homogeneous PDI was dissolved in 50 mM Tris/HCl buffer (pH 8.5). The enzyme solution was adjusted to pH 11 and preincubated at 25 $^\circ\text{C}$. Aliquots were removed and assayed for PDI activities re-activating sulphonated RNAase and denatured prochymosin under conditions as described in Materials and methods section after preincubation for 45 min and 90 min.

Duration of preincubation at pH 11 (min)	Retained PDI activity	
	Re-activation of sulphonated RNAase (RNAase activity, % of original activity)	Renaturation of prochymosin (milk-clotting activity, units/ml)
0	100	50.0
45	50	40.7
90	0	30.8
Without PDI	—	30.8

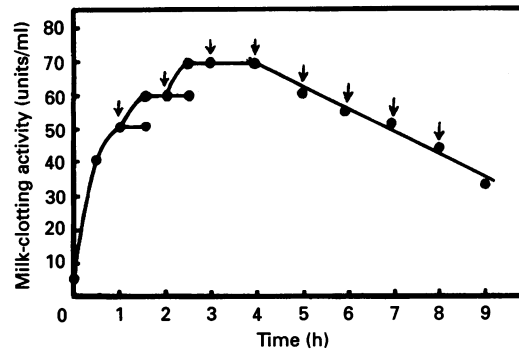


Figure 2 Effect of supplementation of PDI on the renaturation of prochymosin

The procedure described in the legend of Figure 1 was followed except that (1) the refolding solution was left at room temperature for the various times indicated; (2) the supplemented PDI (25 μ g for every 1 ml of refolding solution) was added once every hour during incubation, as indicated by the arrows; and (3) the milk-clotting activity was determined at different time intervals indicated by the data points.

PDI was investigated. As shown in Figure 1, at pH values of about 8 or 9 in the absence of PDI no active chymosin was detected, indicating that the unfolded polypeptide itself was unable to refold correctly and to undergo further autoactivation. However, when PDI was supplemented, the milk-clotting activity was apparent, though rather low. With an increase in pH the active protein steadily increased to a maximum at pH 11. This result demonstrated that PDI was unequivocally capable of facilitating the formation of the native conformation of prochymosin at the pH tested.

To our knowledge the pH used for PDI-catalysed reactions has never exceeded 8.7. It seemed surprising to find an optimum pH as high as around 11. To verify this finding in more detail the following experiments were undertaken. First we confirmed that the enhancement of the renaturation of prochymosin resulted from the catalytic function of PDI, rather than some general effect of the protein. When boiled PDI was included in the renaturation system no increase in the activity of chymosin was detected compared with spontaneous renaturation. The rate of enhanced formation of native prochymosin increased linearly with the quantity of PDI over the range 0–20 μ g (data not shown). Secondly, we examined whether PDI maintained its activity at alkaline pH values. The stock solution of PDI was incubated at pH 11 and 25 $^\circ\text{C}$. At various time intervals aliquots were withdrawn to determine the degree of re-activation of sulphonated RNAase and denatured prochymosin under standard conditions. Although the assay pHs are different (pH 7.5 versus pH 11), there is good correlation between them (Table 1). Incubation for 45 min results in only partial loss of activities towards both substrates, suggesting that PDI is active at pH 11 within a period of approx. 1 h. The results illustrated in Figure 2 support this argument. Every additional supplementation of PDI leads to a further increase of renaturation until the third supplementation. At later time intervals, the resultant chymosin activity gradually decreases even after replenishment of PDI. This is probably due to the inactivation of prochymosin, as Foltmann [11] has proved that prochymosin is unstable at alkaline pH values. Thirdly, we ascertained at which stage of the refolding course PDI exerted its function. As indicated in the Materials and methods section, the refolding process consists of two stages, pH 11 and pH 8. It is necessary to clarify this question. To do

Table 2 Effect of PDI added at different stages of the refolding process on renaturation of prochymosin

The refolding process consisted of two stages as described in the legend of Figure 1. Refolding at pH 11 and neutralization followed by dialysis at pH 8, refer to the first and second stages respectively. Milk-clotting activities are given as the means of triplicate assays \pm S.D.

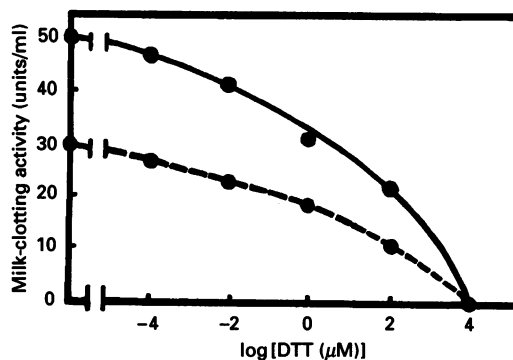
Addition	Milk-clotting activity (units/ml)
No PDI	32.5 (\pm 1.0)
PDI added at the first stage	51.0 (\pm 2.0)
PDI added at the second stage	39.0 (\pm 1.0)

this, aliquots of PDI were added to the renaturation system at different stages; Table 2 shows that the clotting activity of the sample supplemented with PDI at pH 8 is higher than that of the control (with no PDI) but less than that of the system containing PDI throughout. Taking the half-life of PDI at pH 11 ($t_{1/2} = 45$ min) into consideration, it is reasonable to consider that after incubation for 1 h at pH 11 PDI is unlikely to play an important role in the second stage.

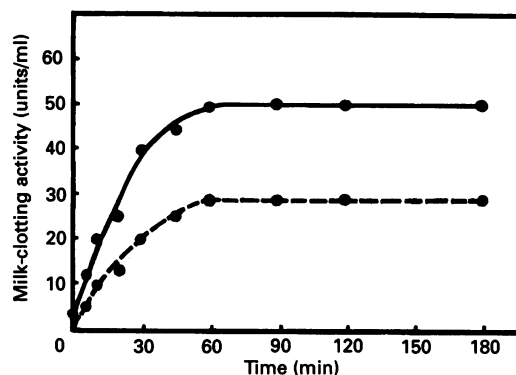
All the results mentioned above fully demonstrated that thiol/disulphide interchange of prochymosin, for both the uncatalysed and PDI-catalysed reaction, occurs mainly at values of approx. pH 11. Since the essential thiol groups of PDI, Cys-35 and Cys-379, have an unusually low pK of 6.7 [12], it is unlikely that, compared with a pH value of 8, an alkaline pH would significantly enhance their reactivity during catalysis, therefore other factors must be considered. It is widely accepted that for a disulphide bond to be formed the corresponding cysteine residues must have their α carbon atoms within 4–9 Å of each other and the adjoining backbones must also be in the proper orientation. Therefore, it is reasonable to assume that the alkaline pH which is favourable for spontaneous refolding of prochymosin, may be helpful in providing the proper substrate for PDI to initiate catalysis. As for the lower pH, which is unfavourable for spontaneous refolding, PDI may assist the correct pairing of disulphide bonds by changing the substrate conformation in addition to catalysing thiol/disulphide interchange, although the efficiency is not high enough. This is consistent with the hypothesis of Creighton et al. [13]. In this situation we prefer to conclude that (1) PDI is active over a broad range of pH values and (2) that the optimum conditions for PDI catalysis differ from protein to protein. As a catalyst PDI preferentially enhances the rate of reaction under conditions favourable to spontaneous refolding.

Effect of DTT on renaturation

Since the thiol form of PDI is essential for catalysing thiol/disulphide exchange [14], PDI is usually assayed in the presence of thiol reagent [7]. Moreover whether cysteine residues are linked in disulphide bonds depends not only on the conformation of the protein but also on the redox potential of the environment. Therefore, it is necessary to enquire into the effect of thiol reagent on the renaturation of prochymosin catalysed by PDI. Figure 3 indicates that like the uncatalysed reaction the PDI-catalysed reaction does not require the presence of DTT and is in fact inhibited by DTT. Marston et al. [5] reported that thiol reagents such as DTT or mixtures of reduced and oxidized glutathione were not required for spontaneous renaturation of prochymosin, and proposed that in the absence of the exo-

**Figure 3** Effect of DTT on the renaturation of prochymosin

The procedure described in the legend of Figure 1 was followed except that the pH value was 11 and different concentrations of DTT were supplied, as indicated, at the first stage of the refolding process. (—), PDI present; (---), PDI absent.

**Figure 4** Kinetics of renaturation of prochymosin

The procedure described in the legend of Figure 1 was followed except that the refolding solution was left at room temperature at the first stage for various times as indicated. (—), PDI present; (---), PDI absent.

genously added thiols the free thiol groups in prochymosin might promote the thiol/disulphide interchange and in turn the rearrangement of disulphide bonds. According to the result described here, it can further be argued that the free thiol groups in prochymosin are sufficient to reduce the inactive disulphide form of PDI to its active form; moreover, the more oxidizing standard redox potential of the PDI active-site [15] is probably responsible for it readily undergoing reduction.

Enhancement of prochymosin renaturation by PDI

On the basis of the optimum conditions for PDI-catalysed reaction (as determined above) the time course of prochymosin renaturation in the presence of PDI was monitored. From Figure 4 it is evident that both the refolding rate and renaturation efficiency are boosted by the addition of PDI. Forty units of active chymosin were recovered in 30 min, while in the absence of PDI it took 1 h to attain the recovery level of 30 units. Although the catalytic efficiency is unspectacular, it is consistent with the inherent property of PDI and comparable with the data

of catalytic-centre activities for re-activation of scrambled RNAase [16] and for the oxidation of reduced bovine pancreatic trypsin inhibitor [13]. The lower catalytic activity of PDI is believed to be due to the reactivation of scrambled protein requiring formation of a great number of native disulphide groups via an unknown number of individual thiol/disulphide exchange steps.

Considerable evidence has indicated that protein concentration is of great importance in determining the refolding efficiency. The higher the concentration, the lower the yield of active protein. From a practical point of view it is difficult to handle large amounts of solution with low concentrations of protein for further processing. Therefore it is rational to choose conditions which may permit proper refolding at higher concentrations of protein. Experiments indicated that PDI was capable of assisting prochymosin renaturation at a protein concentration of 0.5 mg/ml (containing 40% prochymosin), achieving a renaturation efficiency of 40–50%. Most likely this is due to the acceleration of thiol/disulphide interchange catalysed by PDI enhancing formation of the native conformation of prochymosin, leading to decrease of self-aggregation or aggregation with contaminating proteins during refolding.

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