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Studies on the Binding of α–Crystallin to Recombinant Prochymosins and Chymosin

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Purpose: To further investigate the binding of α–crystallin to other proteins as part of its chaperone-like activity, we studied interactions of α–crystallin with recombinant calf prochymosins and chymosin.

Methods: Recombinant calf prochymosin B and one C-terminal mutant (PC+2, with two additional residues, Histidine-Glycine) were expressed as inclusion bodies in E. coli. Native and mutant proteins were denatured in 8 M urea before being refolded by dilution slowly in phosphate buffer, pH 10.7, in the presence and absence of α–crystallin at different concentration ratios. After dialysis, the folded proteins were converted to the active chymosin by acidification. The resulting enzyme activities at standard protein concentrations were determined by a microtitre milk-clotting assay.

Results: Refolding of 1.0 mg/ml of protein inclusion bodies diluted in phosphate buffer at 0.32 M urea in the presence of α–crystallin resulted in enhanced chymosin activity relative to the control without α–crystallin. When lower inclusion body concentrations were used, enzyme activity was not enhanced relative to the control. The mutant enzyme (PC+2) showed no conversion to the active form in the presence of α–crystallin. α–Crystallin formed a complex with refolded prochymosin, as well as with prochymosin during refolding, but not with active chymosin. Removal of the 43-residue propeptide resulted in loss of α–crystallin binding. The addition of two residues (Histidine-Glycine) to the prochymosin C-terminus resulted in precipitation of the mutant prochymosin-α-crystallin complex and loss of enzyme activity.

Conclusions: Our experiments show that even under stringent refolding conditions, α–crystallin, which retains its gross oligomeric integrity, can bind to unfolded proteins in inclusion bodies and enhance the apparent yield of chymosin activity from high concentrations of inclusion bodies. α–Crystallin shows some specificity for binding to its target protein; this specificity may be based on steric considerations as well as residue-specific interactions.

Recombinant proteins can be produced at high level in various heterologous expression systems. However, in bacterial systems such as *E. coli*, these highly expressed proteins may be produced in the form of insoluble inclusion bodies, therefore a refolding step may be essential to obtain active proteins [1]. Among various refolding mechanisms studied, molecular chaperones have been considered to play an important role in the folding of proteins in vivo.

α-Crystallin is one of the major proteins (30-40% of soluble proteins) of the eye lens. It is considered necessary for the stability and refractive properties of the lens [2]. It is composed of 2 subunits, αA and αB, of approximately 20 kDa in mass each, but the protein exists as a large aggregate of varying mass between 300 and 1000 kDa [3]. There is close sequence homology between α–crystallin and the small heat shock proteins (HSP) [4,5]. α–Crystallin suppresses aggregation of various enzymes and structural proteins [6-14]. Unlike GroEL, in an α–crystallin system, the release of the folded or refolded protein is not governed by the hydrolysis of ATP nor the binding of the smaller co-chaperone, GroES [6,8]. Like GroEL, α–crystallin interacts with a variety of unfolded proteins to form soluble, high-molecular weight (HMW) complexes. However, it does not recognize denatured proteins at early stages of the refolding pathway [7], nor does it interact with unfolded, hydrophobic, but stable proteins (e.g. reduced and carboxymethylated α–lactalbumin and α–casein) [13,14].

In this paper we report on functional investigations into the binding and chaperone-like action of α–crystallin on the refolding of recombinant prochymosin. Prochymosin is a zymogen of chymosin, which is a milk-clotting enzyme with low proteolytic activity and an important enzyme for the cheese industry. Chymosin activity determinations rely mainly on determination of clotting activity on milk. The initial action of the enzyme is restricted to the cleavage of the peptide bond between phenylalanine 105 and methionine 106 of κ-casein [15,16]. Generally, the activity of chymosin is expressed as a value of milk clotting activity per proteolytic activity (C/P).

METHODS

Bacterial strains and Plasmid— The competent cells of *E. coli* strain DH5α (F-, ϕ 80 dlacZδ M15 δ (lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK-, mK+) supE44 λ thi-1 gurA96 relA1) obtained from Bethesda Research Laboratories (Gibco-BRL, Paisley, UK) were used as the host for all DNA

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manipulation. The recipient strain for expression of prochymosin was *E. coli* BL21(DE3) [F-, ompT hsdSB(rB-, mB-) dcm gal(DE3)] from Novagen (Cambridge, U.K.), as described [16]. The complete calf prochymosin B cDNA was obtained as an insert in the plasmid pGRG3 [17], kindly donated by Dr. Ward, Genecor Technology (California, USA). Other strains and media used were as described [16].

The construction of the expression plasmids— We have constructed and expressed a wild-type calf prochymosin (PC) and a C-terminal mutant with two additional amino acid residues, histidine and glycine (PC+2), in *E. coli*. Preparation of competent cells and transformations into *E. coli* DH5α cells were as described [16,18]. PCR amplification using primers containing NcoI sites was used to prepare native and mutant amplicons of calf prochymosin B. After PCR amplification and subsequent purification, the calf prochymosin B amplicons were blunt-ended with Klenow fragment DNA polymerase, phosphorylated with DNA kinase, then cloned into EcoRV-cut pBlueScript SK. The pBS-SK was then cut with NcoI, liberating calf prochymosin B cDNAs flanked by NcoI sticky ends. These were then inserted into the NcoI site of pET 3D [19]. Recombinant plasmids were identified and orientated by Pst1 digests. Plasmid DNA was propagated and purified by standard methods [18].

Preparation of inclusion bodies— Inclusion bodies of wild-type prochymosin B and its mutant (PC+2) were produced. *E. coli* BL21 (DE3) cells carrying pET-PC plasmid were aerobically grown at 37 °C in Luria broth supplemented with carbenicillin (100 μg/ml). When the culture reached an OD_{600} of 0.6-1.0, target protein production was induced by the addition of IPTG (isopropyl-β-D-thiogalacto-pyranoside) at a final concentration of 0.4 mM. After an 8 h induction period, the cells were harvested by centrifugation at 6,500 g for 10 minutes. After cell lysis by the lysozyme/deoxycholate procedure [20], the inclusion body pellets were washed and centrifuged as before. The resultant pellets were purified using 0.5% Triton X-100. Pellets were resuspended in 9 volumes (v/v) of buffer containing 0.5% (v/v) Triton X-100 and 10 mM EDTA. After incubation for 5 min at room temperature, washed inclusion bodies were collected by centrifugation

The expression system resulted in prochymosin production of about 30% that of the total protein. Further purification from the inclusion bodies resulted in prochymosin of about 80-90% purity, estimated from SDS gel electrophoresis. Protein concentrations were determined using the BCA (bicinchoninic acid) Protein Assay (Pierce, Rockford, IL, USA) method on a microtitre plate [16].

Solubilization and renaturation— The procedure developed by Marton et al [20] was followed except that α–crystallin was included in the refolding system in some experiments. Washed inclusion pellets were solubilized in 8 M urea buffer, pH 8. The urea mixture was incubated at 25 °C for 1 h before the insoluble molecules were removed by centrifugation. The urea solution was then diluted in a high pH buffer (pH 10.7) for renaturation of prochymosin. In experiments without the addition of α–crystallin, we found that the yield of enzyme was maximal when the urea mixture was diluted 25 fold (0.32 M final urea concentration), and the final concentration of protein 0.25 mg/ml [15]. Using this procedure, approximately 3.0 mg of enzymatically active chymosin was obtained from 1 g wet weight biomass.

After the insolubilization in 8 M urea, the inclusion body solution was diluted with phosphate buffer pH 10.7, bovine α–crystallin (a mixture of αA- and αB-crystallin) [18] was added in varying ratios to the concentration of proteins in the phosphate buffer, as shown in Figure 1. The solution was incubated at 25 °C for 1 h and then adjusted to pH 8 and incubation was continued at 25 °C for 1 h. The solution was transferred to dialyze against buffer (20 mM Tris/HCl pH 8.0, 50 mM NaCl, 1 mM EDTA) at 4 °C overnight. The folded prochymosin were activated by acidification to pH 2, and

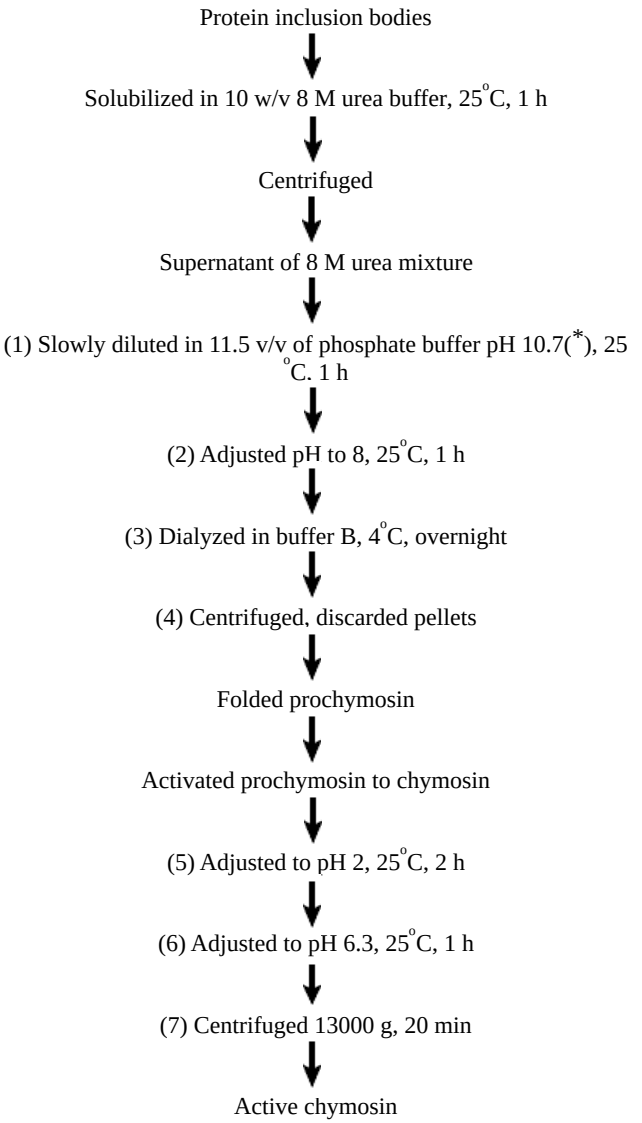

Figure 1. Refolding and activation of prochymosin and chymosin. The (*) indicates the step of the refolding process where α–crystallin was added, after dilution with phosphate buffer. Steps 1 through 7 are the refolding process. See text for details.

incubated for 2 h at 25 °C. Then the pH was adjusted to 6.3 and the mixture incubated for 1 h at 25 °C. The refolding yield was determined by a microtitre-plate milk-clotting assay (see below). Activities were expressed as units/mg of refolding mixture.

Milk-clotting activity assay— This was measured by the method of Emtage et al. [21], modified as follows. Fifty µl volumes of 0.05 M Na_2HPO_4/NaH_2PO_4, pH 6.3 were placed into the wells of a microtitre plate. A 24% (w/v) solution of dried skimmed milk in 0.04 M $CaCl_2$ was prepared and gently mixed for 15 min. During this period, 50 µl of sample was placed into the first well and was serially diluted by a factor of 2 using a Finnpipette Digital 50-200 µl 8 channel pipette (Labsystems (UK) Ltd.). A known mass of authentic purified calf chymosin (Sigma, UK) was used as a standard for the assay and buffer was used as a negative control. The microtitre plate was covered with Nesco film (Fisons Scientific Apparatus, Loughborough, UK) to prevent evaporation and incubated with the milk solution for 15 min at 37 °C. The film was removed and 50 µl of milk solution was pipetted into each well and covered with Nesco film. The microtitre plate was incubated for a further 25 min at 37 °C. The Nesco film was removed and the plate was inverted on tissue paper. Following plate inversion, milk that had not clotted drained out to leave the wells containing clotted milk. The number of clotted wells for each sample could then be compared with that of the standard. One unit of activity is equivalent to the activity of 1 µg of the authentic calf chymosin.

Polyacrylamide gels and Western blotting— These were performed as described previously [16,18,23].

RESULTS

Stability of α–crystallin in refolding conditions— The stability of α–crystallin throughout the refolding conditions was examined. α–Crystallin was dissolved in phosphate buffer (pH 10.7) to give a final concentration of 1 mg/ml and assayed for refolding. It was observed that ≤10% of α–crystallin precipitated out at the refolding stage and at the activation stages; however, protein analysis and Western blotting showed that the majority of the protein was still soluble in the final solution. The stability of the α–crystallin is shown in Figure 2. The effect of α–crystallin on the milk-clotting activity was also examined. The assay used microtitre-plate procedures (see above) with α–crystallin as the sample and authentic chymosin as a standard. α–Crystallin did not influence milk-clotting activity.

Milk clotting activity of native and mutant chymosins— Comparison of the milk-clotting activity of the native chymosin B and of the mutant after being refolded and activated (Figure 3) showed that the addition of histidine and glycine residues at the C-terminus reduced the catalytic activity (C) of this enzyme. The activity decreased from 909.1 units/mg to 151.5 units/mg (1 unit of the activity is the activity equal to of 1 µg of authentic chymosin). Proteolytic activity (P) of the mutant was also lower than of the native, from 149.3 units/mg to 50.5 units/mg (1 unit of the activity is the amount of enzyme that causes an increase in the absorbance of the haemoglobin filtrate of 1.0 at 280 nm). Therefore, the C/P ratio of the mutant was decreased by half from the wild-type recombinant enzyme. In steady-state kinetic experiments for the hydrolysis of the hexapeptide, Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe, by the recombinant wild-type and mutant chymosins [16], there were no significant differences between the K_m values for either protein (0.38 mM recombinant wild-type, 0.67 mM PC+2). There was a difference, however in k_{cat} values; that for the PC+2 mutant was 2.53 s^{-1} relative to 18.9 s^{-1} for the recombinant wild type enzyme [16]. These results suggest that the structure of the PC+2 mutant may have been altered in a way that did not greatly affect substrate binding, but significantly lowered catalytic activity.

Effect of α–crystallin on the refolding of wild-type prochymosin— The effect of α–crystallin on the refolding of

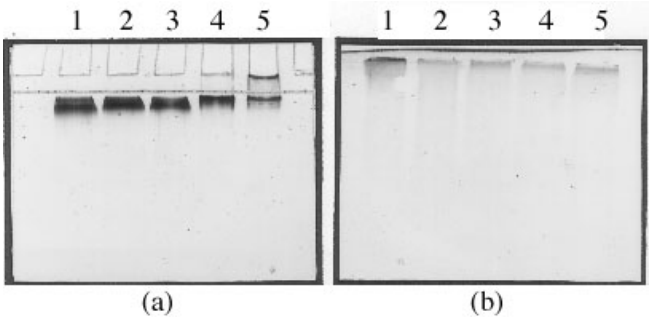

Figure 2. Stability of α–crystallin under refolding conditions. Panel (a): Non-denaturing PAGE gel; Panel (b): Western blot probed with polyclonal anti-α-crystallin antibodies. Lane 1, α–crystallin after incubation in phosphate buffer, pH 10.7; Lane 2, α–crystallin after incubation in phosphate buffer, pH 8.0; Lane 3, α–crystallin after dialysis; Lane 4, α–crystallin after incubation at pH 2.0; Lane 5, α–crystallin after incubation at pH 6.3 and centrifugation.

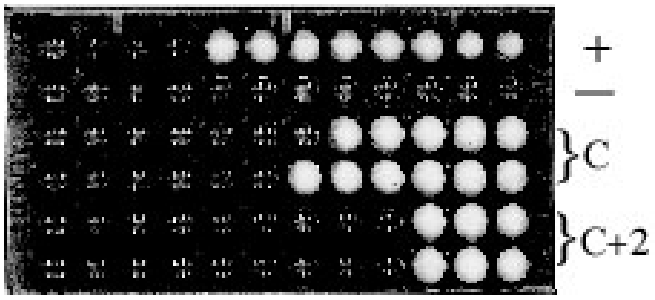

Figure 3. Comparison of milk clotting activity of chymosin and mutant using microtitre plate assay. Twenty-five µl of activated chymosin and mutants were 1:1 serial diluted with 12% skim-milk solution and incubated at 37°C, 25 min. Activity calculates from the number of clotted-milk-wells after inverting the plate. Row " +": 4 µg of authentic chymosin in the first well, Row "-": negative control, Row "C": wild-type chymosin, Row "C+2": the mutant PC+2-chymosin. See text for details.

wild-type prochymosin is shown in Figure 4. Each plate shows the activity of refolded recombinant prochymosin at different ratios of inclusion body protein to α–crystallin. Plate A is using 1.0 mg/ml starting inclusion body concentration, plate B, 0.63 mg/ml, and plate C, 0.25 mg/ml. Assays used the same initial protein concentration. Ratios of inclusion body-to-α–crystallin were from 1:0 to 1:4. The greatest yield of active prochymosin was from a starting inclusion body concentration of 1.0 mg/ml, with an inclusion body: α–crystallin ratio of 1:2 (Figure 4a). This activity was higher than any found by the optimal refolding procedure found in our experiments (see Methods and Chitpinityol & Crabbe [15]). At higher concentrations of α–crystallin there was no increase in reactivation yields. However, when the starting concentration of proteins was 0.63 mg/ml (found to be the optimal inclusion body concentration in the absence of α–crystallin), or 0.25 mg/ml, α–crystallin had no effect on the yield of active prochymosin (Figure 4b and c). These results indicate that α–crystallin aided the refolding of prochymosin at high concentrations of inclusion bodies.

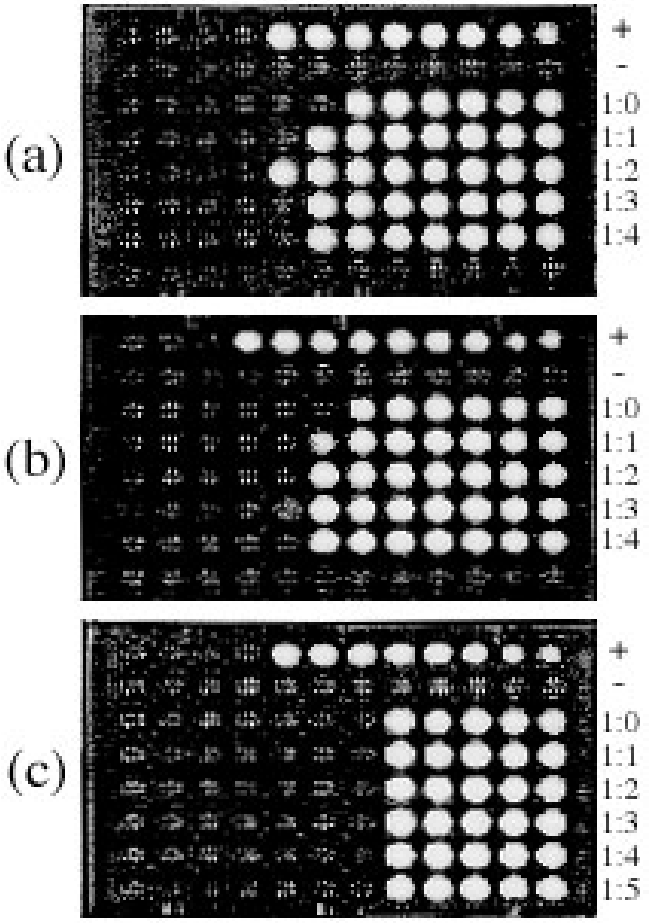

Figure 4. Effect of α–crystallin on the refolding of prochymosin. The milk clotting assay demonstrates the effect of concentrations of α–crystallin added on the refolding of wild-type prochymosin at different initial *E. coli* protein concentrations in phosphate buffer. The milk-clotting assay is described in Materials and Methods. Panel (a): 1.0 mg/ml initial protein. Panel (b): 0.63 mg/ml initial protein. Panel (c): 0.25 mg/ml initial protein. Row " +": authentic chymosin, 4 μg in the first well. Row "-": negative control. For the remaining rows, the amount of α–crystallin added is shown as the ratio of initial protein to α–crystallin (numbers to the right of figures.) Enzyme activity is shown by the white wells containing clotted milk; the larger the number of wells containing clotted milk, the greater the enzyme activity, and so the greater the efficiency of refolding. See text for details.

Interactions between α–crystallin and prochymosin— The studies on the interaction of α–crystallin with unfolded and folded prochymosin (Figure 5 and Figure 6) showed the formation of a soluble HMW complex. The non-denaturing gel showed that in the refolding system containing α–crystallin, a band corresponding to prochymosin was not observed, only a large molecular mass of protein was observed on the top of the gel (see Figure 5, "PC-a"). This large molecular mass was absent in the normal refolding system. Since α–crystallin formed a large macromolecular complex with a molecular mass of approximately 800 kDa visible at the top of the non-denaturing gel, the complex of this interaction could not be identified from its mass (see Figure 5, "a"). Therefore, the interaction between prochymosin and α–crystallin was analyzed by Western-blotting using anti-chymosin antibody and anti-α–crystallin antibody. The large molecular mass band showed sensitivity to both anti-chymosin and anti-α–crystallin antibodies suggesting the interaction of prochymosin or chymosin with α–crystallin. Using this method, it could be demonstrated that this interaction occurred with both unfolded and folded prochymosin. However, α–crystallin did not form a complex with active chymosin.

Interestingly, α–crystallin was cleaved during the activation stage (see Figure 5, "C-a"). The absence of the band corresponding to α–crystallin and the presence of smaller proteins shown in Figure 5 "C-a" were clearly observed using SDS-PAGE gels and non-denaturing gels. This hydrolysis did not occur when α–crystallin was incubated with active chymosin (see Figure 6, "C+a"). The activation steps did not alter α–crystallin oligomerization (Figure 2), so our result suggests that the α–crystallin structure may have been subjected to a conformational change whilst it was bound to prochymosin, thus rendering it susceptible to digestion by the enzyme. This conformational change did not affect, however, the chaperone-like activity of the α–crystallin when incubated with 1.0 mg/ml inclusion bodies (Figure 4a).

Effect of α–crystallin on the refolding of mutant prochymosin— Inclusion bodies of the mutant prochymosin PC+2 were refolded in the presence and absence of α–crystallin. The refolding experiments were performed in the same manner as for the native protein. The majority (>80%) of the protein formed a PC+2-α–crystallin complex during activation that precipitated out of solution, and no enzyme activity could be detected by the milk clotting assay. This result suggests that the 2 additional amino acids (His and Gly) played a role on the binding of this zymogen to α–crystallin, since under identical conditions there was no precipitation or loss of activity with the native recombinant enzyme. Indeed, addition of α–crystallin resulted in increased activity of refolded enzyme at 1.0 mg/ml inclusion body concentration (Figure 4a). Histidine can be uncharged or positively charged

depending on its local environment, thus altering the net charge on the protein. The additional histidine may be involved in the binding of the two proteins, thus strengthening their interaction, and so possibly preventing release of α-crystallin during the acidic activation.

DISCUSSION

The in vitro activity measurements that characterize a protein as a molecular chaperone include (i) the ability to suppress aggregation and assist refolding of non-native proteins and (ii) the ability to protect from aggregation during protein denaturation under stress conditions. In this study, our experiments demonstrated that α–crystallin could increase the yield of active chymosin (Figure 4a), presumably by binding to unfolded proteins, and so influencing the folding process. This result is in accord with data from Jakob et al. [6], where α–crystallin increased the refolding yields of citrate synthase and α–glucosidase. In those experiments, as in ours, the refolding process was not carried out under optimum conditions for the target proteins' refolding. Carver et al. [13,14] have suggested that α–crystallin acts as an anionic surfactant by interacting with the partially unfolded form of the protein about to precipitate out of solution. By binding to the unfolded proteins, α–crystallin could suppress the aggregation of those proteins by heat or chaotropic agents [10,24].

In our study, the formation of HMW complexes of α–crystallin both with unfolded and folded prochymosin appear to be ATP-independent, as suggested previously [6]. The capacity to bind unfolded proteins is limited by the amount of α–crystallin and the number of sites for binding per molecule [11,25]. However, in our study, the complex was destroyed when the zymogen was activated to active enzyme, chymosin. α–Crystallin did not interact with active chymosin. Similar results were obtained from work previously reported by Carver et al. [13].

The substrate specificity of α–crystallin is significantly different from other chaperones. The majority of known chaperones can recognize and bind non-native structures formed in vitro during protein folding reactions [11,25,26]. No affinity for the intermediates formed during our experiments was observed during the refolding reaction in vitro. Similar results were shown by Das & Surewicz [27] using rhodanese as a model substrate protein.

It was notable that the C-terminal addition prochymosin mutant, PC+2, behaved in a different way from the native protein when incubated in the presence of α–crystallin. PC+2 was bound to α–crystallin during the refolding process and formed a complex. However, it seemed that PC+2 was not

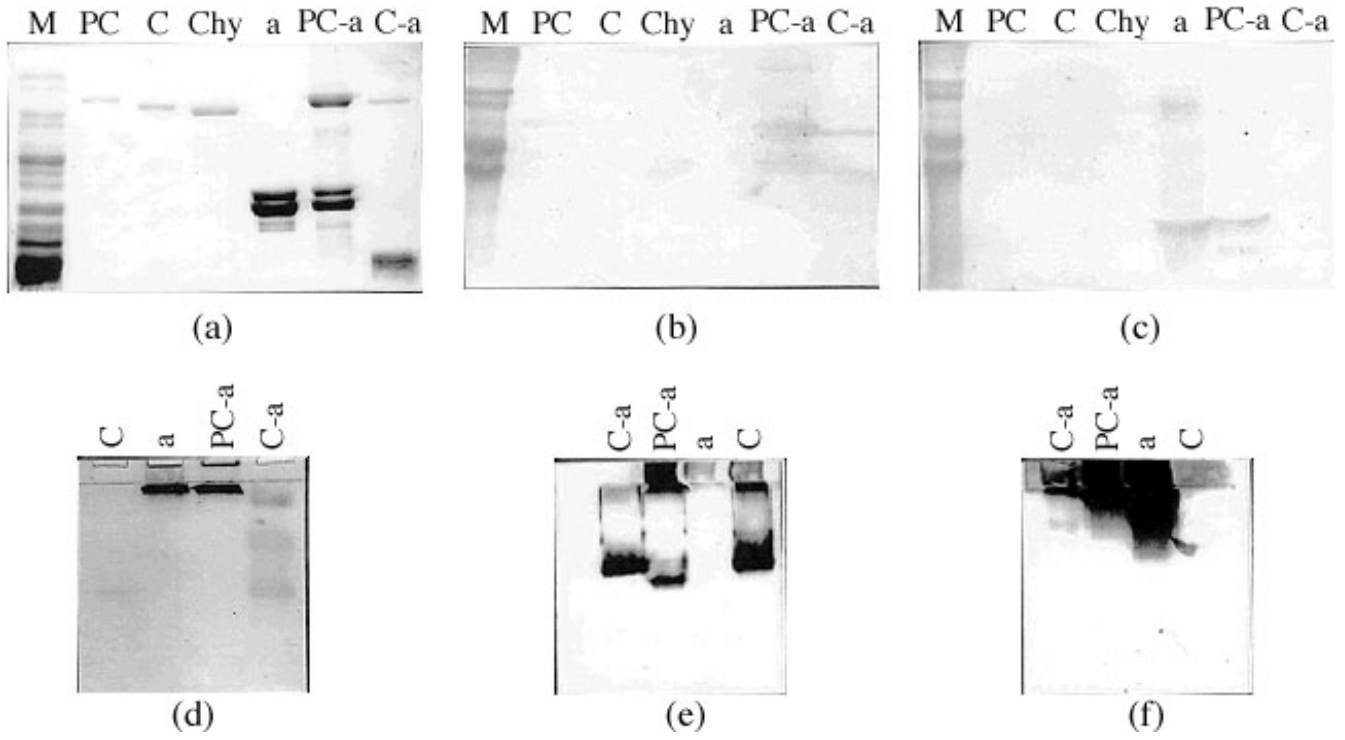

Figure 5. Refolding of wild-type prochymosin in the presence of α–crystallin. Coomassie blue stained SDS-PAGE and Western blot analysis of the refolding of wild-type prochymosin in the presence of α–crystallin. The experiment of panel (a) is identical to that of (b) and (c) whereas (d) is identical to (e) and (f). (a): 10% SDS-PAGE. (d): 10% nondenaturing gel. (b), (e): Western blots probed with polyclonal antichymosin antibodies. (c), (f): Western blots probed with polyclonal anti-α-crystallin antibodies. The lanes are: molecular weight marker ("M"), authentic chymosin ("Chy"), α–crystallin ("a"), prochymosin ("PC"), chymosin ("C"), prochymosin refolded with α–crystallin ("PC-a"), and chymosin from prochymosin folded with α–crystallin ("C-a").

released from this HMW complex and could not convert into active chymosin. This suggests that the inserted amino acid residues may play a part in this interaction. It may have increased the strength of the bonding between α–crystallin and PC+2 and/or modified the structure of the complex such that it was not suitable for the autocatalytic conversion. The histidine residue may play the major part of this affect by which it could be uncharged or positively charged depending on its local environment and may alter the net charge of the protein. The increasing interactions may have been too strong and so did not allow α–crystallin to be released during the acidic activation, resulting in precipitation of, the complexed proteins during the neutralization step. The hydrophobic rich region and the charged lysine groups at the C-terminus of α–crystallin have been shown to be important in protein binding and chaperone-like activity of α–crystallin [18]. Itzhaki et al. [28] have suggested that hydrophobic and positively charged side chains of the target protein tend to interact favorably with the bacterial chaperonin, GroEL whereas negatively charged side chains tend to resist. In addition, the refolding efficiency was reduced when the strength of GroEL-complexed protein increased.

Post-translational modification of α–crystallin causes protein crosslinking and loss of chaperone-like function; both can be prevented by ibuprofen [29]. We have suggested that the C-terminal arm of α–crystallin could interact with unfolded proteins via charge-charge interactions, and then link the substrate proteins further via hydrophobic interactions with the exposed phenylalanine-rich domain near the N-terminus [18]. This has been reinforced by recent findings on α–crystallin in human age-related nuclear cataract [30]. We found that the prochymosin-α–crystallin complex was destroyed during zymogen activation treatment which resulted in degradation of α–crystallin, and α–crystallin may bind to the pro-region (which is rich in basic residues) or to the substrate-binding pockets of prochymosin.

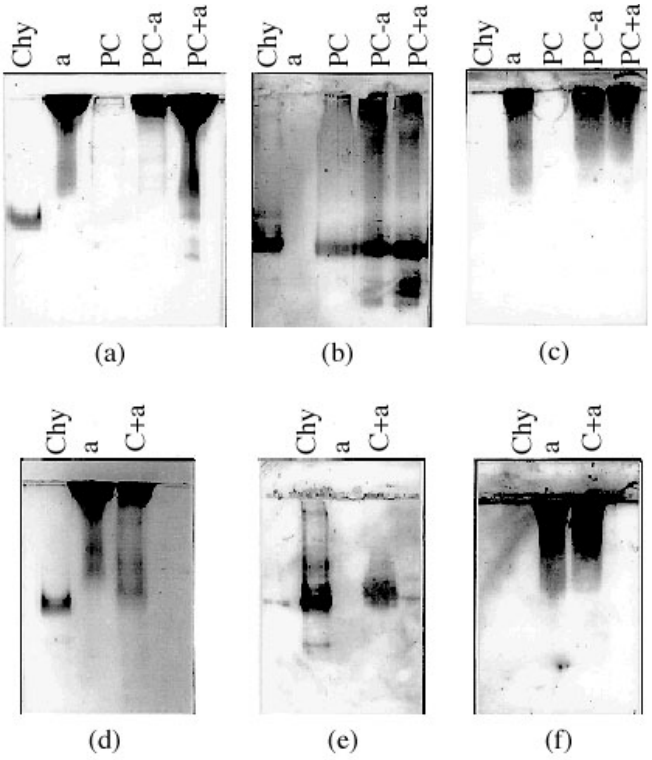

Figure 6. Binding of α–crystallin with folded prochymosin. The nondenaturing gel and Western blots demonstrate the binding of α–crystallin with folded prochymosin (a, b, c) and chymosin (d, e, f). α–Crystallin was added and incubated with the protein at 25°C overnight. (a), (d): Coomassie blue stained 10% nondenaturing gel. (b), (d): Western blots probed with polyclonal antichymosin antibodies. (c), (f): Western blots probed with polyclonal anti-a-crystallin antibodies. The lanes are: authentic chymosin ("Chy"); wild type prochymosin ("PC"); prochymosin refolded with α–crystallin, 1:4 ("PC-a"); folded prochymosin incubated with α–crystallin ("PC+a"); chymosin incubated with α–crystallin ("C+a").

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