Denaturation studies on natural and recombinant bovine prochymosin (prorennin)

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1. Prochymosin in solution in the presence of 8 M-urea is fully unfolded, as indicated by its fluorescence spectrum, fluorescence quenching behaviour and far-u.v. c.d. spectrum. 2. Equilibrium studies on the unfolding of prochymosin and pepsinogen by urea were carried out at pH 7.5 and pH 9.0. The results indicate that the stabilization energies of the two proteins are identical at pH 7.5, but that at pH 9.0 pepsinogen is significantly less stable than prochymosin. 3. Kinetic studies on the unfolding of prochymosin and pepsinogen indicate that the processes can be described by a single first-order rate constant, and that at any given value of denaturant concentration and pH the rate of unfolding of prochymosin is significantly greater than that of pepsinogen. 4. Unfolding of prochymosin by concentrated urea is not fully reversible, unlike that of pepsinogen. Kinetic analysis of the refolding of the proteins suggests the presence of a slow process following unfolding in urea; for pepsinogen this process leads to a slowly refolding form, whereas for prochymosin the slow process in urea leads to a form that cannot refold on dilution of the denaturant. 5. The results provide a rationale for an empirical process for recovery of recombinant prochymosin after solubilization of inclusion bodies in concentrated urea. 6. In all respects studied here, natural and recombinant bovine prochymosin were indistinguishable, indicating that the refolding protocol yields a recombinant product identical with natural prochymosin.

INTRODUCTION

Recombinant DNA technology has allowed a wide variety of mammalian proteins to be synthesized as recombinant products in microbial host cells. This has been particularly important for non-glycosylated proteins that are required on a bulk industrial scale, such as bovine growth hormone and prochymosin. Calf prochymosin, the zymogen of chymosin, a milk-clotting aspartic proteinase used in cheese production, has been studied here as a model of such proteins. Prochymosin cDNA has been cloned and expressed in *Escherichia coli* (Harris *et al.*, 1982; Emtage *et al.*, 1983); like many other animal proteins expressed in *E. coli*, recombinant prochymosin forms intracellular protein aggregates, referred to as inclusion bodies or refractile granules (Emtage *et al.*, 1983; Nishimori *et al.*, 1984; Schoemaker *et al.*, 1985). Formation of such aggregates can simplify the early stages of concentration and purification of the recombinant products, but, in general, problems are encountered in the recovery of biologically active proteins from them in acceptable yield. In the case of recombinant prochymosin, a successful solubilization and refolding protocol has been devised (Marston *et al.*, 1984); the inclusion bodies are solubilized and denatured in urea and then renatured by a process involving alkaline treatment at high protein dilution. Comparable methods have been devised for other recombinant proteins (reviewed in Marston, 1986), but no general systematic procedures are available. Solubilization/refolding protocols are individually tailored to the protein product, and the mechanisms underlying these protocols have rarely been described in depth.

In order to rationalize the empirical procedure devised for the recovery of recombinant prochymosin, it is essential to have some understanding of the unfolding of prochymosin induced by high concentrations of agents such as urea and by high pH. Little background work has been undertaken on this topic. We here present studies on the properties of prochymosin unfolded in urea, on the unfolding transition and its reversal, and on the

kinetics of unfolding and refolding. Since the unfolding and refolding of prochymosin have not previously been studied, we have included, for comparison, experiments on the homologous zymogen pig pepsinogen, which has been investigated extensively in this respect (Perlmann, 1963; Frattali *et al.*, 1965; McPhie, 1975, 1980, 1982; Ahmad & McPhie, 1978). The work demonstrates that prochymosin and pepsinogen differ in their rates of unfolding and in the processes that they undergo when maintained in concentrated urea; whereas pepsinogen after unfolding in urea slowly converts into a slow-refolding form, prochymosin slowly converts into a form that cannot refold simply by dilution of the denaturant. We further show that natural calf stomach prochymosin and recombinant prochymosin (recovered by solubilization and refolding from *E. coli* inclusion bodies) are identical in all the respects studied here. Preliminary accounts of some of this work have been presented (Sugrue *et al.*, 1985, 1988).

MATERIALS AND METHODS

Materials

Urea (AristaR) and acrylamide (Electran) were obtained from BDH Chemical Co. and ultrapure guanidinium chloride was obtained from Schwarz-Mann.

Pepsinogen (grade 1) was purchased from the Sigma Chemical Co. and authentic prochymosin was prepared by using the method of Foltmann (1970).

Recombinant prochymosin was prepared by using the method of Marston *et al.* (1984). An additional gel filtration on Sephadex G-150 was employed as the final step in the purification protocol. All proteins were homogeneous by SDS/PAGE.

Stock protein solutions were made up in the absence of denaturant (0.5 or 2.0 mg/ml) or in freshly prepared 6 M-urea or 4 M-guanidinium chloride (0.5 mg/ml), buffered either with phosphate (50 mM) or with Tris/HCl (20 mM), and were stored at 4 °C.

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Spectrofluorimetry

Protein fluorescence was recorded on a Perkin-Elmer MPF-3 spectrofluorimeter thermostatically controlled at 25 °C. For fixed-wavelength measurements the excitation monochromator was set at 290 nm (295 nm for quenching studies), and the emission monochromator was set at 330 nm and 340 nm for prochymosin and pepsinogen respectively. Emission spectra were recorded at speeds of either 12.5 or 50 nm/min. The excitation and emission slit widths were adjusted to 6–7 nm band pass.

C.d. spectroscopy

C.d. measurements were made at the Department of Biochemistry, University of Newcastle upon Tyne, Newcastle upon Tyne, U.K., with a Jobin-Yvon Dichrograph IV, which was linked to an Apple 11e microcomputer. All solutions were filtered through a Millipore 0.2 µm-pore-size filter before use, each protein solution having $A_{280} = 1.0$. Cells of 0.01 cm path length were used.

Each scan was measured at 5 nm/min, and ten scans were made for each sample. After each sample was scanned, a baseline reading was measured at the same scan speed and with the same number of rescans.

Potential chymosin activity

Potential milk-clotting activity of prochymosin was determined after acid activation by using a micro-titre plate assay (Emtage *et al.*, 1983).

Protein concentration

Protein concentration was determined by using the Bradford (1976) dye-binding assay.

Quenching studies

Small portions (10 µl) of 8 M-acrylamide were diluted into 3 ml of protein solution (0.2 mg of protein/ml) and the fluorescence change was recorded after each addition. Fluorescence change was corrected for dilution after each addition, but no correction for the inner-filter effect was necessary, since even at the highest

quencher concentration used the A_{295} was < 0.1. Fluorescence data were analysed by the Stern-Volmer equation:

$$F_0/F_Q = 1 + K_{sv}[Q] \quad (1)$$

when F_0 and F_Q are fluorescence emission intensities respectively in the absence of quencher and in the presence of quencher at concentration $[Q]$, and K_{sv} is a constant indicating the accessibility of the fluorophore to the quencher. Where a protein contains several tryptophan residues differing in accessibility (heterogeneous emitters), the relationship between F_0/F_Q and $[Q]$ is not linear (Eftink & Ghiron, 1981). In such cases it is plausible to assume that a fraction of the total fluorescence ($1 - f_a$) is not quenchable under the conditions studied, and that quenching of the remainder (f_a) can be described by a single value of K_{sv} . This model generates the relationship proposed by Lehrer (1971):

$$\frac{F_0}{\Delta F} = \frac{1}{f_a} + \frac{1}{K_{sv}[Q]} \quad (2)$$

where ΔF is $F_0 - F_Q$; this equation reduces to eqn. (1) when $f_a = 1$. Data giving a linear plot of $F_0/\Delta F$ versus $1/[Q]$ indicate a reasonable fit to this model, and the parameters f_a and K_{sv} indicate the fraction of total fluorescence contributed by fluorophores accessible to quencher and the accessibility of those fluorophores.

Equilibrium denaturation studies

Portions of stock enzyme solution (2 mg/ml), prepared in water or 6 M-urea and buffered with 20 mM-Tris/HCl, pH 7.5, were diluted 1/100 into various concentrations of urea (buffered with 20 mM-Tris/HCl to give pH 7.5 or pH 9.0). After an incubation period of 20 min the intrinsic fluorescence was recorded.

Alkaline denaturation was monitored by recording the fluorescence change and pH after sequential additions of a 0.1 M-NaOH solution. The protein concentration was 0.2 mg/ml dissolved in 20 mM-Tris/HCl buffer, pH 7.5.

Kinetic studies

Stock zymogen solutions were rapidly diluted (1/100) into various concentrations of urea by using a rapid manual mixing technique; the denaturant was pre-equilibrated in the fluorimeter light-path, allowing unfolding to be monitored within 3 s after mixing. The denaturant solutions were prepared in either 20 mM-Tris/HCl or 20 mM-sodium phosphate and the final pH was adjusted to between pH 6.0 and 9.0. The change in fluorescence signal was monitored continuously.

To study renaturation, a 'double-jump' technique was employed. Enzyme was added to 6 M-urea/20 mM-Tris/HCl buffer, pH 8.0, and after a variable incubation time a small portion of the denatured protein was rapidly diluted (1/10) into 'renaturation buffer' (20 mM-Tris/HCl buffer, pH 7.5) and the fluorescence change was monitored for up to 20 min.

RESULTS

Properties of prochymosin in 8 M-urea

Incubation of prochymosin in 8 M-urea increased the wavelength of maximum fluorescence emission (from approx. 330 nm to approx. 350 nm) and reduced the fluorescence intensity by approx 50%. These changes are consistent with unfolding of prochymosin, the average tryptophan environment becoming more polar. This was supported by studies in which the tryptophan fluorescence of prochymosin was quenched by acrylamide. Addition of acrylamide produced both a red-shift in the maximum wavelength of emission (λ_{em}) and a downward-curving Stern-Volmer plot (Fig. 1). This is characteristic of proteins

Fig. 1. Fluorescence quenching of prochymosin

Portions of 8 M-acrylamide were added to prochymosin in (curve 1) 20 mM-Tris/HCl buffer, pH 7.5, or (curve 2) 8 M-urea/20 mM-Tris/HCl buffer, pH 7.5. The fluorescence intensity was recorded after each addition, and the data are displayed by a Stern-Volmer plot, where F_0 and F_Q are respectively the fluorescence intensity in the absence and in the presence of quencher at quencher concentration $[Q]$.

Fig. 2. C.d. spectra of prochymosin in the far-u.v. region

—, C.d. spectrum of native prochymosin; ----, c.d. spectrum of prochymosin denatured in 8 M-urea. Measurements were made in 20 mM-sodium phosphate buffer, pH 7.5, at a protein concentration of 1 mg/ml.

whose tryptophan residues are heterogeneous with respect to accessibility to quencher (Eftink & Ghiron, 1976). Incubation of prochymosin in 8 M-urea linearized the Stern–Volmer plot (Fig. 1), suggesting that in the denatured state all the tryptophan residues are equally accessible to solvent. Use of a modified Stern–Volmer equation appropriate to heterogeneous emitters (Lehrer, 1971) indicated that approx. 20% of the fluorescence was inaccessible in the native state only. Since fluorescence at these wavelengths is predominantly from tryptophan residues, of which prochymosin contains four, it is reasonable to suggest that a single internal tryptophan residue, which is buried in the native molecule, becomes accessible to quencher after incubation in 8 M-urea (Table 1).

The unfolded nature of prochymosin in 8 M-urea was confirmed by comparing the characteristics of the far-u.v. c.d. spectrum in the absence and in the presence of denaturant (Fig. 2). In 8 M-urea the ellipticity at 220 nm becomes substantially less negative,

and the far-u.v. c.d. spectrum changes to a type that is characteristic of a random coil (spectrum not shown). These changes indicate loss of ordered secondary structure due to solvent denaturation. In the native state, pepsinogen, natural prochymosin and recombinant prochymosin showed effectively identical far-u.v. c.d. spectra (not shown), and analysis of these spectra indicated a predominance of β -sheet with a small fraction of α -helix, as expected from the known structures of aspartic proteinases.

Equilibrium and kinetic studies of the unfolding of prochymosin

The unfolding transition was followed by measuring the decrease in fluorescence intensity as a function of denaturant concentration or pH, after equilibration for 20 min. The unfolding transitions of prochymosin induced by high urea concentration (Fig. 3a) or alkaline pH (Fig. 3b) showed a single sharp transition between the native and unfolded conformations, implying a co-operative two-state transition during unfolding with no intermediates. The alkaline denaturation of pepsinogen (Fig. 3c) was more complex, with a minor transition at pH 8–9. At pH 7.5 prochymosin (natural and recombinant) and pepsinogen were similar in sensitivity to urea denaturation, the unfolding transition showing a mid-point at approx. 2.7 M-urea (Table 2). The proteins were also similar in sensitivity to guanidinium chloride (results not shown), the mid-point of the single co-operative unfolding transition being at 1.3 ± 0.1 M-guanidinium chloride in each case. Increasing the pH from 7.5 to 9.0 increased the sensitivity of the proteins to urea denaturation, pepsinogen being affected more, so that it is markedly more readily denatured by urea at the higher pH than is prochymosin. The change in fluorescence intensity between the native and denatured state was not altered by the pH change.

Equilibrium constants for the transition between the folded and unfolded states were derived at each urea concentration, and converted to give the free energy change on unfolding (ΔG_u) at each urea concentration. Stabilization energies (ΔG_u^0), i.e. free energies of unfolding in absence of denaturant, for prochymosin and pepsinogen were obtained from these data by linear extrapolation (Greene & Pace, 1974; Pace, 1975) (Fig. 4). At pH 7.5, no significant difference in ΔG_u^0 between prochymosin and pepsinogen was observed (Table 2), and the value obtained approximates the literature value for pepsinogen of 27.2 kJ/mol (Ahmad & McPhie, 1978). At pH 9.0 the stabilization energies were reduced, but significantly more so for pepsinogen than for prochymosin.

To obtain kinetic data, the fluorescence change on denaturation was monitored continuously after rapid addition of aqueous solutions of protein to denaturant; data could be collected within 3 s of mixing. Under all conditions studied here, the entire fluorescence change was represented by a single exponential curve from which a first-order rate constant (k_1) was derived (Fig. 5); there was no unresolved faster phase. The value of k_1 increased markedly both with increasing urea concentration and with pH (Fig. 6a). A 10-fold increase in k_1 can be generated either by an increase of pH by 1 unit at constant urea concentration or by an increase of urea concentration by 2 M at constant pH. Combinations of high urea concentration and high pH produced unfolding processes that were complete within the mixing time, and hence could not be measured by using the methods employed here. Each rate constant was obtained from triplicate experiments, and the reproducibility was in the range $\pm 5\%$ to $\pm 15\%$. When natural and recombinant prochymosin were compared under identical conditions, the values of k_1 observed agreed to within $\pm 10\%$ (Fig. 6a). Within the error, the natural and recombinant proteins are identical in their kinetics of denaturation.

Table 1. Quenching data for prochymosin and pepsinogen

The quenching data, which were obtained in 20 mM-Tris/HCl buffer, pH 7.5, were plotted according to the equation of Lehrer (1971) and generated linear plots. The parameters K_{sv} (Stern–Volmer constant) and f_a (fraction of fluorescence from residues accessible to quencher) were obtained from the slope and intercept. See the Materials and methods section for further background.

Protein	K_{sv} (M^{-1})	f_a
Natural prochymosin	9.94 ± 0.15	0.80 ± 0.01
Recombinant prochymosin	9.97 ± 0.10	0.83 ± 0.01
Pepsinogen	8.89 ± 0.23	0.81 ± 0.01
Natural prochymosin + 8 M-urea	9.11 ± 0.67	1.0 ± 0.01
Pepsinogen + 8 M-urea	9.45 ± 0.21	1.0

Fig. 3. Denaturation of prochymosin and pepsinogen

(a) Urea-induced unfolding of prochymosin. Natural (● and ○) and recombinant (▲ and △) prochymosin were incubated in solutions of various urea concentrations, and the fluorescence intensities were recorded after 20 min incubation at 25 °C. The protein concentration was 20 µg/ml, in 20 mM-Tris/HCl buffer, pH 7.5 (● and ▲), and in 20 mM-Tris/HCl buffer, pH 9.0 (○ and △). (b) Alkaline denaturation of recombinant prochymosin. Portions of 0.1 M-NaOH were added to a recombinant prochymosin solution in 20 mM-Tris/HCl buffer, pH 7.25, and the pH and fluorescence changes were recorded after each addition. The protein concentration was 200 µg/ml. (c) Alkaline denaturation of pepsinogen. Details were as for (b), with pepsinogen in place of prochymosin.

Table 2. Equilibrium studies on unfolding of prochymosin and pepsinogen by urea

[Urea]_{0.5} is the molar concentration of urea corresponding to the mid-point of the unfolding transition (derived from Fig. 4; intercept at $\Delta G_u = 0$). ΔG_u^0 is the stabilization energy of the native state to unfolding in the absence of denaturant (derived by linear extrapolation as in Fig. 4; intercept at [urea] = 0).

	[Urea] _{0.5} (M)		ΔG_u^0 (kJ/mol)	
	pH 7.5	pH 9.0	pH 7.5	pH 9.0
Natural prochymosin	2.7	2.2	27.0 ± 1.0	14.9 ± 0.9
Recombinant prochymosin	2.6	2.2	26.9 ± 1.2	14.5 ± 0.6
Pepsinogen	2.9	1.7	26.6 ± 1.0	11.4 ± 0.7

The rates of denaturation of prochymosin and pepsinogen differed considerably, even at pH 7.5, where their equilibrium denaturation behaviour was similar. At this pH the rate of pepsinogen unfolding was approx. 10-fold lower than that of prochymosin (Fig. 6b). At pH 9.0 this difference in rate was smaller, but still significant; this is consistent with the equilibrium studies, which showed pepsinogen denaturation to be more sensitive to the pH increase.

Studies on the kinetics of unfolding in guanidinium chloride led to the same conclusions as those in urea. A single first-order process was detected, and the observed k_1 increased sharply with denaturant concentration. Rate constants for authentic and recombinant prochymosin were essentially identical and 6–10-fold greater than those for pepsinogen in the same conditions (results not shown).

Fig. 4. Estimation of stabilization energies of (a) natural prochymosin, (b) recombinant prochymosin and (c) pepsinogen

Equilibrium constants (K_u) for the interconversion of unfolded and native conformations were determined at particular urea concentrations using equilibrium denaturation data (as in Fig. 3a). Stabilization energies (ΔG_u) were then determined at particular urea concentrations by using $\Delta G_u = -RT \cdot \ln K_u$, where R and T are the universal gas constant and temperature in K. Extrapolation to [urea] = 0 allowed the estimation of the stabilization energy in aqueous solution (ΔG_u^0). Measurements were made at pH 7.5 (●) and at pH 9.0 (▲).

Renaturation of prochymosin

Previous studies have shown that pepsinogen is fully able to regain its native conformation after renaturation from high concentrations of urea (McPhie, 1980, 1982). Prochymosin,

Fig. 5. Kinetics of urea denaturation of prochymosin

Portions of stock protein were rapidly diluted into solutions of various urea concentrations and pH values, and the changes in fluorescence intensity were measured continuously. The data were transformed into a semi-logarithmic plot, which allowed the estimation of a first-order rate constant (k_1) for unfolding. The unfolding data shown were observed for natural prochymosin diluted in 3.5 M-urea/20 mM-sodium phosphate buffer, pH 7.5.

unlike pepsinogen, does not show full reversibility with respect to urea denaturation. Rapid dilution into buffer of a urea-denatured prochymosin solution resulted in a refolded product that has regained only part of the native fluorescence (Fig. 7) and approx. 20% of the native potential enzyme activity. This lack of reversibility, which was observed over a wide range of prochymosin concentrations, suggested that a two-state mechanism was insufficient to explain the urea-induced unfolding of prochymosin.

The kinetics of refolding were therefore studied by using

Fig. 7. Reversibility of urea denaturation of prochymosin

Natural prochymosin was incubated in either 20 mM-Tris/HCl buffer, pH 7.5 (●), or 8 M-urea/20 mM-Tris/HCl buffer, pH 7.5 (▲), and after 20 min incubation at 25 °C small portions were diluted into various concentrations of urea (in 20 mM-Tris/HCl buffer, pH 7.5) and the fluorescence intensities were recorded.

double-jump experiments, in which protein was incubated in high concentrations of urea for various times before renaturation by rapid dilution into aqueous buffer. The refolding process was monitored by measuring the increase in fluorescence as a function of time.

The refolding kinetics of pepsinogen are well characterized (McPhie, 1980, 1982) and involve two kinetic phases. A fast phase could not be monitored by our manual procedure, but a slow phase having a first-order rate constant of $0.008 \pm 0.001 \text{ s}^{-1}$ was observed (Fig. 8), which compares with the published value of $0.005 \pm 0.001 \text{ s}^{-1}$ (McPhie, 1982). The amplitude of the slow phase increased with the time of exposure to urea; when

Fig. 6. Effects of pH and urea concentration on the rate of unfolding (k_1) of (a) natural prochymosin (●) and recombinant prochymosin (▲) and of (b) natural prochymosin (●) and pepsinogen (○)

The protein concentration was 20 $\mu\text{g/ml}$, in 20 mM-sodium phosphate buffer, pH 6–8, or 20 mM-Tris/HCl buffer, pH 9.0.

Fig. 8. Refolding of pepsinogen from 6 M-urea at pH 8.0

Pepsinogen (2 mg/ml) was denatured in 6 M-urea at pH 8.0 for 300 s, after which the denaturant was removed by dilution (1/100) into denaturant-free buffer. The change in the fluorescence yield as a function of time was recorded, and the data were transformed into a semi-logarithmic plot (inset) to determine a first-order rate constant (k_d) for the slow phase in the refolding process.

Table 3. Recovery of potential chymosin activity and native fluorescence in refolded prochymosin as a function of incubation time in 6 M-urea/50 mM-sodium phosphate buffer, pH 8.0

The denatured protein was diluted 25-fold to a final protein concentration of 40 μ g/ml and equilibrated for 20 min before recoveries of fluorescence and potential chymosin activity were determined.

Incubation time in 6 M-urea (s)	Activity recovered (%)	Native fluorescence recovered (%)
0	100	100
10	81	67
20	62 $\pm$ 5	48
30	30 $\pm$ 6	34
40	26 $\pm$ 2	20
50	24	15
60	24	11

renaturation was initiated after less than 20 s in urea, the 'slow' fraction of the fluorescence increase was negligible, but, when the exposure to urea was for 300 s or more, approx. 25% of the fluorescence increase on renaturation was 'slow'.

Comparison of the refolding kinetics of prochymosin with those of pepsinogen revealed significant differences. Applying the double-jump procedure to prochymosin showed that no resolvable slow phase could be detected during refolding. It was also found that the degree of regain of native fluorescence depended on the incubation time in urea (Table 3). After a short incubation time in urea, most of the fluorescence and potential enzyme activity could be recovered after dilution of urea. However, both final fluorescence and enzyme activity were substantially decreased when longer incubation times in urea were employed.

DISCUSSION

The denaturation of prochymosin has been studied and compared with that of the closely related zymogen pepsinogen. Denaturation by urea was studied by fluorescence emission,

fluorescence quenching and far-u.v. c.d. The changes in spectroscopic properties were characteristic of the transfer of tryptophan side chains to more polar environments, the transfer of one tryptophan residue from a position inaccessible to acrylamide to a more exposed situation and the loss of ordered polypeptide backbone structure. In equilibrium studies, denaturation of prochymosin by urea, monitored by fluorescence, was shown to approximate to a two-state transition, as previously observed for pepsinogen, and stabilization energies for the native states of the two proteins at neutral pH (pH 7.5) were found to be identical. Direct studies of the thermodynamics of pepsinogen denaturation by scanning microcalorimetry have demonstrated the two-state behaviour to be an approximation (Mateo & Privalov, 1981); the denaturation behaviour is best represented as that of two homologous lobes, each of which consists of two highly interdependent folding units (Privalov *et al.*, 1981). The two homologous lobes are sufficiently similar in thermodynamics for no intermediate unfolded state to be detected in a titration experiment, but a consequence is that stabilization energies calculated from such titrations will correspond to the energies required to destabilize the less stable of the lobes. The value observed here for both prochymosin and pepsinogen at pH 7.5 (approx. 27 kJ/mol) agrees closely with the 26 kJ/mol quoted by Privalov *et al.* (1981) for the less stable lobe of pepsinogen, derived from calorimetry (at pH 6.5).

Both prochymosin and pepsinogen are destabilized by a shift to pH 9.0, as judged by sensitivity to urea denaturation, but this effect is distinctly more pronounced for pepsinogen. This finding may be rationalized by consideration of the alkaline-denaturation titrations of the two proteins. The alkaline denaturation of prochymosin, reported here, is essentially a single transition centred on pH 10.2, with little impact on the tryptophan environments at pH 9.0. However, the alkaline denaturation of pepsinogen is more complex, and previous work on pepsinogen has identified a conformational change above pH 8.5 that is distinct from the gross denaturation that begins above pH 9 and is centred at pH 10.2 (Frattali *et al.*, 1965; McPhie, 1975; Ahmad & McPhie, 1978). This more limited conformational change in pepsinogen gave a conformation characterized by inability to be converted into active pepsin on exposure to low pH, and was interpreted as a titration of the basic *N*-terminal region resulting in an open conformation for the zymogen.

A second difference between pepsinogen and prochymosin was that whereas denaturation of pepsinogen by urea was fully reversible by simple dilution of urea, prochymosin denaturation was not; this could be accounted for by a slow irreversible process occurring in urea (see below). Denaturation of both proteins by guanidinium chloride appears to be fully reversible (results not shown).

Kinetic studies showed further clear differences between the homologous proteins. At all concentrations of urea studied, the rate of unfolding of prochymosin was considerably greater than that for pepsinogen; this discrepancy was particularly marked at pH 7.5, where the proteins showed identical folding transitions at equilibrium. This kinetic difference between prochymosin and pepsinogen was independent of the denaturant, as it was also observed in studies of the kinetics of unfolding by guanidinium chloride (results not shown). Since prochymosin and pepsinogen have superimposable equilibrium denaturation titrations at pH 7.5, this kinetic difference must arise from a difference in the free energy of the transition state for unfolding. Although it is not possible at this stage to identify the structural basis for this difference, it should now be possible to approach this experimentally by site-directed mutagenesis.

A further noticeable difference was in the kinetics of renaturation from urea. Pepsinogen showed characteristics that

have previously been observed for several proteins and can be described by the kinetic model:

Denaturation gives rise to two thermodynamically similar unfolded states that are in slow equilibrium. Immediate renaturation leads to rapid refolding, but with increasing time in denaturant the proportion of 'slow-folding' form increases, giving rise to biphasic refolding kinetics, the extent of the slower phase increasing with denaturation time. The slow process occurring in the unfolded state may be *cis-trans* isomerization of prolyl peptide bonds, but there is no direct evidence on this point.

The behaviour of prochymosin was clearly distinct in that no slow refolding process was observable, but the proportion of protein that refolded rapidly decreased with increasing denaturation time. This behaviour can be rationalized by two experimentally indistinguishable models, as follows.

Model I: the two unfolded states U_f and U_s exist as for pepsinogen, but U_s is rapidly converted irreversibly into a further state:

Model II: the two unfolded states U_f and U_s exist as for pepsinogen, but the conversion of U_s into U_f is infinitesimally slow, so that the conversion U_f into U_s is effectively irreversible:

The clear difference in renaturation kinetics between pepsinogen and prochymosin explains the differences in reversibility of urea denaturation observed in the equilibrium titration studies.

The existence of this slow process in urea, which leads to the conversion of unfolded prochymosin into a form that cannot be renatured by simple dilution, provides a partial rationalization for the empirical protocol for recovery of recombinant prochymosin from inclusion bodies. This protocol involves initial solubilization in urea, then dilution into high-pH buffer (pH 10.75) followed by neutralization. The very high pH was observed to be critical (Marston *et al.* 1984), which makes it most unlikely that the role of the high-pH step is to facilitate thiol/disulphide exchange, since this process would not increase in rate significantly above pH 9.5. Hence it can be proposed that the dilution of urea-solubilized inclusion bodies into pH 10.75 buffer converts prochymosin 'irreversibly' denatured by urea into a form of the protein denatured by high pH that more readily refolds to the native conformation. Recent work (e.g. Goto & Fink, 1989) has established that proteins denatured by extreme pH may be in a

state distinct from the fully unfolded state in dynamics and conformation.

Throughout this work the denaturation and renaturation behaviour of natural and recombinant prochymosin have been found to be indistinguishable; where quantitative parameters have been determined (rate constants, stabilization energies, K_{sv} values), the values have been identical within the experimental error. This identity of natural and recombinant prochymosin is clearly meaningful; the methods used are sensitive to differences in the denaturation/renaturation of proteins and have established the existence of such differences between prochymosin and pepsinogen.

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REFERENCES

- Ahmad, F. & McPhie, P. (1978) *Biochemistry* **17**, 241–246
 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
 Eftink, M. R. & Ghiron, C. A. (1976) *Biochemistry* **15**, 672–680
 Eftink, M. R. & Ghiron, C. A. (1981) *Anal. Biochem.* **114**, 199–227
 Emtage, J. S., Angal, S., Doel, M. T., Harris, T. J. R., Jenkins, B., Lilley, G. & Lowe, P. A. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3671–3675
 Foltmann, B. (1970) *Methods Enzymol.* **19**, 421–436
 Frattali, V., Steiner, R. F. & Edelhoch, J. (1965) *J. Biol. Chem.* **240**, 112–121
 Goto, Y. & Fink, A. L. (1989) *Biochemistry* **28**, 945–952
 Greene, R. G., Jr. & Pace, C. N. (1974) *J. Biol. Chem.* **249**, 5388–5393
 Harris, T. J. R., Lowe, P. A., Lyons, A., Thomas, P. G., Eaton, M. A., Millican, T. A., Patel, T. P., Rose, C. C., Carey, N. H. & Doel, M. T. (1982) *Nucleic Acids Res.* **10**, 2177–2187
 Lehrer, S. S. (1971) *Biochemistry* **10**, 3254–3263
 Marston, F. A. O. (1986) *Biochem. J.* **240**, 1–12
 Marston, F. A. O., Lowe, P. A., Doel, M. T., Schoemaker, J. M., White, S. & Angal, S. (1984) *Bio/Technology* **2**, 800–804
 Mateo, P. L. & Privalov, P. L. (1981) *FEBS Lett.* **123**, 189–192
 McPhie, P. (1975) *Biochemistry* **14**, 5253–5256
 McPhie, P. (1980) *J. Biol. Chem.* **255**, 4048–4052
 McPhie, P. (1982) *Biochemistry* **21**, 5509–5515
 Nishimori, K., Shimizu, N., Kawaguchi, Y., Hidaka, M., Vozumi, T. & Beppu, T. (1984) *Gene* **29**, 41–49
 Pace, C. N. (1975) *CRC Crit. Rev. Biochem.* **3**, 1–100
 Perlmann, G. E. (1963) *J. Mol. Biol.* **6**, 452–464
 Privalov, P. L., Mateo, P. L., Khechinashvili, N. N., Stephanov, V. M. & Rovina, L. P. (1981) *J. Mol. Biol.* **152**, 445–464
 Schoemaker, J. M., Brasnett, A. H. & Marston, F. A. O. (1985) *EMBO J.* **4**, 775–780
 Sugrue, R., Lowe, P. A., Marston, F. A. O. & Freedman, R. B. (1985) *Biochem. Soc. Trans.* **13**, 1146
 Sugrue, R., Freedman, R. B., Lowe, P. A. & Marston, F. A. O. (1988) *Biochem. Soc. Trans.* **16**, 59–60

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