Unfolding and Refolding of Phospholipase C from *Bacillus cereus* in Solutions of Guanidinium Chloride

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1. Protein-fluorescence studies indicated that phospholipase C from *Bacillus cereus* is denatured in solutions of guanidinium chloride. The denaturation was not thermodynamically reversible and followed biphasic kinetics. 2. Guanidinium chloride solutions released the structural Zn^{2+} from the enzyme and rendered all histidine residues chemically reactive. In the presence of free Zn^{2+} the enzyme was much more resistant to denaturation. Also, the addition of free Zn^{2+} to the denatured enzyme induced refolding. 3. The Zn^{2+} -free apoenzyme was much more sensitive to guanidinium chloride than was the native enzyme and the denaturation appeared to be thermodynamically reversible. 4. Guanidinium chloride denaturation was associated with a reversible inactivation of the enzyme. Heat-inactivated, coagulated enzyme was substantially re-activated on dissolution in guanidinium chloride solutions followed by dialysis against a Zn^{2+} -containing buffer.

Phospholipase C (phosphatidylcholine choline-phosphohydrolase, EC 3.1.4.3) from *Bacillus cereus* is a monomeric enzyme of molecular weight 23000 (Otnæss *et al.*, 1972) and contains two zinc atoms (Little & Otnæss, 1975). The enzyme contains no disulphide bonds (Otnæss *et al.*, 1977) and is extremely resistant to the effects of urea (Little, 1978). Indeed, the enzyme is catalytically active in 8M-urea. Short periods of exposure to elevated temperatures of the enzyme dissolved in 8M-urea destroy the periodic structure without releasing the zinc atoms from the enzyme (Little, 1978). In order to carry out more extensive denaturation of the enzyme, the effect of guanidinium chloride has been examined.

Materials and Methods

Phospholipase C was isolated from the culture supernatant of *B. cereus* as described by Little *et al.* (1975), and Zn^{2+} -free apoenzyme was made by dialysis of the native enzyme against EDTA (Little, 1977). Guanidinium chloride (puriss. grade) was obtained from Fluka A.G., Buchs, Switzerland, and further purified by the double-recrystallization technique described by Nozaki (1972). Diethyl pyrocarbonate was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. The purity of the sample used was estimated to be 80% (Little, 1977).

Fluorescence measurements were made in a Perkin Elmer MPF-3 fluorescence spectrophotometer with the cuvette compartment thermostatically maintained at 23°C. Protein fluorescence was studied by using an excitation wavelength of 285 nm. The intensity of emission at the emission maximum

in the 340–350 nm region was measured. Zinc was determined by atomic-absorption spectroscopy as described previously (Little & Otnæss, 1975). Enzyme assays were performed by using a crude egg-yolk substrate (Zwaal *et al.*, 1971) at pH 7.5 and 23°C by continuous titration of the acid liberated. Titrations were carried out in Radiometer pH-stat equipment. The number of available histidine residues in the enzyme was defined as the number reacting while enzyme (10 μM) was incubated for 15 min with 4.5 mM-diethyl pyrocarbonate in 0.05M-sodium phosphate buffer (pH 6.0) at 22–23°C. The extent of histidine modification was estimated from the increment in A_{240} of the enzyme solution ($\epsilon = 3200 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) (Ovadi *et al.*, 1967). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, and also by the A_{280} ($\epsilon = 51000 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) (Little, 1977).

Results

Unfolding and reversibility of unfolding of the native and Zn^{2+} -free enzymes

On the basis of changes in the intensity of protein fluorescence, exposure of phospholipase C to solutions of guanidinium chloride in the concentration range 1–2M causes marked denaturation. A slight red shift of about 5 nm in the emission maximum was also observed. This unfolding process did not seem to be fully reversible. The protein fluorescence observed with unfolded enzyme that had been diluted to denaturant concentrations below about 1M was lower than that shown by native enzyme

adjusted directly to the same final denaturant concentration (Fig. 1). This effect was reproduced in four independent experiments. Similar results were obtained by using either 0.15M-sodium acetate buffer (pH 6.0) or 0.05M-sodium phosphate buffer (pH 6.0).

The Zn^{2+} -free enzyme was markedly more sensitive to denaturation by guanidinium chloride, being apparently totally unfolded by denaturant concentrations of just over 1M. With the apoenzyme the unfolding process appeared to be fully thermodynamically reversible on dilution (Fig. 1).

The kinetics of denaturation were studied (Fig. 2) and found to be apparently biphasic between 1.3M- and 2.5M-denaturant. At 3M-denaturant the data fitted more closely with a single phase. The apparent rate constants for unfolding phases were fairly constant between 1.3M- and 1.8M-guanidinium chloride, but were significantly higher in the denatured baseline region (Table 1). The amplitude of the slow phase at denaturant concentrations of 1.3–2.25M was fairly constant at about 40%.

Fig. 1. Equilibrium curves for the unfolding and refolding of native and Zn^{2+} -free phospholipase C in guanidinium chloride

(○), Samples of native enzyme diluted from 0.05M-sodium phosphate (pH 6.0) into guanidinium chloride dissolved in the same buffer. (△), Samples of native enzyme diluted with the same buffer from 2M-guanidinium chloride/0.05M-sodium phosphate (pH 6.0). (●), Samples of Zn^{2+} -free enzyme diluted from 2mM-EDTA/0.05M-sodium phosphate (pH 6.0) into guanidinium chloride dissolved in the same buffer. (▲), Samples of Zn^{2+} -free enzyme diluted with the same buffer from 2M-guanidinium chloride/2mM-EDTA/0.05M-sodium phosphate (pH 6.0). The temperature was 25°C and the final enzyme concentration 5.2 μM . With the native enzyme each point represents the average of three separate experiments, and with the Zn^{2+} -free enzyme each point represents the average of two separate experiments.

Attempts to study the kinetics of denaturation of the apoenzyme were not successful. In 1M-guanidinium chloride the unfolding process was complete within 15s, and hence the process was too rapid to follow by using conventional mixing techniques.

Fig. 2. Kinetics of unfolding of phospholipase C in guanidinium chloride

Enzyme (15 μM final concn.) was dissolved in guanidinium chloride/0.05M-sodium phosphate (pH 6.0). The relative fluorescence (F) was measured at different times. F_∞ is the relative fluorescence obtained 5–6h after mixing. The temperature was 22°C. Denaturant concns. were 1.3M (●), 1.8M (▲), 2M (■) and 3M (○).

Table 1. Kinetics of unfolding at different denaturant concentrations

Enzyme (15 μM) was dissolved in different concentrations of guanidinium chloride in 0.05M-sodium phosphate (pH 6.0). Protein fluorescence was measured at different times and the data were plotted as in Fig. 2. Rate constants were calculated by subtracting the slow from the fast phase. The extent of unfolding is defined as $[(F_\infty - F_D)/(F_N - F_D)] \times 100\%$, where F_∞ is the relative fluorescence of the enzyme in denaturant at equilibrium, F_D the relative fluorescence of enzyme in 3M-denaturant at equilibrium and F_N the relative fluorescence in the absence of denaturant.

Guanidinium chloride concn. (M)	Extent of unfolding (%)	Rate constant for fast phase (min^{-1})	Rate constant for slow phase (min^{-1})
1.3	26	0.19	0.060
1.4	40	0.19	0.069
1.6	80	0.18	0.067
1.8	97	0.18	0.050
2.0	99	0.23	0.077
2.25	100	0.48	0.12
2.5	100	0.57	0.25
3.0	100	0.70	—

Unfolding and the Zn^{2+} content of the enzyme

Exposure of the enzyme to guanidinium chloride resulted in the loss of the structural zinc atoms and the exposure of 'hidden' histidine residues. There seemed to be a fairly good correlation between the decrease in protein fluorescence, the decrease in the zinc content of the enzyme and the increase in the number of chemically reactive histidine residues (Table 2). During the histidine assays, it was observed that in solutions of around 1.4M-guanidinium chloride turbidity formed. This we have observed previously when diethyl pyrocarbonate reacts with enzyme species containing only one of the two structural zinc atoms (C. Little, unpublished work). Under the conditions used for histidine assay, in solutions of guanidinium chloride >1.6M, 6.5 histidine residues/molecule were defined as being reactive. However, it was observed that more prolonged exposure of the denatured enzyme to the histidine reagent resulted in the reaction of about 7.5 mol of histidine/mol of enzyme.

Since enzyme denaturation by guanidinium chloride is associated with the loss of Zn^{2+} from the structure, the influence of added Zn^{2+} on the denaturation process was examined. Because of the very limited solubility of Zn^{2+} in phosphate buffers, the experiment was carried out in 0.15M-sodium acetate buffer (pH 6.0). The results (Fig. 3) show that free Zn^{2+} strongly protects the enzyme against denaturation. In the absence of free Zn^{2+} , the enzyme was fully denatured by 2M-guanidinium chloride, whereas in the presence of 0.1mM- Zn^{2+} , over 2.5M-guanidinium chloride was required for total denaturation, and in 1mM- Zn^{2+} approx. 3M-

guanidinium chloride was required (Fig. 3). No extra stabilization was obtained by using Zn^{2+} concentrations above 1mM.

Zn^{2+} was also able to induce the refolding of denatured enzyme. Thus fluorescence measurements indicated that enzyme that had been denatured 99% in 2M-guanidinium chloride was only about 20% denatured when Zn^{2+} to a final concentration of 0.5mM was subsequently added (Fig. 4).

Fig. 3. Effect of added Zn^{2+} on the equilibrium curves for the unfolding of phospholipase C in guanidinium chloride. Enzyme was diluted from 0.15M-sodium acetate, pH 6.0, into guanidinium chloride dissolved in the same buffer. Zn^{2+} was added before the guanidinium chloride solution. The final enzyme concentration was $3 \mu M$, the temperature $25^\circ C$ and the Zn^{2+} concns. 0 (●), 0.1 mM (○) and 1 mM (Δ). Fluorescence was measured after 6h equilibration.

Table 2. Effect of guanidinium chloride on the histidine reactivity and zinc content of phospholipase C

Enzyme ($10 \mu M$) was dissolved in different concentrations of guanidinium chloride in 0.05M-sodium phosphate (pH 6.0) at $25^\circ C$. After 4h the protein fluorescence and the number of reactive histidine residues were measured (see the Materials and Methods section). Alternatively, the enzyme solutions in guanidinium chloride were dialysed for 4h at ambient temperature ($23-24^\circ C$) against guanidinium chloride solutions of different concentrations dissolved in 0.05M-sodium phosphate (pH 6.0). At the beginning of the experiment, enzyme samples were dissolved in guanidinium chloride of the same final concentration as that in the solutions against which they were subsequently dialysed. The zinc content of the samples was then measured. Symbols F and F_D are explained in the legend to Fig. 5.

Guanidinium chloride concn. (M)	Fluorescence ($F-F_D$) (arbitrary units)	Zinc content (mol/mol of enzyme)	No. of reactive histidine residues (mol/mol of enzyme)
0	65	1.87	1.0
0.5	63	1.87	1.0
1.0	55	1.73	1.3
1.2	48	1.52	1.9
1.4	29	0.49	2.6*
1.6	7	0.14	6.5
1.8	1	0.1	6.5
2.0	0	0	6.5
3.0	0	0	6.5

* Solution became turbid.

Fig. 4. Equilibrium curves for Zn^{2+} -induced refolding of phospholipase C in 2M-guanidinium chloride

Enzyme ($15\mu\text{M}$) was incubated for 2 h at 20°C in 2M-guanidinium chloride/0.15M-sodium acetate (pH 6.0). Zinc acetate ($25\mu\text{l}/2.5\text{ml}$ of enzyme solution) was then added. Fluorescence measurements (F) were made at 22°C after the system had come to equilibrium (6 h). F_D is the fluorescence of the enzyme without added Zn^{2+} in 2M-guanidinium chloride. ($\circ$), Enzyme in denaturant; ($\bullet$), enzyme without denaturant. Each point represents the average of two experiments.

Effect of guanidinium chloride on enzyme activity

Incubation of enzyme in solutions of guanidinium chloride above 0.6M caused enzyme inactivation, the extent of which seemed to correlate fairly well with the decrease in protein fluorescence (Fig. 5). Exposure of the enzyme to 2M-denaturant caused total inactivation. Dilution of such inactivated samples resulted in re-activation. However, denatured enzyme that had been diluted to a certain denaturant concentration was often slightly less active than enzyme that had been adjusted directly to the same denaturant concentration. Enzyme in 2M-guanidinium chloride could be fully re-activated by dilution with 0.05M-sodium phosphate buffer (pH 6.0) to a denaturant concentration of 0.4M (Fig. 5). Re-activation could also be accomplished by dialysis, although the extent of activity regained depended on the particular dialysis buffer used. Dialysis for 5 h at $22\text{--}24^\circ\text{C}$ against the above phosphate buffer produced variable degrees of re-activation, whereas dialysis against the normal storage buffer for the enzyme, 2.9mM-sodium 5,5'-diethylbarbiturate (pH 7.4) containing 1mM- ZnSO_4 , resulted in full re-activation.

Fig. 5. Effect of unfolding and refolding on enzyme activity

Enzyme in 0.05M-sodium phosphate (pH 6.0) was mixed with guanidinium chloride and the enzyme activity ($\bullet$) and relative fluorescence ($\square$) were measured after unfolding equilibrium was reached. Fluorescence is expressed as $F - F_D$, where F is the relative fluorescence of the sample and F_D the relative fluorescence in 3M-guanidinium chloride. Alternatively, samples were diluted with the same buffer from 2M-guanidinium chloride/0.05M-sodium phosphate, pH 6.0, and enzyme activity was measured at refolding equilibrium (Δ). The final enzyme concentration was $3\mu\text{M}$ and the temperature 25°C . Each point represents the average of two separate experiments.

Guanidinium chloride and the thermal stability of the enzyme

Previous results (Little, 1978) showed that the presence of 8M-urea protected phospholipase C from irreversible thermal inactivation and it was found that guanidinium chloride could do the same. Thus samples of enzyme in 2M-guanidinium chloride that had been preheated at 100°C for 5 min were fully active on dialysis against a Zn^{2+} -containing buffer. In the absence of the denaturant, the enzyme was fully inactivated and coagulated by preheating at 100°C for 5 min. Dialysis against Zn^{2+} -containing buffers did not cause re-activation. However, such heat-coagulated samples when first dissolved in 4M-guanidinium chloride regained approx. 80% of their original catalytic activity on subsequent dialysis against 2.9mM-sodium 5,5'-diethylbarbiturate buffer (pH 7.4) containing 1mM- Zn^{2+} .

Discussion

Whereas at room temperature solutions of 8M-urea are unable to denature phospholipase C (Little, 1978), marked denaturation occurs in solutions of guanidinium chloride above about 1M. This dif-

ference in effectiveness of these two denaturants seems larger than normal with the present enzyme. Normally, guanidinium chloride is about twice as effective a denaturant on a molar basis as urea (see, e.g., Russell & Cooper, 1972; Prakash & Nandi, 1977), but with phospholipase C, the former denaturant is at least 8-fold more effective than urea.

Phospholipase C from *B. cereus* has a very similar stability in guanidinium chloride to penicillinase from *S. aureus* (Robson & Pain, 1976) and it is perhaps interesting to point out the similarity between these enzymes in terms of molecular weight, helical content and lack of disulphide cross-links (Robson & Pain, 1976; Otnæss *et al.*, 1972, 1977; Little, 1978). However, whereas the unfolding of the penicillinase is thermodynamically reversible, this seemed not to be the case with native phospholipase C. This difference may well arise from the structural zinc atoms in the phospholipase. Unfolding of the native enzyme is accompanied by the loss of Zn^{2+} from the structure. Unfolding studies on the Zn^{2+} -free enzyme showed that this enzyme form is very much more sensitive to guanidinium chloride than is the native enzyme and that the unfolding process appears to be thermodynamically reversible. It is noteworthy that with native enzyme, the refolding curve except at low denaturant concentrations follows very closely the unfolding curve. Thus, although both the unfolded native enzyme and unfolded apoenzyme contain no Zn^{2+} , their refolding curves are quite dissimilar. Presumably most of the Zn^{2+} released on unfolding of the native enzyme is bound once more on dilution of the system. The lack of complete thermodynamic reversibility of the unfolding of the native enzyme may well arise from problems involving binding of all the Zn^{2+} released on unfolding. This view is supported by the observation that the apoenzyme has a slightly lower fluorescence than has the native enzyme (Little, 1978).

Zn^{2+} seems to play a critical role in the stability of phospholipase C. The structural Zn^{2+} seems responsible for the high conformational stability of the enzyme, and in addition the presence of free Zn^{2+} causes marked stabilization of the structure against both thermal (Otnæss *et al.*, 1977) and guanidinium chloride-induced denaturation (Fig. 3). Thus, in the presence of 1 mM- Zn^{2+} , the enzyme is almost as stable in guanidinium chloride as is ribonuclease (compare Miller & Bolen, 1978).

Furthermore, the addition of free Zn^{2+} was able to induce refolding of the denatured enzyme. However, the concentrations of free Zn^{2+} required to induce refolding seem unexpectedly high when compared with the concentrations of free Zn^{2+} which protect the native enzyme from unfolding (cf. Figs. 3 and 4). Thus Zn^{2+} may play a complex role in the unfolding and refolding mechanisms of the enzyme.

The loss of structural Zn^{2+} from the enzyme closely paralleled the decrease in protein fluorescence and enzyme inactivation in the equilibrium unfolding studies (cf. Table 2 and Fig. 5). Thus the equilibrium data provide no evidence for the formation of thermodynamically stable intermediates analogous to the partially unfolded, non-periodic, Zn^{2+} -containing structures which are formed at elevated temperatures in 8M-urea (Little, 1978).

The kinetic studies showed that, except at a point well into the denatured baseline region, the unfolding was not a single-phase process, but seemed to follow pseudo-first-order biphasic kinetics. Such kinetics have been observed during the denaturation of several other simple proteins [see Brandts *et al.* (1975), Lin & Brandts (1978) and references therein]. Such kinetics have been interpreted in terms of metastable intermediates which are not necessarily on the direct pathway between the native and denatured states (Ikai & Tanford, 1971, 1973; Ikai *et al.*, 1973) and also in terms of an extended two-state model where a *cis-trans* isomerization of proline in the denatured polypeptide chain provides the slower of the observed phases (Brandts *et al.*, 1975; Lin & Brandts, 1978).

With phospholipase C, the pseudo-first-order rate constants for the two phases of denaturation were lower and much less well separated than seems to be usual with other simple proteins. The relatively small separation between the fast and slow phases also means that rate constants presented contain significant errors, since they were calculated by subtracting the slow from the fast phase. Phospholipase C from *B. cereus* contains a proline residue (Otnæss *et al.*, 1977), and the low rate of the slow phase could arise if a bulky aromatic residue adjoins the proline residue in the amino acid sequence (Brandts *et al.*, 1975). The present limited kinetic data are consistent with both of the above models of denaturation.

Enzyme denaturation by guanidinium chloride caused inactivation. Dialysis of the denatured, inactivated enzyme against a Zn^{2+} -containing buffer caused full re-activation, indicating that the denaturation process is completely reversible except in the thermodynamic sense. Guanidinium chloride probably causes complete opening up of the enzyme to a random-coil structure, which on removal of the denaturant and addition of Zn^{2+} is converted back into the native enzyme. This would explain both the resistance to irreversible thermal denaturation of the guanidinium chloride-unfolded enzyme and the observation that thermally coagulated inactive enzyme could be substantially re-activated on dissolution in guanidinium chloride solutions followed by dialysis against a Zn^{2+} -containing buffer.

One interesting aspect of the present work is that the unfolding of the apoenzyme appeared to be thermodynamically reversible. From c.d. studies (Little, 1978) it appears that removal of the structural

Zn^{2+} has only minimal effects on the protein conformation. The reversibility of unfolding of the apoenzyme would suggest that the unfolded enzyme does not require the presence of Zn^{2+} in order to fold back to a conformation which is identical or nearly identical with that of the native enzyme.

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