Conformational Studies on Phospholipase C from *Bacillus cereus*

THE EFFECT OF UREA ON THE ENZYME

By CLIVE LITTLE

Institute of Medical Biology, University of Tromsø, P.O. Box 977, N-9001 Tromsø, Norway

(Received 28 April 1978)

1. When heated in 8M-urea, phospholipase C (EC 3.1.4.3) from *Bacillus cereus* undergoes conformational transitions depending on the temperatures used. These transitions were studied by examining protein fluorescence, iodide quenching of protein fluorescence, u.v. difference spectroscopy, chemical availability of histidine residues in the enzyme, circular dichroism and catalytic activity. 2. Unless simultaneously exposed to elevated temperatures the enzyme appears to be unaffected by 8M-urea. Removal of the two zinc atoms from the enzyme renders phospholipase C very sensitive to denaturation by 8M-urea as indicated by fluorescence emission spectra and circular dichroism. 3. Both the native and the zinc-free enzymes are markedly more resistant to irreversible thermal inactivation in the presence of 8M-urea than in its absence. 4. The response of the enzyme to 8M-urea and the role of zinc in stabilizing the enzyme are discussed.

Our previous studies on phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) from *Bacillus cereus* have indicated that the enzyme is extremely stable despite the earlier reports of Ottolenghi (1969), Kleiman & Lands (1969) and Zwaal *et al.* (1971). Thus the enzyme survives prolonged exposure to high temperatures and to high concentrations of chaotropic agents (Little *et al.*, 1975; Otnaess *et al.*, 1977). In addition, 8M-urea at 25°C appears to be unable to open up the structure and expose inert histidine residues for chemical reaction (Little, 1977a). Hence, phospholipase C appears to possess an extremely stable conformation. This stability cannot be due to the presence of disulphide cross-linking in the structure, since the enzyme possesses no cystine (Otnaess *et al.*, 1977). To investigate further this high conformational stability, the effect of urea on the enzyme has been examined in greater detail.

Materials and Methods

Phospholipase C was isolated from the culture supernatant of *B. cereus* as described previously (Little *et al.*, 1975). 1,2-Dihexanoyl-*sn*-glycero-3-phosphocholine was synthesized by the method of Cubero Robles & Van den Berg (1969) as described by Little (1977b). Urea (analytical grade) was from Merck, Darmstadt, Germany and recrystallized from 96% (v/v) ethanol. Diethyl pyrocarbonate was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. The purity of the sample was estimated to be 80% (Little, 1977a). 8-Anilidonaphthalene-1-sulphonate (magnesium salt) was obtained from Serva, Heidelberg, Germany. Enzyme assays were carried

out at 23°C and pH 7.5 by continuous titration of the acid liberated from the substrate. Crude egg yolk substrate (Zwaal *et al.*, 1971) was used as substrate unless otherwise stated. In one experiment, the above crude egg yolk substrate was replaced by 2mM-1,2-dihexanoyl-*sn*-glycero-3-phosphocholine dispersed by stirring for 15 min at 22°C in aqueous 8M-urea. Titrations were carried out by using Radiometer pH-stat equipment.

Assays of available histidine residues were carried out by incubating the enzyme (10–16 μ M) in 0.05M-sodium phosphate buffer (pH 6.0) for 15 min at room temperature (22–23°C) with 4.5mM-diethyl pyrocarbonate. 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). Absorption measurements were made in a Gilford 240 spectrophotometer.

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, 1977a). Zinc was determined by atomic-absorption spectroscopy as described by Little & Otnaess (1975). Difference spectroscopy was carried out with a Beckman DBG-T spectrophotometer. Fluorescence measurements were made in a Perkin-Elmer MPF-3 fluorescence spectrophotometer with the spectra corrected at 5 nm intervals. Unless otherwise stated, all fluorescence measurements were carried out at 25°C. Quantum yields were obtained by comparison with tryptophan. The quantum yield for tryptophan fluorescence at pH 6.0 and 23°C was taken as 0.14 (Chen *et al.*, 1969). In the fluorescence-quenching experiments, a small amount (about 10 μ M) of sodium dithionite was added to the KI solutions to

prevent the oxidation of iodide and NaCl was used to maintain constant ionic strength.

C.d. spectra were obtained in a Cary model 60 spectropolarimeter equipped with a model 6002 c.d. attachment. The instrument was calibrated with (+)-10-camphorsulphonic acid. All spectra were measured at 27°C by using fused-quartz cells of pathlengths 2mm (far-u.v.-region spectra) or 10mm (near-u.v.-region spectra). Protein concentrations used were 0.071–0.088mg/ml in the far-u.v. region and 0.1–0.88mg/ml in the near-u.v. region. The ellipticity is expressed as the mean residue weight ellipticity:

$$[\Theta] = \frac{M \cdot \psi}{10 \cdot c \cdot l}$$

in degrees·cm²·dmol⁻¹, where *M* is the mean residue weight, *ψ* the observed ellipticity in degrees, *c* the protein concentration in g/ml and *l* the pathlength in cm. The mean residue weight was 115 as calculated from the amino acid composition of the enzyme (Otnaess *et al.*, 1977). The error in ellipticity was 2.5% at 220nm and 8% at 195 nm. The reported curves represent the mean values from at least three recordings.

Results

Urea and the thermal stability of the enzyme

Phospholipase C showed a progressive decline in activity when preincubated in 0.05M-sodium phosphate buffer (pH 6.0) at temperatures above 40°C and assayed after cooling. However, it maintained its full activity when preincubated in 8M-urea and the above buffer at temperatures up to about 80°C (Fig. 1*a*). When activity was measured by using a pH-stat (Fig. 1*b*) it was evident that the phospholipase C that had been heated to 60–80°C in 8M-urea was also initially inactivated, but recovered activity within a few minutes in the assay medium. Above 80°C the inactivation was irreversible. (When the urea concentration was decreased below 5–6M by dialysis the lag phase of reactivation was removed). The reactivation of the enzyme in the assay system was prevented by the presence of 8M-urea.

Fluorescent properties of enzyme samples heated in 8M-urea

Phospholipase C exhibited excitation and emission spectra with maxima at 285 and 340nm respectively. To look for possible conformational transitions in the enzyme, the effect of temperature on the protein fluorescence was examined. Conformational transitions occurred at around 60°C and around 75°C (Fig. 2). In addition, the slight irregularities in the

Fig. 1. Thermal inactivation of phospholipase C (a) Enzyme (30.5 μM) in 0.05M-sodium phosphate buffer (pH 6.0) (●) or in the same buffer containing 8M-urea (○) was exposed to different temperatures for 5 min and then cooled and assayed. In one experiment, the enzyme that had been heated in 8M-urea was assayed in 8M-urea with 1,2-dihexanoyl-*sn*-glycero-3-phospholine as substrate (see the Materials and Methods section) (Δ). (b) pH-stat recorder traces obtained during the assay of enzyme samples that have been heated at 22°C (—) or 78°C (----) in 8M-urea. Equal amounts of protein (about 3 μg) were used in each assay.

fluorescence-temperature curve between 5 and 55°C may suggest additional conformational changes.

Fluorescence emission spectra run at 25°C for enzyme samples that had previously been heated in 8M-urea are shown in Fig. 3(a). Prior heating of the enzyme up to about 55°C had no apparent effect on the fluorescence, whereas previous heating at 80°C markedly decreased the fluorescence quantum yield and caused a red shift of about 10nm in the peak emission wavelength. Heating above 80°C produced no further changes in fluorescence.

The large change in fluorescence produced by preheating the enzyme in 8M-urea at 80°C was almost totally reversed on subsequent dilution of the sample with buffer, whereas phospholipase C samples

exposed in 8M-urea to temperatures above 80°C (i.e. irreversibly inactivated samples) still had modified fluorescence properties even after dilution (Fig. 3b).

Effect of 8M-urea on the reactivity of histidine residues

The chemical reactivity of the histidine residues in the enzyme was used to look for conformational transitions induced by preheating phospholipase C

Fig. 2. Temperature dependence of fluorescence of phospholipase C in 8M-urea

Enzyme (6.5 μ M) in 8M-urea and 0.05M-sodium phosphate buffer (pH 6.0) was used. The excitation wavelength was 285 nm and the emission at 340 nm was measured. The same enzyme sample was used for each determination.

in 8M-urea. Consistent with previous results (Little, 1977a), without preheating, the enzyme in 8M-urea contains a single reactive histidine residue. Enzyme samples that had been preheated at 43°C were markedly less reactive to the histidine reagent, whereas enzyme that had been preheated at 70°C or above contained additional reactive histidine residues (Table 1).

These changes in histidine reactivity caused by preheating the enzyme in 8M-urea were at least partly reversed on subsequent dialysis of the enzyme first against 0.05M-sodium phosphate buffer, pH 6.0, and then against 8M-urea in the same buffer. Enzyme samples that had initially been heated to no higher than about 70°C all now contained one histidine residue able to react at about the same rate with the histidine reagent. Enzyme samples that had been heated above 80°C, i.e. irreversibly inactivated enzyme, contained more than one chemically available histidine residue (Table 1).

Role of Zn^{2+} in the enzyme stability

Phospholipase C contains two zinc atoms whose removal from the structure causes an increase in the number of chemically available histidine residues (Little, 1977a) together with enzyme inactivation (Little & Otnaess, 1975). Thus the increase in the number of chemically available histidine residues and the enzyme inactivation observed above might involve the loss of Zn^{2+} from the enzyme. When this possibility was examined further (Table 2) it was found that even irreversible thermal inactivation of

Fig. 3. Fluorescence emission spectra for phospholipase C after heating in 8M-urea

Enzyme (15 μ M) in 0.05M-sodium phosphate buffer (pH 6.0) and 8M-urea was exposed for 5 min to different temperatures and then cooled to room temperature. Fluorescence emission spectra (excitation wavelength 285 nm) were run at 25°C before (a) and after (b) dilution of the sample with 4 vol. of the above phosphate buffer. The emission slit width on the spectrofluorimeter was increased when the diluted enzyme was analysed. The temperatures to which the enzyme was exposed were: room temperature (a), 43°C (b), 60°C (c), 80°C (d), 100°C (e). Curve f refers to an enzyme sample exposed for 10 min to 100°C.

Table 1. *Effect of heat and 8M-urea on the reactivity of histidine residues in phospholipase C*

Enzyme (12.5–16 μ M) in 8M-urea and 0.05M-sodium phosphate buffer (pH 6.0) was heated for 5 min at different temperatures and then cooled to room temperature (22–23°C). The number of histidine residues reacting in 15 min with diethyl pyrocarbonate (4.5 mM) was then measured in the enzyme either immediately (column A) or after the enzyme sample had been dialysed for 1.5 h against 0.05M-sodium phosphate buffer (pH 6.0) and then for 3.5 h against 8M-urea in this buffer (column B).

Temperature (°C)	Available histidine residues (mol/mol of enzyme)	
	A	B
22	0.92	0.63
45	0.31	0.65
60	0.71	0.68
70	1.58	0.60
80	2.79	0.83
90	3.2	1.9
100	3.9	3.1

Table 2. *Effect of heating phospholipase C in 8M-urea on the activity and zinc content of the enzyme*

Phospholipase C (7 μ M) in 8M-urea and 0.05M-sodium phosphate buffer (pH 6.0) was heated at 100°C and samples were removed at different times. The samples were then dialysed for 3.5 h against the phosphate buffer and the enzyme activity and zinc content were measured.

Time (min)	Activity (%)	Zinc content (mol/mol of enzyme)
0	100	2.1
1.5	100	1.9
3	35	1.8
4.5	10	1.6
6	1.5	1.5
8	0	1.3
10	0	1.2

phospholipase C in 8M-urea caused relatively little Zn^{2+} loss from the enzyme.

It is clear that the zinc atoms are very tightly bound to the protein structure, and to see whether the zinc atoms could be involved in maintaining the structural integrity of phospholipase C, the properties of the Zn^{2+} -free enzyme were examined. Fig. 4 shows that whereas the removal of Zn^{2+} from the enzyme has little effect on the fluorescence properties, major changes in the fluorescence emission spectrum occur when the metal-free enzyme is exposed to 8M-urea at 25°C. Dilution of the system to 1.5M-urea together with the addition of Zn^{2+} to a final concentration of 1 mM restored the fluorescence emission spectrum to

Fig. 4. *Fluorescence emission spectra of zinc-free phospholipase C*

Zinc was removed from phospholipase C by dialysis for 5 h at room temperature against 2 mM-EDTA in 0.05M-sodium phosphate buffer (pH 6.0). Fluorescence emission spectra (excitation wavelength 285 nm) were run at 25°C before (curve a) and after (curve b) dialysis against EDTA. In one experiment (curve c) after dialysis against EDTA, urea to a final concentration of 8M was added. The enzyme concentration was 9 μ M in all cases.

that of the native enzyme and the enzyme was now fully active. When the thermal stability of the Zn^{2+} -free enzyme was studied (Fig. 5), the enzyme appeared to be much less susceptible to irreversible thermal inactivation in the presence of 8M-urea than in its absence. Indeed, comparison of the results in Fig. 5 with those in Fig. 1(a) shows that in 8M-urea the Zn^{2+} -free enzyme is just as resistant to irreversible denaturation as is the enzyme that contains two zinc atoms. The effect of 8M-urea on the Zn^{2+} -free enzyme could not be attributed to contaminating Zn^{2+} in the urea. Zn^{2+} analyses showed that the enzyme sample used contained less than 0.1 mol of Zn/mol of enzyme in either the presence or the absence of urea.

When Zn^{2+} was added to the metal-free enzyme in 8M-urea and the enzyme assayed, a lag phase analogous to that in Fig. 1(b) was noted. This occurred even with enzyme samples exposed to temperatures no higher than 25°C. Dialysis of enzyme samples against Zn^{2+} -containing buffers removed this lag phase (results not shown).

Effect of urea on the environment of the aromatic amino acids

Perturbation difference spectra were run on enzyme samples exposed to 8M-urea and elevated

temperatures. These studies were made at room temperature with enzyme dissolved in 8M-urea, but not preheated as reference. Enzyme samples preheated up to about 60°C showed little or no spectral

Fig. 5. Effect of temperature on the reactivation of zinc-free phospholipase C

After removal of zinc from the enzyme (see legend to Fig. 4), the enzyme (14 μ M) in the presence (○) or absence (●) of 8M-urea was heated to various temperatures for 5 min and then cooled to 0°C, dialysed for 3 h at room temperature against 0.15M-NaCl containing 1mM-ZnSO₄ and buffered at pH 7.4 by 2.9mM-sodium 5,5'-diethylbarbiturate, and then assayed.

changes in the region 210–310nm (Figs. 6a and 6b) whereas preheating to higher temperatures yielded negative difference maxima at about 287 and 283nm with a shoulder at about 278nm. Preheating at 80°C produced the largest spectral changes in the 270–310nm region. Above 80°C these spectral changes were partially reversed (Fig. 6a).

Preheating phospholipase C in 8M-urea also produced spectral changes around 230nm. As noted with other proteins (Glazer & Smith, 1960, 1961; Móra & Elödi, 1968; Donovan, 1969), these changes were more marked than those observed in the 280nm region. It was also found that preheating the enzyme above 80°C produced still larger spectral changes so that the reversal of the spectral changes is confined to the 280nm region (Fig. 6b).

The quenching by small molecules of tryptophan fluorescence provides information on the environment of tryptophan residues in proteins (Steiner *et al.*, 1964; Lehrer, 1975; Eftink & Ghiron, 1976). Iodide quenching of tryptophan fluorescence in phospholipase C was therefore studied. The data are shown in Fig. 7 plotted by the modified Stern-Volmer equation (Lehrer, 1971):

$$\frac{F_0}{\Delta F} = \frac{1}{[x] f_a K_q} + \frac{1}{f_a}$$

where [x] is the molar concentration of quencher, K_q the quenching constant and f_a the fractional maximum accessible protein fluorescence. F_0 is the quantum yield in the absence of quencher and ΔF is the decrease in quantum yield caused by the quencher. Since the quencher decreased the height of the

Fig. 6. U.v.-absorption difference spectra for phospholipase C after being heated in 8M-urea

Enzyme (9.5 μ M) in 8M-urea and 0.05M-sodium phosphate buffer (pH 6.0) was heated for 5 min at various temperatures. Difference spectra were then run at 25°C with the unheated enzyme as reference. The temperatures used were 45°C (a), 60°C (b), 70°C (c), 80°C (d), 90°C (e) and 100°C (f).

Fig 7. Modified Stern-Volmer plots for iodide quenching of tryptophan fluorescence of enzyme after heating in 8M-urea

The experimental conditions were as described in Table 3. Symbols: ■, enzyme without urea; ●, enzyme in 8M-urea, but not heated. Enzyme heated in 8M-urea to 43°C (○), 80°C (△), 100°C (□).

Table 3. Quenching of tryptophan fluorescence of phospholipase C by iodide

Quenching of tryptophan fluorescence (excitation wavelength 295 nm, emission wavelength 340 nm) was carried out in 0.05M-sodium phosphate buffer (pH 6.0) at 25°C by using 15.2 μM-enzyme. Quenching was carried out on the native enzyme in the above phosphate buffer and also on the enzyme in 8M-urea in the phosphate buffer after 5 min exposure to different temperatures. In the latter case, 8M-urea was present during the quenching studies. NaCl was added to maintain constant ionic strength.

Enzyme sample	f_a	K_q (M ⁻¹)
Native	0.8	4.5
22°C, 8M-urea	0.8	3.8
43°C, 8M-urea	0.77	3.7
80°C, 8M-urea	1	3.8
100°C, 8M-urea	1	3.8

emission maxima without altering the shape of the emission spectra, fluorescence intensities were used rather than quantum yields. Linear plots were obtained (Fig. 7), and from the slopes and intercepts f_a and K_q values were calculated and are shown in Table 3. The results indicate that in the native enzyme and enzyme that had not been preheated in 8M-urea about 20% of the tryptophan fluorescence is inaccessible for quenching by iodide. Preheating the enzyme in 8M-urea at 80°C or above produces enzyme forms where all tryptophan residues are equally accessible to the quencher. No changes in K_q , the quenching constant, were noted as a result of preheating the enzyme in 8M-urea.

Enhancement of the fluorescence of 8-anilino-naphthalene-1-sulphonate may also be used to detect conformational changes in proteins (see, e.g., Dodd & Radda, 1969). However, in the present work no detectable enhancement of fluorescence (excitation wavelength 366 nm, scan emission from 400–600 nm) occurred at 25°C when 8-anilino-naphthalene-1-sulphonate up to 8 mM final concentration was added to 10–15 μM-phospholipase C either to the native enzyme or to the enzyme after preheating at any temperature for 5 min at 8M-urea.

Circular dichroism of phospholipase C

Far- and near-u.v. region c.d. spectra of phospholipase C are shown in Fig. 8. For the native enzyme, the far-u.v. region c.d. spectrum (Fig. 8a, curve a) is that of a globular protein with typical bands for α-helix and β-structure (Rosenkranz, 1974). With the isodichroic method of Rosenkranz & Scholtan (1971), it can be calculated that the enzyme contains 30–36% α-helical structure and 24–30% β-structure, with the remainder being irregular non-periodic conformation. In the near-u.v. region (Fig. 8b, curve a) the native enzyme shows a negative band at 276 nm, a shoulder at 287 nm and a positive band at 297 nm. Both tyrosine and tryptophan residues contribute to the spectrum at 276 nm (Beychok, 1966; Strickland, 1974). The shoulder at 287 nm is probably due to tyrosine chromophores. Tryptophan residues exhibit several bands between 290 and 305 nm, and since phenylalanine and tyrosine residues do not contribute significantly to the dichroism above 290 nm (Horwitz *et al.*, 1970; Strickland, 1974) the 297 nm band may be assigned to the tryptophan chromophores.

The c.d. spectra are not altered by exposure of phospholipase C to 8M-urea at 27°C (curve c). The stronger denaturant 7M-guanidine hydrochloride at 27°C has a drastic effect on the enzyme conformation and no c.d. bands are detectable (curve h). This observation supports the conclusion that the far-u.v. region c.d. spectrum arises partly from a true helical structure and not from the simulation of helical structures by other rigid structures in the enzyme (Jirgensons & deHaas, 1977).

Preheating the enzyme at 43°C in 8M-urea caused marked alterations in the far-u.v. region c.d. spectrum (Fig. 8a, curve f), suggesting extensive denaturation. Lesser changes are noted in the near-u.v. region c.d. spectra, where the magnitude of the 276 nm band has decreased somewhat (Fig. 8b, curve f). These spectra suggest an enzyme form of tightly folded aperiodic structure (Beychok *et al.*, 1966). Preheating of the enzyme in 8M-urea at 80 or 100°C abolishes the near-u.v. region bands, and again the far-u.v. region c.d. spectra indicate extensive denaturation (Figs. 8a and 8b, curves d and g).

Fig. 8. C.d. spectra of phospholipase C

The enzyme was dissolved in 0.02M-sodium phosphate buffer with or without 8M-urea. Zn^{2+} was removed from the enzyme by dialysis at room temperature for 5 h against 2mM-EDTA in the above phosphate buffer. Other conditions were as described in the Materials and Methods section. The curves were: a, native enzyme; b, Zn^{2+} -free enzyme; c, native enzyme in 8M-urea; d, native enzyme in 8M-urea after exposure to 80°C for 5 min; e, Zn^{2+} -free enzyme in 8M-urea; f, native enzyme in 8M-urea after exposure to 43°C for 5 min; g, native enzyme in 8M-urea after exposure to 100°C for 5 min; h, native enzyme in 7M-guanidine hydrochloride. (a) Far-u.v. region spectra; (b) near-u.v. region spectra.

The removal of the zinc atoms from phospholipase C appears to have little effect on the secondary structure (Fig. 8a, curve b), but small changes in the near-u.v. region (Fig. 8b, curve b) suggest small conformational alterations, especially in the vicinity of the tyrosine residues. Zinc atoms themselves do not seem to influence near-u.v. region c.d. (Wood *et al.*, 1971). Exposure of the metal-free enzyme to 8M-urea even at 27°C causes drastic changes in the c.d. spectra (Fig. 8a, curve e), suggesting extensive denaturation.

Discussion

The present data indicate that in 8M-urea phospholipase C is converted into several different conformational forms depending on the temperature to which the enzyme is exposed. Since nearly all the studies in the present work were carried out on the enzyme after cooling to room temperature, then the present data refer only to enzyme forms that survive this cooling process. The simplest interpretation of the results involves the existence/formation of four different conformational forms in 8M-urea, depicted as E_1 , E_2 , E_3 , E_4 . E_1 is the enzyme form existing before the solutions in 8M-urea are heated above room temperature. Form E_1 is very similar indeed to the

native enzyme E. Forms E and E_1 both possess full catalytic activity and contain one chemically reactive histidine residue; they have apparently identical fluorescence emission spectra, quantum yields of fluorescence, accessibility of tryptophan fluorescence to quenching by iodide and c.d. spectra. Forms E and E_1 thus appear to be identical, although previous work (Little, 1977a) suggests that the single available histidine residue in form E_1 is more reactive than that in form E. Form E_1 cannot be regarded as a denatured state and can be considered as being no more than a quasi-native form (Tanford, 1968). At around 43°C another conformational form, E_2 , is produced. Forms E_2 and E_1 are very similar in terms of fluorescence properties and u.v.-absorption spectra and both are catalytically active on contact with substrate in either the presence or the absence of 8M-urea. They differ in two respects: the single available histidine residue is significantly less reactive in form E_2 than in E_1 , and unlike form E_1 , E_2 appears to be markedly denatured, as indicated by far-u.v. region c.d. studies. Form E_2 appears to be tightly folded. At around 60–80°C form E_3 is produced. Form E_3 has no catalytic activity, but regains full activity when the urea concentration is lowered. Form E_3 has a much lower quantum yield of fluorescence than forms E_1 or E_2 and in addition the peak wavelength for fluores-

Table 4. *Characteristics of the postulated conformational forms produced by heating phospholipase C in 8M-urea*
The activity of the native enzyme was defined as 100%.

Enzyme form	E ₁	E ₂	E ₃	E ₄
Temperature where formed (°C)	Room temperature	Approx. 43	60–80	>80
Activity (%)	100	100	0	0
Activity on removal of urea (%)	100	100	100	0
Histidine availability (mol/mol of enzyme)	0.92	0.31	2.79	3.9
f_a	0.8	0.77	1	1
Quantum yield for fluorescence	0.19	0.19	0.08	0.08
Secondary structure	As native enzyme	Non-periodic, tightly folded	Non-periodic	Non-periodic

Scheme 1. *Interconversion of the postulated conformational forms of phospholipase C*

cence emission has undergone a red shift of about 10nm relative to that of the native enzyme. These fluorescence changes are fully reversible on lowering of the urea concentration. Form E₃ has about three chemically available histidine residues. The u.v. absorption spectral changes accompanying the transition E₂ → E₃ are consistent with the perturbation of aromatic amino acids, especially tyrosine residues, in which these chromophores are given generally improved access to solvent (see Donovan, 1969). Form E₃ would thus appear to be fairly loosely folded compared with forms E₁ and E₂.

Enzyme form E₄ is produced at temperatures above 80°C. Forms E₃ and E₄ are indistinguishable in terms of protein fluorescence and c.d. spectra. They have different u.v. absorption spectral properties and form E₄ contains a greater number of chemically reactive histidine residues than does form E₃. Form E₄ does not revert to the native enzyme on removal of urea and is irreversibly inactive. Zn²⁺ analyses showed that even form E₄ contained two zinc atoms and so even this form is not fully opened up and unfolded. Some of the characteristics of these four postulated

enzyme conformations are shown in Table 4 and routes for their interconversion are summarized in Scheme 1.

Phospholipase C contains 7 tryptophan residues (Otnaess *et al.*, 1977). Usually with globular proteins, the highly hydrophobic tryptophan side chains are fully exposed only on extensive denaturation. The iodide-quenching experiments indicated that even in the native enzyme about 80% of the fluorescence was accessible for iodide quenching. Since the iodide ion is a charged and heavily hydrated species it appears that most of the tryptophan residues in the enzyme are exposed. The 8-anilinonaphthalene-1-sulphonic acid fluorescence experiments suggest that the enzyme lacks on its surface any hydrophobic sites capable of binding 8-anilinonaphthalene-1-sulphonic acid.

Commercial urea often contains ammonium cyanate. In addition, aqueous solutions of urea, especially at alkaline pH and elevated temperatures, hydrolyse to ammonium cyanate (Hagel *et al.*, 1971), a reagent capable of modifying several side chains in proteins (Stark, 1967; Means & Feeney, 1971). Nearly all the present work was carried out with urea that had been freshly recrystallized twice from 96% ethanol. Urea solutions were prepared immediately before use and enzyme samples usually analysed within 1 h of being exposed to urea. It is difficult to see how the generation of small concentrations of cyanate ions could be involved in these clearly defined and in some cases reversible transitions in the enzyme. Most of the above results were reproduced on three different batches of phospholipase C and so do not represent the behaviour of an atypical preparation.

The present data suggest that phospholipase C is extremely resistant to the chaotropic agent urea. The enzyme was even active when assayed in 8M-urea. This stability probably arises in part from the zinc atoms in the enzyme. In the zinc-free enzyme, even at room temperature, marked fluorescence changes occur on addition of 8M-urea, and the c.d. spectra

indicate the loss of all ordered structure. In addition in the absence of denaturants the zinc-free enzyme is very sensitive to temperatures of even 40°C and is easily converted into a form that does not regain activity on addition of free Zn^{2+} ions. The zinc atoms in the structure would therefore appear to contribute substantially to the general stability of the enzyme.

The effect of 8M-urea on phospholipase C is rather unusual. High concentrations of urea normally denature and inactivate enzymes fairly readily, but in the present case 8M-urea protects the enzyme from irreversible inactivation. Thus the native enzyme is far less susceptible to irreversible thermal inactivation in the presence of 8M-urea than in its absence. Furthermore, with the zinc-free enzyme, irreversible inactivation occurs very much more readily in the absence than in the presence of 8M-urea. Clearly therefore, zinc is not involved in this apparent stabilization of the enzyme by urea. There is evidence that the high heat stability of the zinc-free enzyme in 8M-urea may involve the formation of a conformation similar to E_3 . At room temperature, the zinc-free enzyme forms in 8M-urea a species that like form E_3 has no c.d. bands above 220nm, has a fluorescence emission spectrum similar to that of E_3 and also on addition of Zn^{2+} ions is initially inactive in the assay system; removal of the urea by dialysis after addition of Zn^{2+} ions produces an active enzyme form. It is possible that phospholipase C contains some core of stable structure, possibly a particularly hydrophobic region, which is further stabilized by high urea concentrations and which serves as a nucleation centre for refolding of the polypeptide chain after denaturation. Studies on synthetic block co-polypeptides have shown that α -helix conformation in blocks of hydrophobic amino acids such as L-alanine and L-leucine is not destroyed by 8M-urea (Auer & Doty, 1966; Jones *et al.*, 1976). In addition, with pancreatic phospholipase A_2 , an enzyme that is extremely resistant to denaturation by 8M-urea (Scanu *et al.*, 1969), the high conformational stability has been attributed in part to hydrophobic interactions in the enzyme (Jirgensons & deHaas, 1977). The observation that form E_3 gives no c.d. bands characteristic of ordered structure rules out the possible existence of any significant extent of residual periodic structure. Thus if the postulated nucleation centre or centres are based on periodic structure they must be small. Alternatively non-periodic structure may be involved. It is hoped that the X-ray crystallographic studies that have just begun on this enzyme (Hough *et al.*, 1978) may give an answer to these unusual stability properties of phospholipase C.

I am deeply indebted to Hermann Rosenkranz, Bayer A. G., D-509 Leverkusen, Germany for measurements and discussion of the circular dichroism. The skilled technical

assistance of Liv Melvær and Sissel Johansen is gratefully acknowledged.

References

- Auer, H. E. & Doty, P. (1966) *Biochemistry* **5**, 1716–1725
- Beychok, S. (1966) *Science* **154**, 1288–1299
- Beychok, S., Armstrong, J. McD., Lindblow, C. & Edsall, J. T. (1966) *J. Biol. Chem.* **241**, 5150–5160
- Chen, R. F., Edelhoch, H. & Steiner, R. F. (1969) in *Physical Principles of Protein Chemistry* (Leach, S. J., ed.), part A, pp. 171–244, Academic Press, New York and London
- Cubero Robles, E. & Van den Berg, D. (1969) *Biochim. Biophys. Acta* **187**, 520–526
- Dodd, G. H. & Radda, G. K. (1969) *Biochem. J.* **114**, 407–417
- Donovan, J. W. (1969) in *Physical Principles of Protein Chemistry* (Leach, S. J., ed.), part A, pp. 102–170, Academic Press, New York and London
- Eftink, M. R. & Ghiron, C. A. (1976) *Biochemistry* **15**, 672–680
- Glazer, A. N. & Smith, E. L. (1960) *J. Biol. Chem.* **235**, PC 43
- Glazer, A. N. & Smith, E. L. (1961) *J. Biol. Chem.* **236**, 2942–2947
- Hagel, P., Gerding, J. J. T., Fiegggen, W. & Bloemendal, H. (1971) *Biochim. Biophys. Acta* **243**, 366–373
- Horwitz, J., Strickland, E. H. & Billups, C. B. (1970) *J. Am. Chem. Soc.* **92**, 2119–2129
- Hough, E., Little, C. & Jynge, K. (1978) *J. Mol. Biol.* **121**, 567–570
- Jirgensons, B. & deHaas, G. H. (1977) *Biochim. Biophys. Acta* **494**, 285–292
- Jones, M. N., Patrick, C. P. & Phillips, M. C. (1976) *J. Colloid Interface Sci.* **55**, 116–125
- Kleiman, J. H. & Lands, W. E. M. (1969) *Biochim. Biophys. Acta* **187**, 477–485
- Lehrer, S. S. (1971) *Biochemistry* **10**, 3254–3263
- Lehrer, S. S. (1975) in *Concepts in Biochemical Fluorescence* (Chen, R. & Edelhoch, H., eds.), vol. 2, pp. 515–544, Marcel Dekker, New York
- Little, C. (1977a) *Biochem. J.* **167**, 399–404
- Little, C. (1977b) *Acta Chem. Scand. Ser. B* **31**, 267–272
- Little, C. & Otnaess, A.-B. (1975) *Biochim. Biophys. Acta* **391**, 326–333
- Little, C., Aurebekk, B. & Otnaess, A.-B. (1975) *FEBS Lett.* **52**, 175–179
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Means, G. E. & Feeney, R. F. (1971) *Chemical Modification of Proteins*, pp. 84–89, Holden-Day, San Francisco
- Móra, S. & Elödi, P. (1968) *Eur. J. Biochem.* **5**, 574–582
- Otnaess, A.-B., Little, C., Sletten, K., Wallin, R., Johnsen, S., Flengsrud, R. & Prydz, H. (1977) *Eur. J. Biochem.* **79**, 459–468
- Ottolenghi, A. C. (1969) *Methods Enzymol.* **14**, 188–197
- Ovadi, J., Libor, S. & Elödi, P. (1967) *Acta Biochim. Biophys. Acad. Sci. Hung.* **2**, 455–458
- Rosenkranz, H. (1974) *Z. Klin. Chem.* **12**, 415–422
- Rosenkranz, H. & Scholtan, W. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 896–904

- Scanu, A. M., van Deenen, L. L. M. & deHaas, G. H. (1969) *Biochim. Biophys. Acta* **181**, 471–473
- Stark, G. R. (1967) *Methods Enzymol.* **11**, 590–594
- Steiner, R. F., Lippoldt, R. E., Edelhoch, H. & Fratteli, V. (1964) *Biopolymers* **1**, 355–366
- Strickland, E. H. (1974) *Crit. Rev. Biochem.* **2**, 113–175
- Tanford, C. (1968) *Adv. Protein Chem.* **23**, 122–282
- Wood, E., Dagleish, D. & Bannister, W. (1971) *Eur. J. Biochem.* **18**, 187–193
- Zwaal, R. F. A., Roelefsen, B., Comfurius, P. & van Deenen, L. L. M. (1971) *Biochim. Biophys. Acta* **233**, 474–479