Biophysical Chemistry 19 (1984) 1–11
Elsevier

BPC 00821

THERMODYNAMICS OF THE BINDING OF Ca^{2+} TO PORCINE PANCREATIC PHOSPHOLIPASE A_2

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Received 7th July 1983
Accepted 18th August 1983

Key words: Phospholipase A_2; Calcium binding; Thermodynamics

The binding of Ca^{2+} to porcine pancreatic phospholipase A_2 was studied by batch microcalorimetry. Enthalpies of binding at 25°C were determined as a function of Ca^{2+} concentration in buffered solutions at pH 8.0 using both the Tris-HCl and Hepes-NaOH buffer systems. The calorimetric results indicate that protons are released on calcium binding and that in addition to the binding of the active-site calcium, there appears to be weak binding of a second Ca^{2+}. Results from potentiometric titrations indicate that this proton release on binding Ca^{2+} arises from a change in pK of a histidine(s) functional group. The thermodynamic functions ΔG^0, ΔH^0 and ΔS^0 for calcium binding to phospholipase A_2 have been determined. These results are compared with literature data for Ca^{2+} complex formation with some small molecules and also the protein troponin-C.

1. Introduction

Phospholipase A_2 (EC 3.1.1.4) is an enzyme which catalyses the specific hydrolysis of the 2-acyl ester linkage of 3-*sn*-phosphoglycerides [1]. This small ($M_r \approx 14000$) water-soluble enzyme is found in high concentrations in mammalian pancreatic tissue and also in snake and bee venoms [2]. The mammalian enzyme is secreted from the pancreas as a zymogen, prophospholipase A_2, and is converted into the active enzyme by tryptic cleavage of the N-terminal heptapeptide [3]. Although both the zymogen and active enzyme hydrolyse monomeric substrates, only the active enzyme can hydrolyse phospholipids in aggregated structures [4].

One interesting feature of the pancreatic phospholipases A_2 is their nearly absolute requirement of Ca^{2+} for catalytic activity. Other group IIA ions, some transition metal and lanthanide cations have been investigated as possible activators of phospholipase A_2 [5,6]. To date, only Gd^{3+} can replace Ca^{2+} with some retention of activity, albeit small (4% of the activity found with Ca^{2+} under identical conditions). This high calcium specificity of pancreatic phospholipase A_2 suggests that the metal ion has a function in the catalysis. A hypothesis for the catalytic role of Ca^{2+} has been reported [7].

Studies using equilibrium gel filtration, ultraviolet difference spectroscopy [5] and ^{43}Ca-NMR [8] indicate that porcine phospholipase A_2 and its zymogen possess only one high-affinity Ca^{2+}-binding site per protein molecule. From the determined association constants it is apparent that the zymogen binds Ca^{2+} with about the same affinity as the active enzyme. In addition to the binding of this catalytically important Ca^{2+}, studies at relatively high calcium concentrations and alkaline pH

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Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

indicate that porcine pancreatic phospholipase A_2 possesses an additional low-affinity calcium-binding site [9]. An association constant for this site has been determined [9]. The porcine zymogen, however, does not possess this low-affinity site. Moreover, high concentrations of Ca^{2+} change the apparent pK of the N-terminal amino group of the active enzyme. From these observations it has been suggested that this second low-affinity Ca^{2+}-binding site in phospholipase A_2 is located close to the N-terminus of the protein molecule [9].

Although association constants have been determined for calcium binding to the porcine enzyme, no data are available to enable a determination of the thermodynamic parameters for these calcium-binding steps. In this paper we report the results of a calorimetric, potentiometric and CD spectroscopic study of calcium binding to porcine pancreatic phospholipase A_2. The thermodynamic quantities ΔG^0, ΔH^0 and ΔS^0 are reported for the binding of Ca^{2+} to the enzyme at 25°C.

2. Experimental

2.1. Materials

Porcine pancreatic phospholipase A_2 was obtained from Sigma Chemical Co. and used without further purification. On arrival, the 3.2 M $(NH_4)_2SO_4$ suspensions from individual vials were combined together and stored at 4°C. Calorimetric, spectroscopic and activity measurements indicated no deterioration in this stock suspension during a 3 month period. The enzyme was found to be electrophoretically homogeneous on a 12 : 1 acrylamide gel in 0.2% SDS, Tris-glycine buffer, pH 8.5.

Enzyme solutions for calorimetric measurements were prepared by initially removing most of the salt by the passage through a short column of Sephadex G-25, using as eluant a buffer at pH 8.00 containing 0.05 M Tris-HCl (or Hepes-NaOH) and 0.10 M NaCl. The eluate was then dialysed exhaustively against the buffer, using dialysis tubing that had previously been treated with approx. 0.1 M $NaHCO_3$ solution containing 2 mM EDTA and rinsed extensively with distilled deionised water. Enzyme solutions for pH titrations were prepared using the same procedure except that the eluant was a solution of 0.10 M NaCl. Protein concentrations were determined by absorbance measurements at 280 nm using an extinction coefficient of 1.30 $cm^3\ mg^{-1}$ [5]. Calcium contamination of the enzyme solutions was monitored by atomic absorption using a Perkin Elmer 303 spectrophotometer. The calcium concentrations in the enzyme solutions were the same, within experimental error, as those of the stock buffers, i.e., 0.15 ppm. At the enzyme concentrations used in the calorimetric experiments, this buffer calcium concentration corresponds to 0.02 mol total Ca^{2+} per mol enzyme.

A stock $CaCl_2$ solution was prepared using Fisher certified ACS $CaCl_2 \cdot 2H_2O$. The calcium concentration was determined by complexometric titration with EDTA standardised using Aldrich 99.999% $CaCO_3$ as the primary standard [10].

All other chemicals used were of the highest purity available. All solutions were prepared using glass-distilled deionised water.

2.2. Calorimetric measurements

Calorimetric measurements were made at 25°C using an LKB 10700-2 batch microcalorimeter equipped with gold reaction cells. The calibration of the instrument was carried out electrically and the accuracy checked by determining the heat of neutralisation of NaOH by HCl [11].

The enthalpy of binding of Ca^{2+} to phospholipase A_2 was determined from the heats of mixing of solutions of enzyme in buffer with solutions of $CaCl_2$ in buffer at the same pH and NaCl concentration. Heats of dilution of the enzyme and $CaCl_2$ solutions were determined separately and subtracted from the measured heat of mixing to obtain the heat of reaction. The observed heats of reaction were in the range -1.3 to -6.2 mJ. To determine whether there is a concomitant proton uptake or release on calcium binding to phospholipase A_2 at pH 8.0, the experiments were carried out in both Tris-HCl and Hepes-NaOH buffer systems. In the mixing and dilution experiments, the calorimeter reference cell contained aliquots of buffer chosen to match closely the

volumes in the reaction cell. The amount of solution added to each compartment of the calorimeter cell was determined by weighing the dispensing glass syringes before and after loading. Prior to loading the calorimeter, the pH at 25°C for both the enzyme and calcium solutions was measured using a Beckman model 3560 digital pH meter with a Fisher microprobe combination electrode. After the calorimetric experiment, the pH of the mixed solution was also determined. The change in pH on mixing was never greater than 0.01 pH unit.

Enzymatic activity [12] and ultraviolet spectroscopic measurements, before and after storage of an enzyme solution in the calorimeter reaction cell, indicated that for the duration of a mixing experiment there was no enzyme degradation. However, the enzyme did 'stick' to the walls of the gold reaction cell and consequently extreme care was taken in the cleaning of the calorimeter reaction cell at the end of each experiment.

2.3. Potentiometric titrations

pH titrations were carried out using a Radiometer recording titration system comprising a PHM82 digital pH meter (readability 0.01 pH unit), a TTT80 titrator, an ABU80 autoburette and an REC80 servograph chart recorder. The Radiometer G222C glass and K4112 calomel electrodes used were standardised using Fisher certified pH 4.00 and pH 7.00 standard buffers. The titration cell was maintained at 25.00 ± 0.05°C using a Forma Scientific model 2006 circulating water bath.

Titration curves were generated by titrating, with standard 0.01 M NaOH, 2.1-cm^3 aliquots of a 0.11 mM enzyme (or enzyme + calcium) solution at pH 4.5 in 0.1 M NaCl. Nitrogen gas saturated with water vapor was flushed over the solution during the titration.

2.4. CD spectra

Circular dichroism measurements were made on a Carey model 60 spectropolarimeter equipped with a CD attachment. All recordings were made at ambient temperature (26 ± 1°C in the sample cell). The ellipticity curves were constructed from an average of three separate scans. Cells of 0.1 cm path length were used in the range 210–250 nm while for the near ultraviolet (250–300 nm) 1-cm cells were used. No attempt was made to collect data below 215 nm because of the high level of instrument noise in this region. The optical activity is presented as the mean residue ellipticity calculated using the standard expression [3] with a value of 112 for the mean residue molar mass.

3. Results

3.1. Calorimetry

The observed enthalpies of reaction, ΔH_{obs}, of Ca^{2+} with phospholipase A_2 in both Tris and Hepes buffers are shown as a function of the total Ca^{2+} concentration in fig. 1. The differences in ΔH_{obs} for the two buffer systems indicate that there is a net change in protonation of the enzyme on binding Ca^{2+}. The enthalpies of protonation of Tris and Hepes buffers are, respectively, -47.44 kJ mol^{-1} [11] and 20.9 kJ mol^{-1} [14]. As the ΔH_{obs} values in Hepes buffer are less exothermic than those in Tris buffer, it follows that protons are released from the enzyme on binding Ca^{2+} at pH 8.0.

The first approach used in analysing the ΔH_{obs} data was to assumed that Ca^{2+} binds to the enzyme with 1 : 1 stoichiometry. The apparent equilibrium constant, K_1', and enthalpy change, $\Delta H_1'$, for the association at pH 8.0 are given by

$$K_1' = \frac{[CaE]}{[Ca][E]} \tag{1}$$

and

$$\Delta H_{obs} = \frac{Q_{obs}}{n_E} = \frac{n_{CaE}}{n_E} \cdot \Delta H_1' \tag{2}$$

where CaE and E represent, respectively, the calcium-enzyme complex and the calcium-free enzyme, n_E is the total number of moles of enzyme, n_{CaE} the number of moles of calcium-enzyme complex and Q_{obs} the observed heat change. The analysis of eqs. 1 and 2 to obtain best-fit values for K_1' and $\Delta H_1'$ was carried out using a generalised non-

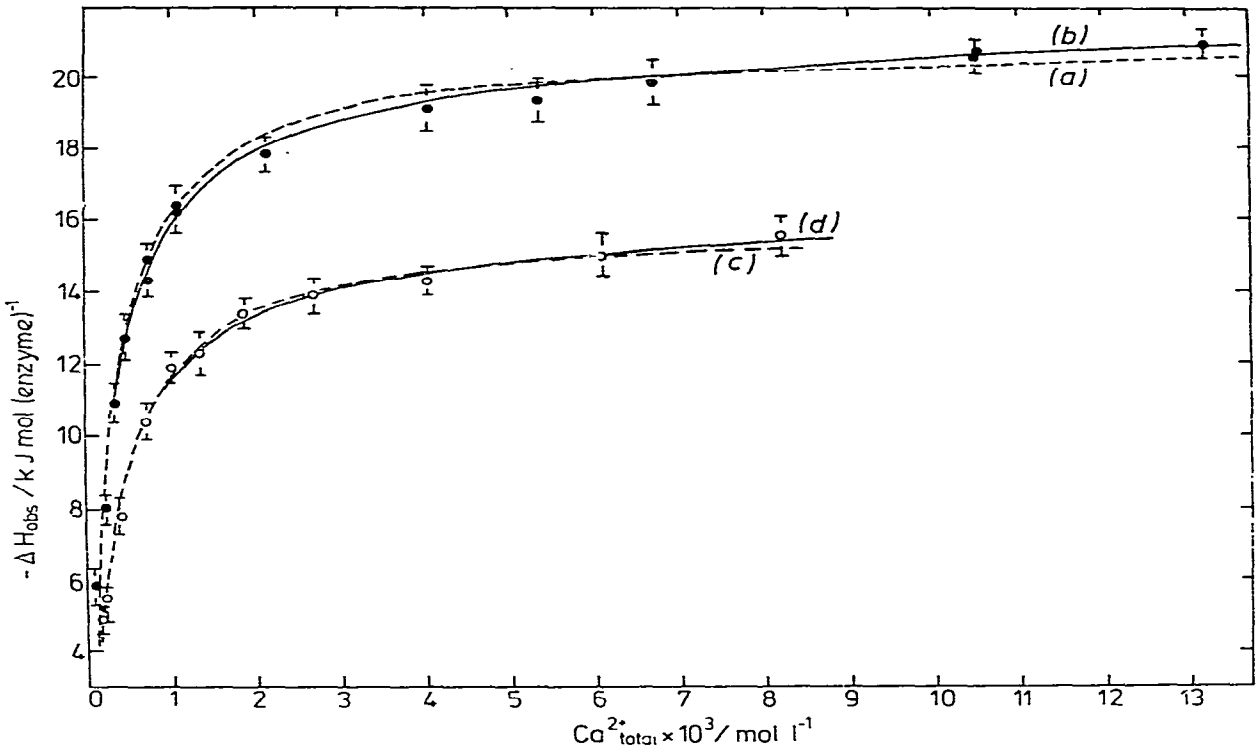

Fig. 1. The observed enthalpy of reaction of Ca^{2+} with phospholipase A_2 as a function of the total calcium concentration; pH 8.00, 0.10 M NaCl, 25°C; [enzyme] = 5.6×10^{-5} M; (●) 0.05 M Tris buffer; (○) 0.05 M Hepes buffer. The error bars for each experimental point represent the estimated uncertainties in ΔH_{obs}. Curves a-d were calculated as described in the text.

linear least-squares computer program [15]. For each ΔH_{obs} data point, a weighting factor inversely proportional to the square of the estimated uncertainty in ΔH_{obs} was included in the calculation. The K_1' and $\Delta H_1'$ results for each buffer system together with their estimated standard deviations are listed in table 1. The difference between the values of K_1' in each of the buffers is greater than

Table 1

Apparent thermodynamic quantities for the binding of Ca^{2+} to phospholipase A_2

Conditions: 0.1 M NaCl, 0.05 M buffer, pH 8.00, 25°C. Model I: one Ca^{2+}-binding site assumed. Model II: two binding sites assumed. R is a least-squares goodness-of-fit index: $R = [\Sigma_i w_i (Y_i^{obs} - Y_i^{calc})^2 / \Sigma_i w_i (Y_i^{obs})^2]^{1/2}$ where Y_i^{obs} and Y_i^{calc} are, respectively, the observed and calculated functions for the i-th observation and w_i its weighting factor. Estimated standard deviations were calculated using the least-squares computer program.

Model	Buffer	K_1'	$\Delta H_1'$ (kJ mol^{-1})	K_2'	$\Delta H_2'$ (kJ mol^{-1})	R
I	Tris	3507 ± 220	−20.9 ± 0.3	–	–	0.034
I	Hepes	2829 ± 134	−15.8 ± 0.2	–	–	0.020
II	Tris	3930	−19.7 ± 0.5	50	−3.5 ± 2	0.028
II	Hepes	3200	−15.0 ± 0.5	50	−2.9 ± 2	0.021

the combined standard deviations. We could find no evidence to suggest that this difference arises from specific interactions of the buffer components with either enzyme or Ca^{2+}. *

The ΔH_{obs} vs. calcium concentration curves calculated using these values of K_1' and $\Delta H_1'$ are shown in fig. 1 (curves a and c). For the data in Tris buffer, the overall agreement between the calculated curve and the experimental points is almost within the experimental uncertainty, however, a trend in the data from 2 to 13 mM calcium is to be noted. The data in Hepes buffer do not extend to high enough total calcium concentrations for such a trend to be discernible. The trend in the Tris buffer data can be interpreted in terms of the weak binding of a second Ca^{2+} to the enzyme. This is consistent with results from equilibrium dialysis and ultraviolet difference spectroscopic studies at high Ca^{2+} concentrations [9].

The binding of this second Ca^{2+} can be expressed using the apparent equilibrium constant

$$K_2' = \frac{[Ca_2E]}{[CaE][Ca]} \tag{3}$$

where Ca_2E represents the calcium-enzyme complex with a second Ca^{2+} bound in the weak binding site. If $\Delta H_2'$ is the apparent enthalpy change for this second binding step then,

$$\Delta H_{obs} = \frac{n_{CaE}}{n_E} \cdot \Delta H_1' + \frac{n_{Ca_2E}}{n_E} \cdot \Delta H_2' \tag{4}$$

where n_{Ca_2E} is the number of moles of calcium-enzyme complex with two Ca^{2+} bound. The ΔH_{obs} data were further analysed using eqs. 1, 3 and 4. The approach used was to fix the values of K_1' and K_2' and calculate best-fit values of $\Delta H_1'$ and $\Delta H_2'$ using the method of least squares. For the data in Tris buffer, the values used for K_1' and K_2' were 3930 and 50, respectively. The value for K_1' was obtained from an analysis, assuming one binding site, using ΔH_{obs} data in the total calcium concentration range 0.09–4.05 mM. This value agrees very favorably with reported constants [9,5] determined in Tris buffer at the same pH and ionic strength. The value used for K_2' was that determined in Tris buffer at pH 7.5 [9]. The $\Delta H_1'$ and $\Delta H_2'$ values and their estimated uncertainties are shown in table 1. The smaller value of the goodness-of-fit index, R, indicates that the incorporation of a second weak binding site improves the overall fit to the ΔH_{obs} data. This is also indicated in fig. 1 where the ΔH_{obs} vs. total calcium concentration curve is shown (curve b). The uncertainties in the $\Delta H_i'$ values were estimated from both the experimental errors in the ΔH_{obs} data and the uncertainties in the K_i' values.

The enthalpy data in Hepes buffer were also analysed using a two-site model. The results obtained (table 1) were used to calculate the ΔH_{obs} vs. total calcium concentration curve as shown in fig. 1 (curve d). Although there is a very slight improvement in the fit in the range 3–8 mM calcium, the overall fit to the experimental data is about the same as for the one-site model. For consistency with the data in Tris buffer, we have assumed the validity of the two-site model for the Hepes buffer system.

As protons are involved in the first Ca^{2+}-binding step, the calculated apparent enthalpy change $\Delta H_1'$ is made up of three terms, the enthalpy change on binding Ca^{2+}, ΔH_1^0, the enthalpy change associated with proton ionisation from the enzyme, ΔH_{EH^+}, and the enthalpy change on protonation of the buffer, ΔH_B. Thus, $\Delta H_1'$ is given by

$$\Delta H_1' = \Delta H_1^0 + \frac{n_{H^+}}{n_{CaE}} \cdot \Delta H_{EH^+} + \frac{n_{H^+}}{n_{CaE}} \cdot \Delta H_B \tag{5}$$

where n_{H^+} is the number of moles of protons associated with Ca^{2+} binding at pH 8.0. Using eq. 5 along with the $\Delta H_1'$ and ΔH_B data for each buffer system the calculated value of the ratio n_{H^+}/n_{CaE} is 0.18 ± 0.04. In order to obtain a value for ΔH_1^0 it remains to determine ΔH_{EH^+}, the apparent enthalpy change associated with proton ionisation from a specific group(s) on the enzyme when a Ca^{2+} binds at pH 8.0. A value for ΔH_{EH^+} was estimated as follows. The enthalpy change on mixing a solution of unbuffered enzyme at pH 8.0 in 0.1 M NaCl with HCl in 0.1 M NaCl was determined by batch microcalorimetry. Heats of dilution of the reactants were shown to be negligi-

* Potentiometric titrations of Tris-HCl and Hepes solutions in the presence and absence of Ca^{2+} indicate that the interaction of Ca^{2+} with the buffer components is negligible.

ble. From three experiments where the mole ratios of HCl to enzyme were 0.73, 0.64 and 0.38 the observed enthalpy changes were -29.9, -27.9 and -29.4 kJ mol^{-1}, respectively. The observed enthalpy change is the sum of the enthalpies of protonation of a number of different functional groups on the enzyme. These groups are probably the N-terminal amino group and imidazole side chains of the three histidine residues (vide infra). It would appear, therefore, that the enthalpies of protonation of the functional groups involved in these protonation experiments are approximately the same. We have assumed a value for ΔH_{EH^+} of $+29 \pm 1$ kJ mol^{-1}. The value of ΔH_1^0 calculated using eq. 5 is reported in table 2.

The apparent equilibrium constant at pH 8.0, K_1', can be corrected for the contribution from the associated proton ionisation on calcium binding using the relationship

$$K_1 = K_1'(1 + [H^+]/K_{EH^+}) \quad (6)$$

where K_1 is the calcium-binding constant and K_{EH^+} the effective ionisation constant for the enzyme function group(s) involved. In deriving this relationship it has been assumed that Ca^{2+} does not bind to the form of the enzyme with this functional group(s) protonated. Using the Henderson-Hasselbach equation and the n_{H^+}/n_{CaE} ratio at pH 8.0, the effective pK_{EH^+} is calculated to be 7.3 ± 0.1. Using this pK_{EH^+}, K_1 calculated using eq. 6 is 4714 ± 600. It is worth noting that using these values for K_{EH^+} and K_1 the calculated values for K_1' at pH 7.5 and 7.0 are in excellent agreement with those determined experimentally [5].

The estimated uncertainty in $\Delta H_2'$ of ± 2 kJ mol^{-1} is too large to enable a reliable determination of whether there is a concomitant net change in protonation of the enzyme on binding a second Ca^{2+} at pH 8.0. From the $\Delta H_2'$ results it would appear that the net change in protonation is close to zero. A calorimetrically observable change might have been expected, based on the results of a recent ^{13}C-NMR study [9]. For a sample of porcine enzyme modified by replacing the N-terminal amino acid L-alanine by a 90% enriched L-[3-^{13}C]alanine there appears to be an increase in the apparent pK of the α-NH_3 group from 8.4 to 9.4 in high concentrations of calcium. At levels of calcium just sufficient to saturate the strong binding site, the pK remains the same as for the calcium-free enzyme. If the α-amino group is the only one with a change in pK on binding the second Ca^{2+}, then a net change in protonation should be clearly evident from the $\Delta H_1'$ data. As this is not the case, perhaps there are changes in the protonation of other functional groups involved in the binding of this second Ca^{2+}. It is also worth noting that as a consequence of the modification procedure used, the phospholipase A_2 sample used in the NMR study [9] had the ϵ-NH_2 groups of all lysine residues protected by acetamidation and so was different from the native enzyme. Assuming the net contribution of the involvement of any protons in the binding of the second Ca^{2+} is zero, then the thermodynamic quantities for the second binding step at 25°C and in 0.1 M NaCl are: $\Delta G_2^0 = 9.7 \pm 0.7$ kJ mol^{-1}, $\Delta H_2^0 = -3 \pm 2$ kJ mol^{-1}, $\Delta S_2^0 = +23 \pm 9$ J K^{-1} mol^{-1}.

3.2. Potentiometric titrations

Fig. 2 shows the titration curves of phospholipase A_2 in the pH range 5–9, obtained in the absence and presence of Ca^{2+}. The data have been plotted in the form of the experimental pH versus $\bar{r}_H$, the average number of H^+ removed per mole of enzyme. The values of $\bar{r}_H$ were calculated using an arbitrary reference pH taken as the pH at the start of each titration. For the region of the titration curve pH < 6, where carboxyl ionisation might be expected [16], there is a large shift to lower pH in the presence of an excess of Ca^{2+}. From chemical modification studies [17] and refinement of the X-ray structure of the bovine enzyme [18], a carboxyl side chain has been identified as one of the groups involved in the coordination of the active-site calcium (vide infra). The observed displacement of the titration curve in the carboxyl region is consistent with carboxylate coordination to the calcium. The region of the titration curve where imidazole ionisation occurs is also displaced to lower pH in the presence of Ca^{2+}. We have looked more closely at this pH range 6.5–8 because the functional groups which titrate in this region will be those that participate in the release

of protons on binding Ca^{2+} to phospholipase A_2 at pH 8.0. The porcine enzyme has three histidine residues and one α-amino group [19] that would be expected to be involved in proton ionisation in the pH range 6.5–8. This region of each titration curve was analysed as follows. For a tetraprotic acid H_4A the average number of protons bound to A, $\bar{\upsilon}_H$, is given by

$$\bar{\upsilon}_H = \frac{\sum_{i=1}^{4} i(a_H)^i / \prod_j^i K_j}{1 + \sum_{i=1}^{4} (a_H)^i / \prod_j^i K_j} \qquad (7)$$

where a_H is the activity of H^+ and K_j the acid dissociation constant for H_jA. For a given set of K_j, a curve of $\bar{\upsilon}_H$ versus pH can be constructed and compared with the phospholipase titration curve over the same pH range. The values of K_j are then adjusted to give the best fit to the shape of the enzyme titration curve. These calculations using eq. 7 were performed with the aid of a modified version of the computer program COMICS [20]. For the titration of phospholipase A_2 in the absence of Ca^{2+} the region of the curve from pH 6.5 to 8 can be fitted using four pK values of 8.4, 7.3, 6.7 and 6.6. The fit to the titration curve is shown in fig. 2. These acid dissociation constants are stoichiometric constants [21] and consequently it is not possible to assign these unequivocally to individual functional groups on the enzyme. The acid dissociation with an ascribed pK of 8.4 probably has a dominant contribution from amino group ionisation. The apparent pK of the α-$\overset{+}{N}H_3$ group determined by NMR [9] is 8.4, which is in good agreement with the titration result. The other three pK values will be predominantly associated with ionisations from the imidazole side chains of His-17, His-48 and His-115.

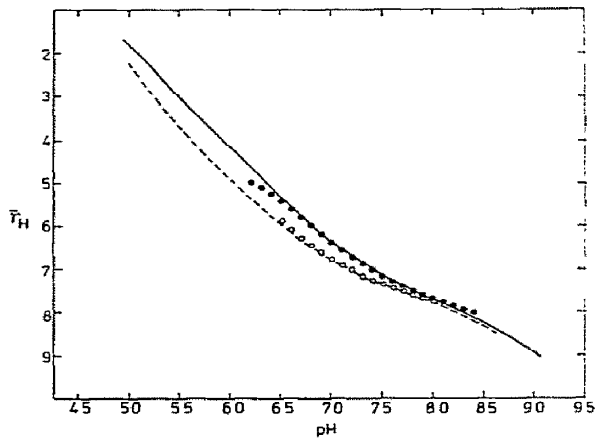

Fig. 2. Potentiometric titrations of phospholipase A_2 in 0.1 M NaCl, 25°C; (———) no Ca^{2+}, (- - - - - -) 9.2 mM Ca^{2+}, (●———●) and (○- - - - - -○) are the calculated curves based on the ionisation of four functional groups as described in the text.

For the titration curve in the presence of 9.2 mM Ca^{2+}, a good fit to the neutral pH region is obtained using pK values of 8.3, 7.0, 6.5 and 6.2, as shown in fig. 2. The major difference between these dissociation constants and those obtained in the absence of Ca^{2+} is the decrease in the three pK values that are associated with histidine ionisations. From an ^{1}H-NMR study of pancreatic phospholipases A_2 [22], apparent intrinsic pK^* values of the histidine residues have been determined both in the presence and absence of Ca^{2+}. The NMR study was carried out at 40°C and in ^2H_2O so the absolute pK^* values obtained cannot be used to analyse further the stoichiometric acid dissociation constants obtained in this study. On binding Ca^{2+} to phospholipase A_2, the NMR results indicated no change in the intrinsic pK^* value for either His-17 or His-115 but there was a considerable drop in the pK^* for the active-site histidine, His-48 [22]. This result is consistent with the general lowering of the three pK values associated with histidine ionisation obtained from analyses of the pH titration curves.

The quantity n_{H^+}/n_{CaE}, the number of moles of protons dissociated from the enzyme per mole of calcium-enzyme complex formed at pH 8.0, was shown from our calorimetric data to be 0.18 ± 0.04. This ratio was also calculated using the pK values obtained from the above analysis. For each set of four pK values the fraction of enzyme in each of the forms H_jE ($j = 1$–4) at pH 8.0 was calculated. From the differences between these fractions without Ca^{2+} and in the presence of 9.2 mM Ca^{2+}, the fraction of protons ionised on forming the calcium-enzyme complexes was 0.14. Under the

conditions of 9.2 mM Ca^{2+} and a total enzyme concentration of 0.1 mM, the first binding site is saturated so the ratio n_{H^+}/n_{CaE} is 0.14 which is in good agreement with that obtained from the calorimetric data. It should be stressed that this analysis does assume that the addition of a second Ca^{2+} to form Ca_2E does not result in a net change in the protonation of the enzyme.

3.3. CD spectra

The CD spectra of porcine phospholipase A_2 are shown in fig. 3. In the far-ultraviolet region a negative band was observed at 223 nm. The addition of Ca^{2+} has no effect on the position or shape of this band. This observation suggests that the binding of Ca^{2+} does not induce any significant changes in the secondary structure of the enzyme. In the near-ultraviolet region, the CD spectrum of the apoenzyme shows positive bands at 283, 265 and 255 nm. These band positions and their amplitudes are in good agreement with those reported by Jirgensons and De Haas [23]. Porcine phospholipase A_2 contains seven disulfide bridges, one tryptophan, eigth tyrosine and five phenylalanine residues [19] which could contribute to the near-ultraviolet CD spectrum. On the basis of CD spectra of other proteins and polypeptides [24,25], the

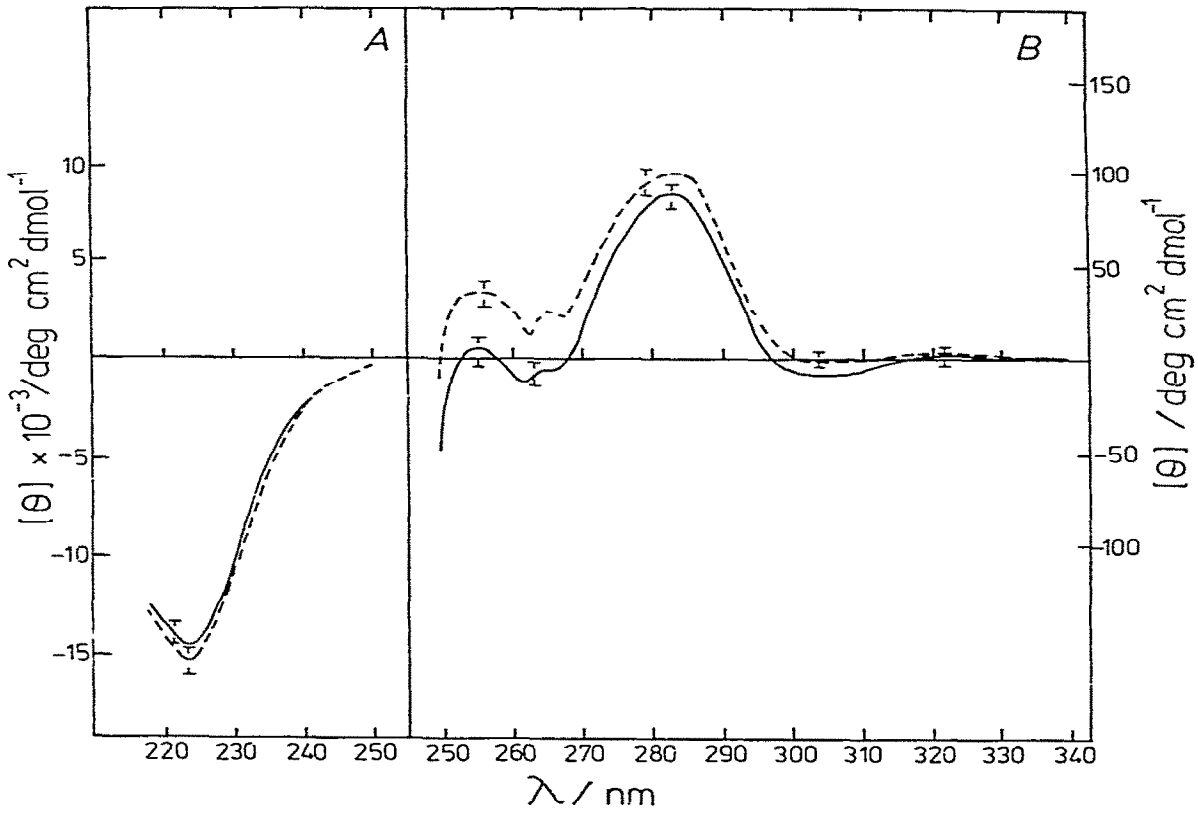

Fig. 3. CD spectra of phospholipase A_2 in 0.05 M Hepes-NaOH buffer, pH 8.0, 0.10 M NaCl. Panel A: (- - - - - -) 0.10 mg cm^{-3} enzyme, (———) 0.10 mg cm^{-3} enzyme + 11.3 mM $CaCl_2$. Panel B: (- - - - - -) 0.95 mg cm^{-3} enzyme, (———) 0.95 mg cm^{-3} enzyme + 6.3 mM $CaCl_2$. The error bars represent the noise level in the spectra.

positive band at 283 nm will be predominantly associated with tyrosine side chains while the bands in the 250–270 nm range probably arise from a combination of the disulfide bridges and the phenylalanine side chains.

Small changes in the near-ultraviolet CD spectrum of phospholipase A_2 are observed on the addition of Ca^{2+}. There is a slight decrease in the ellipticity of the positive band at 283 nm while in the 250–270 nm region the changes, although still small, are more significant.

4. Discussion

The thermodynamic data shown in table 2 for the binding of the active-site Ca^{2+} to phospholipase A_2 indicate that the enthalpy change makes a very significant contribution to the overall free energy change. In order to interpret these data it is useful to look at some typical calcium-small molecule complexes. A Ca^{2+} is classified as being hard or class (a) [26] and consequently has a preference for complex formation with ligands possessing hard donor atoms, for example, oxygen donors [26,27]. The majority of known calcium complexes both in the solid state and solution have oxygen donors [28,29]. Thermodynamic data for complex formation in water of Ca^{2+} with some oxygen donor ligands are shown in table 2. The entropy changes are large and positive while the enthalpy changes are small and either exo- or endothermic, the latter being more common. The interaction between Ca^{2+} and an oxygen donor is predominantly electrostatic in nature and the energy gained on union of the metal ion and ligand is offset by the energy required to disrupt hydration, which is generally quite strong for hard acceptors and donors [30]. The net effect is a small, often endothermic, enthalpy change. The dehydration of the metal ion and ligand on complex formation causes liberation of electrostricted water molecules which results in a large gain in entropy and so ΔS^0 is large and positive. It is clear from the data in table 2 that Ca^{2+} complex formation with phospholipase A_2 does not follow the pattern established with small carboxylic acid-type ligands. One important difference between a macromolecular ligand like phospholipase A_2 and a small multidentate ligand is that in the metal ion-binding region of the macromolecule, the donor groups are often prearranged in a way ideally suited for complex formation. This is not the situation for a flexible small ligand. Moreover, the metal-binding region of a protein might be expected to have a decreased accessibility to the solvent compared with a hypothetical open-chain small molecule containing the same donor groups. This being the case, the degree of hydration of the ligand groups would be considerably reduced and as a consequence, metal ion complex formation would lead to a more exothermic ΔH and less positive entropy change than are found for a typical small ligand. The ΔH^0 and ΔS^0 data for Ca^{2+} binding to phospholipase A_2 are consistent with a reduced hydration of the donor functional groups in the metal-binding region, compared with an analogous small molecule as a ligand. There is some support for this hypothesis arising from studies of complex formation with macrocyclic polyethers [31]. The cyclic nature

Table 2

Thermodynamic data at 25°C for 1:1 Ca^{2+} complex formation with various ligands

The standard state is the hypothetical ideal solution of unit concentration in water or the appropriate ionic medium.

Ligand	ΔG_1^0 (kJ mol^{-1})	ΔH_1^0 (kJ mol^{-1})	ΔS_1^0 (J K^{-1} mol^{-1})
Phospholipase A_2	-21.0 ± 0.4	-16.4 ± 1.4	$+15 \pm 6$
$CH_3N(CH_2COO^-)_2$ [a,d]	-22.0	-5	$+57$
SO_4^{2-} [b,e]	-13.2	$+6.4$	$+66$
$CH_2(COO^-)_2$ [b,f]	-14.3	$+8$	$+75$
Troponin-C [a,e]	-32.2	-32.2	0

[a] $\mu = 0.1$ M. [b] Zero ionic strength. [c] Ref. 33. [d] Ref. 37. [e] Ref. 38. [f] Ref. 39.

of these ligands means that the ether donor oxygens are in a prearranged orientation ideally suited for complex formation and also that these donor groups have a reduced access to the solvent compared with an open-chain ligand. Although there are no thermodynamic data available for Ca^{2+}-polyether complex formation in water, for coordination of the chemically closely related Sr^{2+} with 18-crown-6 [32] the enthalpy change is large and exothermic while the entropy change is very small. This is very similar to what is observed for Ca^{2+} binding to phospholipase A_2.

The affinity of phospholipase A_2 for Ca^{2+} is much lower than for other calcium-binding proteins like tropin-C and parvalbumin [28]. Thermodynamic data have been determined for Ca^{2+} binding to both the Ca^{2+}-Mg^{2+} sites and Ca^{2+}-specific sites of rabbit skeletal muscle troponin-C [33]. The results for binding to the Ca^{2+}-specific sites are shown in table 2. The greater affinity for Ca^{2+} of these sites compared with the active site of phospholipase A_2 results from a much larger exothermic enthalpy change. As enthalpy changes are the quantities most directly related to changes in bond strengths, the enthalpy data suggest overall stronger bonding of the Ca^{2+} in troponin-C compared with phospholipase A_2. The X-ray structure has recently been reported [18] of bovine pancreatic phospholipase A_2. Structural refinement from a resolution of 0.24 nm [34] to 0.17 nm has produced a more detailed description of the Ca^{2+}-binding site in the molecule. The Ca^{2+}-binding site in the porcine enzyme is expected to be very similar to that of the bovine species. This is supported by the high degree of homology between the porcine and bovine enzymes [19], especially in the Ca^{2+}-binding loop, and also by the marked similarities in the CD spectra of the enzyme from the two sources [23]. The Ca^{2+} coordination geometry is approximately octahedral with the carboxyl of Asp-49, the peptide carbonyls of Tyr-28, Gly-30 and Gly-32, and two water molecules as the coordinating groups [18]. For troponin-C the coordination geometry in the Ca^{2+}-specific sites has been predicted to be octahedral with three or four of the donors being carboxyl groups [35]. The smaller exothermic ΔH^0 for Ca^{2+} binding to phospholipase A_2 compared with that for troponin-C is consistent with the lower number of negatively charged donor groups.

On binding of calcium to porcine phospholipase A_2 small changes in the near-ultraviolet CD spectra of the enzyme are observed. These observed changes arise because of changes in the local environment of some of the side-chain chromophores which absorb in the near-ultraviolet region [25]. As there is no significant alteration in the enzyme secondary structure on calcium binding, these small spectral changes probably arise from chromophores near the calcium-binding region of the enzyme. From the three-dimensional structure [18] one of the Ca^{2+}-binding functional groups is the peptide carbonyl oxygen of Tyr-28. It is quite conceivable that the environment of the aromatic ring of this tyrosine residue will change when a Ca^{2+} binds to the enzyme. This is supported by the fact that a tyrosine ultraviolet difference spectrum is observed on binding of the active-site Ca^{2+} [5,9]. Moreover, residues 27 and 29 are both cystines linked to residues 123 and 45, respectively. A change in the orientation of the peptide carbonyl of Tyr-28 on coordination to the Ca^{2+} could alter either the dihedral angle of the disulfide or the C–S–S bond angle which would lead to changes in the CD spectrum [25]. Changes in the environment of tyrosine and disulfide chromophores that are in very close proximity to the Ca^{2+} could account for the observed small changes in the CD spectrum of phospholipase A_2 on binding calcium.

Our calorimetric study has produced further evident in support of the proposed weak binding of a second Ca^{2+} to phospholipase A_2 [9]. The results of a recent ^{43}Ca-NMR study of calcium binding to porcine phospholipase A_2 and prophospholipase A_2 also provide evidence for a low-affinity site on the enzyme. For prophospholipase A_2 the linewidth data could be fitted to a model of a single calcium binding equilibrium [8] while for phospholipase A_2 a second low-affinity binding site had to be included to account for the linewidth data [36]. With such a low binding constant for this second Ca^{2+} it is difficult to obtain reliable thermodynamic data. Assuming no net involvement of protons in the binding step, the thermodynamic quantities obtained indicate that both the

enthalpy and entropy changes contribute significantly to the overall free energy change. These data are consistent with the binding to a site on the enzyme that is exposed to the solvent, perhaps on the enzyme surface. The biochemical significance of this weak binding site is not yet certain. It has been suggested [9] that this second calcium stabilises the interface recognition site and enables more effective catalysis at alkaline pH.

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