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Hydrogen-Ion-Titration Studies of Pancreatic Phospholipase A and Its Zymogen

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The titration curves of porcine pancreatic phospholipase A an its zymogen were measured in the pH region 2.0 to 9.5 at 25 °C.

In the pH region 2 to 6 the titration curves were measured using an Ag-AgCl electrode as reference electrode, at ionic strength 0.5. It was found that the titration of the carboxyl groups could be described with the Linderstrøm-Lang model using an intrinsic pK of 4.5 and an electrostatic interaction factor w of 0.073. The side-chain carboxyl group of the second (*viz.* glutamyl) residue in prophospholipase appeared to have an abnormally low pK of 3.7. The maximum positive charge of the two proteins as determined by titration agreed well with the values predicted by the amino acid composition.

From an analysis of the titration curves covering the region 4.5 to 9.5, which were measured with a calomel electrode at ionic strength 0.1, the following could be concluded. The intrinsic pK and w of the carboxyl groups appeared to be at this ionic strength 4.7 and 0.114, respectively. In both phospholipase and prophospholipase the number of titratable carboxyl groups was found to be 3 higher than given by the amino acid analysis. All 3 histidines present were titratable with an intrinsic pK of 6.6 and w near zero. The terminal amino group which is free only in phospholipase appeared to have an apparent pK of 8.3.

Phospholipase A acts on 3-*sn*-phosphoglycerides with specific hydrolysis of fatty acid ester bonds at the glycerol C-2 position [1]. In pancreatic juice of man [2], rat [3] a d pig [4] the enzyme has been shown to occur not in its active form, but as an enzymatically inactive precursor. Limited proteolysis catalysed by trypsin converts the zymogen into the active enzyme. The precursor of porcine pancreatic phospholipase A has been shown to be a single-chain protein consisting of 130 amino acids [4] and the complete amino acid sequence has been reported [5,6]. The molecule, being intramolecularly cross-linked by six disulfide bridges contains pyroglutamic acid as N-terminal amino acid. Activation of the zymogen is initiated by the tryptic hydrolysis of the Arg-7—Ala-8 bond resulting in the release of the amino terminal heptapeptide Pyroglu-Glu-Gly-Ile-Ser-Ser-Arg [7] and active enzyme. Previous attempts to detected conformational differences between the prophospholipase and the active enzyme by applying optical rotatory dispersion and circular dichroism techniques [8] indicated for both proteins a high content in α-helix but only minor conformational changes during the activation could be observed even in the presence of high concentrations of urea or guanidine-HCl. In order to obtain additional information on the structure of the zymogen and the active enzyme, hydrogen ion titration curves of both proteins were measured and analysed.

Enzyme. Phospholipase A (EC 3.1.1.4).

MATERIAL AND METHODS

Phospholipase and prophospholipase were isolated from porcine pancreas as described earlier [1,4]. The lyophilized enzymes were dissolved in water and subsequently deionized by passing through a mixed-bed ion-exchange column using a recycling system. The titration equipment has been described extensively earlier [9].

However, for the titration towards low pH we used instead of a calomel an Ag-AgCl electrode of the electrolytic type [10]. This electrode can serve for our purposes as a reference electrode provided that the activity of the chloride ions remains constants during titration. The titration procedure was as follows. First the pH meter equipped with the calomel electrode was calibrated with standard 0.05 M

phosphate and 0.05 M phthalate buffers [10]. Subsequently these standard buffers were made 0.5 M in KCl. It was found that due to the change in ionic strength the pH of the phosphate buffer changed from 6.865 to 6.665 and the phthalate from 4.008 to 3.698 at 25 °C. These high ionic strength buffers were used for calibrating the pH meter when the Ag-AgCl electrode was used as reference electrode. In that case the protein solutions and the titrant (HCl) were made 0.5 M in Cl^- too by the addition of KCl. A blank titration to low pH of a 0.5 M KCl solution was performed to correct for the concentration of free H^+ ions. The advantages of the Ag-AgCl electrode compared with the calomel electrode are the higher reproducibility and the more rapid response.

Protein concentrations were measured spectrophotometrically at 280 nm with $A_{1\,cm}^{1\%}$ 14.8 for phospholipase and 14.2 for prophospholipase at pH 8, as determined by amino acid analysis.

The titrations at low pH were performed at 25 °C and at an ionic strength of 0.5 (KCl). The protein concentrations were about 0.4% (4 ml). As titrant we used 0.07 M HCl. The isoionic point was taken as starting point. No backward curves were measured,

The titrations in the neutral region were performed at 25 °C and at an ionic strength of 0.1 (KCl). The protein concentrations were about 0.9% (3 ml). In this case too the titrations started from the isoionic point. The forward and back titration curves showed no irreversibility.

The curves presented are the mean of measurements on two different samples which showed no significant differences. The calculations were based on a molecular weight of 13900 for phospholipase and 14600 for prophospholipase as can be derived from the amino acid composition [5, 6].

ANALYSING PROCEDURE

For the analysis of the titration data we followed the principles outlined earlier [9, 11]. We wish to mention shortly the equation used in this paper.

$$\mathrm{pH} = pK_i + \log(\alpha_i/1 - \alpha_i) - 0.868\, wZ \qquad (1)$$

where K_i is the intrinsic dissociation constant of the n_i groups of class i, α_i their degree of dissociation and w the electrostatic interaction factor. As usual we assume that $Z = \bar{Z}_H$, where $\bar{Z}_H$ is the mean charge of the protein caused by binding of protons only. For $\bar{Z}_H$ we have the relation

$$\bar{Z}_H = Z_{max} - \Sigma\, n_i\, \alpha_i \qquad (2)$$

where Z_{max} is the maximum positive charge given by

$$Z_{max} = n_{His} + n_{\alpha\text{-amino}} + n_{Lys} + n_{Arg} \qquad (3)$$

Equation (2) can be written as

$$\bar{Z}_H = Z_{max} - n_{His}\,\alpha_{His} - \Sigma'\, n_i\, \alpha_i \quad \text{or}$$

$$n_{His}\,\alpha_{His} = Z_{max} - \bar{Z}_H - \Sigma'\, n_i\, \alpha_i \qquad (4)$$

For $-\mathrm{dpH}/\mathrm{d}\bar{Z}_H$ which represents the slope of a normal titration curve and which can be viewed as the reciprocal of the buffer capacity, we have the relation

$$-\mathrm{dpH}/\mathrm{d}\bar{Z}_H = 1/[2.303\, \Sigma\, n_i\, \alpha_i\, (1 - \alpha_i)] + 0.868\, w \qquad (5)$$

A plot of $-\mathrm{dpH}/\mathrm{d}\bar{Z}_H$ *versus* $\bar{Z}_H$ will be called a differential titration curve. Such a curve normally shows two peaks, one near pH 6 and the second near pH 9. The position of the second peak on the $\bar{Z}_H$ axis is given by

$$Z_{II} = n_{Lys} + n_{Arg} - n_{COOH}\,. \qquad (6)$$

This equation may be obtained by combining Equations (2) and (3) assuming that at the pH of the second peak the degree of dissociation of the carboxyl, histidyl and terminal amino groups is one, and zero for all other groups. If the α-amino groups are not fully dissociated at the pH of the second peak then Equation (6) becomes

$$Z_{II} = n_{Lys} + n_{Arg} - n_{COOH} - n_{\alpha\text{-amino}} \cdot (1 - \alpha_{\alpha\text{-amino}})\,. \qquad (7)$$

RESULTS AND DISCUSSION

Titration in the pH Region 2 to 6

Fig. 1 shows the normal titration curve of phospholipase and prophospholipase in the pH region 6 to 2. Near pH 2 both curves tend to a plateau because the degree of dissociation of all groups goes to zero

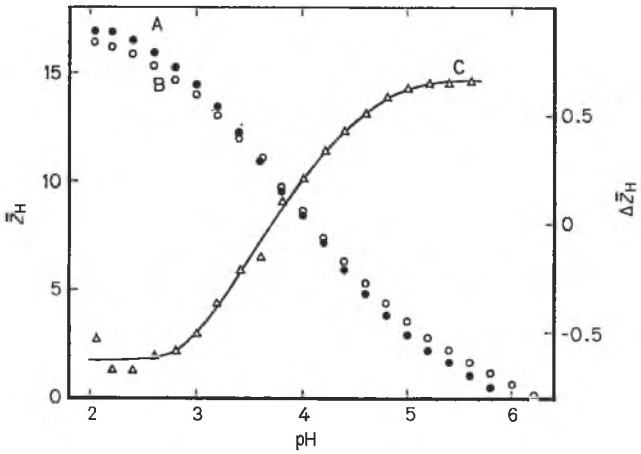

Fig. 1. *Normal titration curve of prophospholipase (A) and phospholipase (B).* Curve C gives the difference between curve B and A

Table 1. *Partial amino-acid composition of phospholipase and prophospholipase [5,6]*
Prophospholipase has a N-terminal pyroglutamic acid residue [7]; since this residue does not contain a free α-amino group its has been left out in this table

Nature of groups	Prophospholipase	Phospholipase
α-COOH	1	1
Asparagine	17	17
Asparatic acid	6	6
Glutamine	1	1
Glutamic acid	7	6
Histidine	3	3
α-Amino	—	1
Lysine	9	9
Tyrosine	8	8
Arginine	5	4

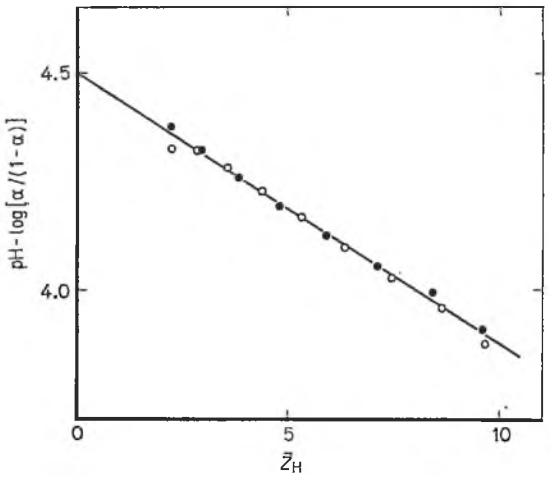

Fig. 2. *Determination of* pK_i *and* w *for the carboxyl groups of (●) prophospholipase and (○) phospholipase*

and so $\bar{Z}_H$ goes to Z_{max} (Equation 2). At pH 2 $\bar{Z}_H$ amounts to 16.9 for prophospholipase and 16.3 for phospholipase, whereas the expected value of Z_{max} is 17 for both of them (see Table 1). As regards phospholipase the value of 16.3 might indicate that even at pH 2 $\bar{Z}_H$ is not yet equal to Z_{max}. On the other hand this value lies very probably within the experimental accuracy by which these numbers can be estimated.

Fig. 1 also shows the difference titration curve of the two proteins in this pH region. This figure corresponds with the titration curve of a single group. Studying Table 1 it is likely that this curve can be attributed to the side-chain carboxyl group of a glutamic acid residue which the prophospholipase has in excess. From this curve we estimated an apparent pK of about 3.7 for the carboxyl group of this glutamic acid which is the second amino acid in prophospholipase. Such a pK is abnormally low. This might mean that this particular residue is linked to or at least in the neighbourhood of a positively charged group.

Fig. 2 gives a plot of pH — log (α/1 — α) *versus* $\bar{Z}_H$, where α represents the degree of dissociation of the carboxyl groups; α was calculated as $\alpha = (Z_{max} - \bar{Z}_H)/n_{COOH}$. For prophospholipase we substituted $n_{COOH} = 17$ and for phospholipase a value of 16, using $Z_{max} = 17$ for both. These values for the number of carboxyl groups are in contrast with the numbers given by the amino acid analysis but this discrepancy is discussed below. As can be seen in the figure both proteins seem to obey Equation (1) in the region shown. From the slope and intercept we calculated at ionic strength 0.5 $w = 0.073$ and $pK_i = 4.5$ for both proteins. As at constant temperature and ionic strength w only depends on the radius of the protein we conclude that both proteins have, as expected, the same radius. From similar experiments at ionic strength 0.1 we estimated $w = 0.114$ and $pK_i = 4.7$ for both proteins. This value of w may be compared with the w of the carboxyl groups of ribonuclease (which has a similar molecular weight) *viz.* 0.102 at ionic strength 0.15 [12]. The calculated w amounts to 0.059 at ionic strength 0.5 and to 0.086 at ionic strength 0.1. For these calculations we assumed a partial specific volume of 0.73 and a hydration of 0.2 [13]. Thus the experimental values of w are higher than the theoretical ones.

The difference of 0.2 in pK_i at the two ionic strengths reflects very probably a specific ionic strength dependence of pK_i not described by the Linderstrøm-Lang model [14]. The Debye-Hückel theory predicts a difference of about 0.05 to 0.1 (depending on the assumption about the radius of the ion) in pK_i at the two ionic strengths used in our experiments [15]. It should be noted in this respect that the experimental value of pK_i of the carboxyl groups seems to be fairly high since from experiments on model compounds Tanford and Nozaki estimated a pK_i of 4.1 for aspartyl and 4.5 for glutamyl carboxyl groups at zero ionic strength [16]. In addition to that the question can be put forward as to what the effect is of the presence of two classes of carboxyl groups on the linearity of a plot as shown in Fig. 2 where all groups were assumed to have the same pK_i. Therefore we calculated a number of titration curves with two classes of carboxyl groups with different pK_i. For the number of glutamic acids we used a value of 6, for that of the aspartic acids a number of 9; the glutamyl carboxyl groups were assumed to have the higher pK_i. The number of groups and the pK values of the other classes was chosen as described later. Considering these calculated curves as being experimental, a plot as shown in Fig. 2 was constructed from it assuming only one class of carboxyl groups. Varying the pK difference between the two classes of carboxyl groups from 0.4 to 0.7 a number of plots was obtained which were

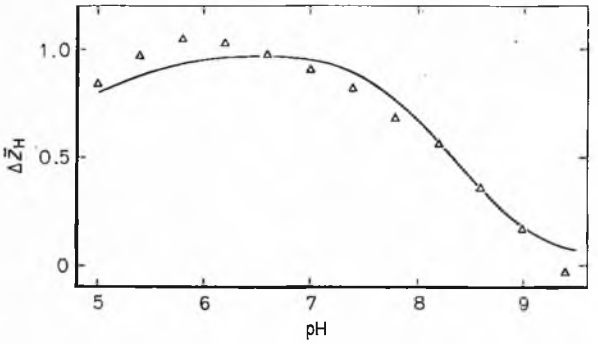

Fig. 3. *Difference titration curve of phospholipase and prophospholipase.* The curve represents the titration of the α-amino group. Drawn line has been calculated as described in the text using $pK = 8.3$ and $w = 0$

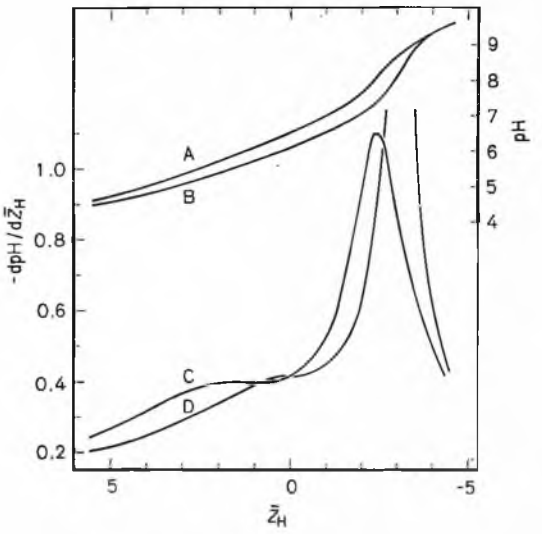

Fig. 4. *Normal and differential titration curves of phospholipase (A and C) and prophospholipase (B and D).* The maximum of $-dpH/d\bar{Z}_H$ for prophospholipase has not been designated in the figure but it amounts to 2.0

linear from about $\bar{Z}_H = 2$ to almost Z_{max} so the fact that actually two classes of carboxyl groups were involved could not be detected. The pK_i found from this curve appeared to be the mean of the values used. Moreover w as estimated from the slope of this curve was increased by 5 to 16% using a pK_i difference between the two classes of carboxyl groups of 0.4 to 0.7. This could explain, at least partially, the difference between the theoretical w and the w as obtained from the plot in Fig. 2. As regards the pK_i of the carboxyl groups our results suggest a pK_i for the carboxyl groups which is fairly high as compared with expected or experimental values.

Titration in the pH Region 4.5 to 9.5

In Fig. 3 the difference titration curve of prophospholipase and phospholipase in the neutral pH region is shown. This curve can be interpreted as follows. If the pK of the histidines in prophospholipase and phospholipase do not differ too much it can easily be shown using Equation (2) and the data of Table 1 that $\Delta Z = \alpha_{COOH} - \alpha_{\alpha\text{-amino}}$. Therefore in the pH region 5 to 6 ΔZ reaches a maximum of 1 and decreases at higher pH because of the increasing dissociation of the α-amino group reaching a value of zero at pH 9. From this curve we estimated an apparent pK of 8.3 which is a reasonable value for a terminal amino group.

Fig. 4 gives the normal and differential titration curves of phospholipase and prophospholipase. The striking difference in shape of the peaks in the differential curve can be explained as being mainly caused by the absence of a titratable α-amino group in prophospholipase, which give rise to a very sharp peak at $Z_{II} = -3.1$ in this protein. From this value applying Equation (6) and using for $n_{Lys} + n_{Arg}$ the data from Table 1, we find $n_{COOH} = 17.1$ while the amino acid analysis gives a number of 14. Following the same procedure for phospholipase we should take in consideration the observed pK of 8.3 for the terminal amino group. Therefore we have to use Equation (7) instead of Equation (6). Since the pH of the second peak (at $Z_{II} = -2.4$) is about 8.1, α for the α-amino group will be about 0.4 and n_{COOH} for phospholipase becomes according to this analysis 16.0 while Table 1 shows a value of 13. So both for phospholipase and prophospholipase the titration procedure results in a value for the number of titratable side-chain carboxyl groups which is 3 larger than listed in Table 1. It is very likely that in both proteins the same residues which are supposedly present as amides actually are in the acid form. In the elucidation of the primary structure of the enzyme the assignment of the amide or acid form has been based on direct identification by thin-layer chromatography of the amino acids liberated in the Edman degradation. This technique in some cases might give difficulties especially for sequences such as -Asn-Asn-.

The first peak in the differential titration curves is not well resolved so a direct estimation of the number of titratable histidines from this curve is not possible. Therefore we applied Equation (4), using for Z_{max} 17 and for the carboxyl and α-amino groups the numbers and pK values mentioned above. The lysines and tyrosines were considered as one class with a pK_i of 10.3. The results shown in Fig. 5 indicates clearly that all three histidines in both proteins are titratable with an apparent pK of 6.6. This pK will be nearly equal to the intrinsic pK because the charge at pH 6.6 is about zero for phospholipase and about -1 for prophospholipase. From a similar

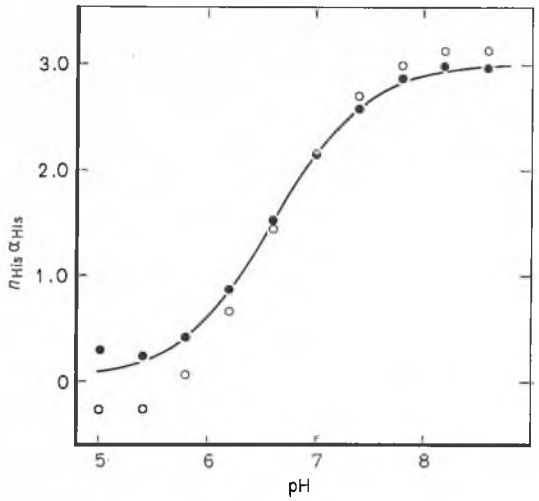

Fig. 5. *Titration of histidines in prophospholipase (●) and phospholipase (○).* Drawn line has been calculated assuming 3 histidines with pK 6.6 and $w = 0$

plot as shown in Fig. 2 we found for the titration of the histidines w near zero. This might mean that the three histidines are located on the protein at remote positions so that the dissociation of one histidine will not affect the dissociation of the two others and *vice versa*. This also explains our failure to obtain a reasonably fitting calculated differential titration curve, using Equation (5), where a constant value of w has to be assumed for all classes.

In conclusion we want to say that the major differences between prophospholipase and phospholipase in the pH region 2 to 9.5 as judged by the here applied titration technique seem to be the absence of a free α-amino group in prophospholipase, present in phospholipase with a normal pK and the presence of a glutamyl residue with an abnormal low pK in prophospholipase. Whether there is a relation between these titrimetric differences and the difference in enzymatic activity is not yet clear.

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