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Ionization Behavior of Papaya Peptidase A and Its Succinylated Derivative

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By upscale titration in 0.15 M KCl at 25 °C, the titration curve of papaya peptidase A in the pH-range 2.0–11.6 and that of its succinylated derivative in the pH-range 4.2–11.6 have been obtained.

Curve fitting for the ionization constants found in the titration curves yields values which can precisely reconstruct the experimental curves. For both proteins, the experimental curves are adequately described on the basis of six group sets, the stoichiometry and pK values of which are reported.

For papaya peptidase A, the observed titratable groups are in excellent agreement with those expected from previously published amino acid analyses of the enzyme and reveal the presence of 16 titratable carboxyl groups, the pK values of which fall into two categories. For succinyl-papaya peptidase A, the actually titratable groups accord well with those expected from recently reported chemical determinations of the number and identity of acylated groups.

The latex of the growing papaya fruit contains at least four discrete thiol proteases, *i.e.* papain (EC 3.4.22.2),[1] chymopapains A[2] and B[3] (jointly designated as EC 3.4.22.6), and papaya peptidase A.[4,5] Among these, only papain has been extensively characterized, for a review see Ref. 6.

Though these enzymes are very much alike in terms of common biophysical parameters, differences are apparent, notably concerning the isoelectric points. Thus, papaya peptidase A has a larger excess of basic amino acids and exhibits a higher isoelectric point than any of the other thiol proteases in the latex.[4,5,7] Recently, measurements of the free electrophoretic mobility as a function of pH established the isoelectric point of papaya peptidase A as 11.7 and that of the succinylated enzyme as 3.8.[8] Since the difference in amino acid composition constitutes a major difference between the thiol proteases of the latex, we decided to investigate the hydrogen ion dissociation behavior of papaya peptidase A and that of the succinylated enzyme.

The fact that protein titration curves differ substantially from the sums of the unperturbed titrations of the constituent acidic and basic groups, and that this difference is generally observed to be dependent on the ionic strength has traditionally been ascribed to electrostatic interactions between the titratable groups. A theoretical treatment of the influence of electrostatic interaction on the ionization behavior of proteins was introduced by Linderstrøm-Lang[9] and later refined by Tanford and Kirkwood.[10] The theory of Tanford and Kirkwood requires knowledge of the coordinates and free energy of interaction of the ionic groups. Since that information is generally not available the interpretation of titration curves ultimately rests on the agreement between calculated and experimental data (*e.g.* Refs.11–13). The demonstration[14] that the potentiometric titration curve of a protein does not contain the information necessary to provide meaningful values for electrostatic interaction led Marini *et al.*[15] to suggest that the curves should be analyzed by the much simpler approach of Levy.[16] Levy considered that the ionic side chains of protein ionize independently of the charge on the molecule and could be described with a so-called group set ionization constant. This constant reflects the individual

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environment of the residue and is a resultant of all the forces which influence the ionic properties of the group. The groups with similar dissociation constants may be treated as a set regardless of chemical identity, and the protein titration curve may be described by the selection of the proper ionization constants.

We present here the potentiometric titration curves of papaya peptidase A and succinyl-papaya peptidase A. The curves are analyzed on the basis of the model proposed by Levy,[16] as specifically applied by Marini *et al.*,[15] *i.e.* we have assigned each residue to its identified chemical set and determined the ionization constants, K_i, by curve fitting.

MATERIALS AND METHODS

Dried papaya latex (crude type 1, lot 127C-0334) was obtained from Sigma, St. Louis, Missouri, USA. Procedures for the preparation of papaya peptidase A and its succinylated derivative as well as extinction coefficients for the spectrophotometric determination of their concentration have been given previously.[8]

All titrations were performed in 0.15 M KCl at 25 °C. Prior to a titration experiment a sample of lyophilized protein was dissolved in 0.15 M KCl and ultrafiltrated exhaustively in 0.15 M KCl employing an Amicon ultrafiltration cell equipped with a PM 10 membrane.

In a typical titration run, 4 ml of a 1 % protein solution in 0.15 M KCl was acidified by addition of 200 μl 0.5 M HCl. Upscale titration was performed using the automatic titration equipment available at Chemistry Laboratory III, H. C. Ørsted Institute, Copenhagen. The equipment is developed by Dr.'s Preben Graae Sørensen and Ole J. Heilmann at that laboratory. pH was measured by a PHM 64 research pH meter from Radiometer A/S, Copenhagen, equipped with a glass electrode G 2040 C and a calomel electrode K 101/K.

The titrant was 0.5 M NaOH, and a titration curve represents 700 measurements of pH as a function of the volume of base added.

Prior to each protein titration a blank titration of the solvent was run. The data was compiled on a RC 4000 computer which calculated the difference between protein and blank titrations expressed as the number of protons bound per protein molecule as a function of pH.

The pH-range of titration was 2.0–11.6 for papaya peptidase A. In the case of the succinylated enzyme, the pH-range was limited by the low solubility of the modified protein in acidic medium.[8] Therefore, a smaller amount of 0.5 M HCl was added prior to titration and data obtained only in the range of pH 4.2–11.6.

The time needed for ultrafiltration was about one hour, and the subsequent titration was complete in about 30 minutes. This is well within the range of time in which papaya peptidase A retains its enzymatic activity, and since the titrations were performed on unactivated preparations, autolysis was ignored.

Data treatment. The mathematical model used to fit the potentiometric curve is

$$\bar{r}=C-\sum_i \frac{N_i \cdot K_i}{10^{-pH}+K_i}$$

where $\bar{r}$ is the average number of protons bound per protein molecule, N_i is the group set number, and K_i is the ionization constant per group set N_i. The ordinate scale was arbitrarily chosen so that the alkaline end point of titration represents the point in which no dissociable protons are bound, *i.e.* $\bar{r}\equiv 0$ for pH=11.6. C represents the maximal number of dissociable protons which can be bound to the protein in the pH range studied. Thus C is a constant dependent on the number of dissociable protons in the protein or protein derivative as well as on the accessible pH range, and therefore differs for the native and succinylated papaya peptidase A.

The computer-assisted evaluation of the ionization constants was performed using a non-linear algorithm which adjusts the parameter values of the function to minimize the sum of squares of the deviation between experimental and calculated points. Initial estimates of the number of groups in each set, N_i, were assigned from the amino acid analysis. For papaya peptidase A, three independent amino acid analyses have been published.[4,5,7] From these reports the titratable groups of papaya peptidase A are expected to be as follows: carboxyl groups of aspartic acid (+aspargine) 13; carboxyl groups of glutamic acid (+ glutamine) 18–20; imidazole groups of histidine 4; thiol groups of cysteine 0–1; ε-amino groups of lysine 16–23; phenolic groups of tyrosine 11–14; and guanidyl groups of arginine 11. In addition, the enzyme is expected to

possess a single *C*-terminal carboxyl and a single *N*-terminal amino group. Based on chemical methods, a number of 24.8±1.5 amino groups per molecule of papaya peptidase A has been reported.[8] Therefore, the highest among the reported numbers of lysine residues in papaya peptidase A (*i.e.* 23) is considered the most reliable.

In assigning an optimal curve-fit it is essential to compare the experimental error with the fact that the standard deviation of a fit decreases when the number of parameters in the model increases. The titration curves presented here are considered to be reproducible within 0.5 groups. Therefore, it is not meaningful to attempt to improve a curve-fit to a standard deviation which is far below this limit.

RESULTS

When the experimental titration points for papaya peptidase A are evaluated on the basis of 6 group sets, the data of Table 1 are obtained. Mean ± standard deviation of the difference between the experimental and calculated points amounts to 0.0±0.2 groups. Using the data of Table 1 the titration curve of Fig. 1 is calculated. In order to facilitate visual judgement of the fit of the calculated curve to the experimental points, only the experimental points for each 0.2 pH-unit increment are shown in the figure.

The carboxyl ionizations of papaya peptidase A fall into two groups (*cf.* Table 1, group sets 1 and 2). The data for the carboxyl ionizations failed to fit a curve calculated on the basis of a single ionization constant as evidenced by a significant increase in the standard deviation (by at least a factor of 5) of the fit. On the other hand, no significant improvement of the fit was obtained by including an extra group set, representing the C-terminal carboxyl group, into the model. The intermediate region is represented by group set 3 (6 groups of pK 7.31, *cf.* Table 1) and, somewhat unexpected, it was found necessary to include group set 4 (4 groups of pK 8.55) to account for the transition between the neutral and alkaline regions. Finally, the alkaline region is represented by group sets 5 and 6 (20 and 9 groups of pK 10.15 and 10.97, respectively).

Evaluation of the titration points for succinyl-papaya peptidase A on the basis of 6 group sets yields the data of Table 2. Using these data the titration curve of Fig. 2 results. Again the experimental points (only one point per 0.2 pH-unit increment is shown) fall perfectly on the calculated curve in agreement with the value of 0.0±0.2 for the mean ± standard deviation of the difference between experimental and calculated points.

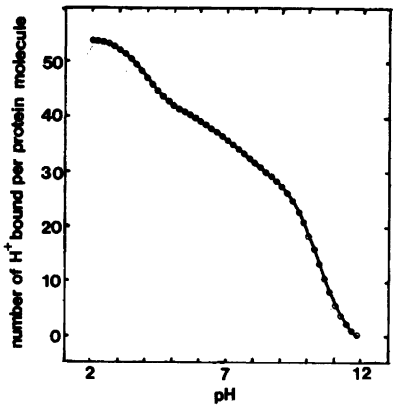

Fig. 1. Hydrogen ion titration curve of papaya peptidase A in 0.15 M KCl at 25 °C. The solid line is theoretically calculated on the basis of the parameters of Table 1. The circles represent experimental points (only one point per 0.2 pH-unit increment is shown).

Comparison of the titration curves shown in Figs. 1 and 2 demonstrates that the highly basic

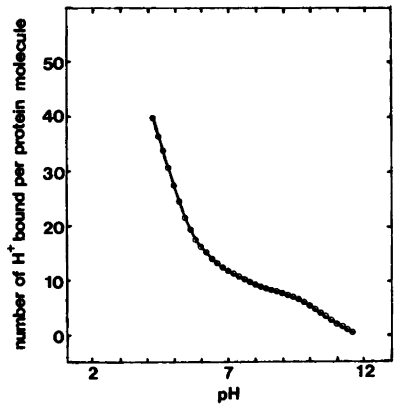

Fig. 2. Hydrogen ion titration curve of succinyl-papaya peptidase A in 0.15 M KCl at 25 °C. The solid line is calculated on the basis of the parameters of Table 2. The circles are experimental points (only one point per 0.2 pH-unit increment is shown).

enzyme papaya peptidase A is converted into a highly acidic enzyme upon succinylation. The acidic region of the titration curve of the modified enzyme is characterized by group sets 1 and 2 (11 and 18 groups of pK 4.70 and 4.92, respectively, *cf.* Table 2). In the region of transition between the acidic and neutral part of the curve 5 groups of pK 6.33 now appear. The neutral region is represented by 4 groups of pK 8.28, whereas in the alkaline region 3 groups of pK 10.20, and 4 groups of pK 10.98 titrate. As for papaya peptidase A, none of the regions of the titration curve of succinyl-papaya peptidase A can be satisfactorily described on the basis of fewer group sets as evidenced by a significant increase in the standard deviation of the fit (from 0.2 to at least 0.8 groups).

DISCUSSION

The experimental titration curve of papaya peptidase A as well as that of the succinylated derivative is adequately described on the basis of six group sets and no electrostatic interaction.

The carboxyl ionizations of papaya peptidase A fall into two groups (sets 1 and 2 of Table 1). A similar separation of carboxyl ionizations into two or more classes have been reported for a number of proteins, *e.g.* lysozyme,[17–19] ovalbumin,[20] reduced and oxidized cytochrome c,[21] and chymotrypsinogen.[22] In the examples cited, the total number of carboxyl groups titrated is close to the number expected from the amino acid analyses. In general, however, there is no agreement between the stoichiometry of each subclass of carboxylic groups and that of aspartic and glutamic acid residues. Furthermore, as in the above cited examples, the difference between pK values of the two subclasses in Table 1 is much larger than the difference between pK values of aspartyl and glutamyl carboxylic groups in model compounds.[23] This may indicate that local effects are more important than the nature of the residue bearing the carboxyl group.

The published amino acid analyses of papaya peptidase A[4,5,7] do not discriminate between aspartic and glutamic acid on the one side and their corresponding amides on the other. The total number of these residues is about 32,[4,5,7] and from Table 1 it is seen that about 15 of these residues contain titratable carboxyl groups (the *C*-terminal amino acid not mentioned). Since the corresponding sum of aspartic and glutamic acid residues in papain is 14,[24] it may now be concluded that the factor responsible for the extreme basicity of papaya peptidase A relative to that of papain is the larger content of basic amino acids in the former enzyme rather than a different content of acidic amino acids.

According to the amino acid analyses[4,5,7] papaya peptidase A should possess about six groups which titrate at approximately neutral pH, *i.e.* 4 imidazoles, 0–1 thiol and a single *N*-terminal amino group. Since it was not considered appropriate to attempt to improve the curve-fit by introducing additional group sets, we will not discriminate between their ionization constants but shall tentatively assign these six groups to group set 3 of Table 1. The potentially titratable groups which can account for the constituents of group sets 4–6 of Table 1 are, according to the amino acid analyses, about 23 ε-amino groups of lysine, 11–14 phenolic groups of tyrosine, and 11 guanidyl groups of arginine. Among these groups, ε-amino ionizations are the most obvious candidates for the four groups of set 4. Though their pK of 8.55 is rather low, similar pK values have been reported for ε-amino ionizations of RNase[25] and chymotrypsinogen.[22] The total of 29 groups in sets 5–6 undoubtedly represent ε-amino and phenolic titrations, since the guanidyl groups of arginine are not expected to titrate in the pH-range studied. Assuming (1) that the higher number of tyrosines (*i.e.* 14) is the correct one (and there is reason to believe so, since two of the three amino acid analyses state

Table 1. Curve-fit parameters for the potentiometric titration of papaya peptidase A.

Group set	N	pK
1	11	3.84
2	5	5.52
3	6	7.31
4	4	8.55
5	20	10.15
6	9	10.97

mean ± standard deviation of the difference between experimental and calculated points=0.0±0.2, C=53.7.

Table 2. Curve-fit parameters for the potentiometric titration of succinyl-papaya peptidase A.

Group set	*N*	p*K*
1	11	4.70
2	18	4.92
3	5	6.33
4	4	8.28
5	3	10.20
6	4	10.98

mean ± standard deviation of the difference between experimental and calculated points=0.0±0.2, *C*=45.0.

that number[5,7]), and (2) that guanidyl ionizations do not contribute to the group set 6, this would mean that four out of fourteen phenolic groups of tyrosine in papaya peptidase A are not titrated at a pH where normal phenolic groups have been converted almost 100 % to their anionic form. The traditional explanation for such an observation is that these groups are buried in the hydrophobic interior of the enzyme and thus inaccessible to the solvent.[23] By spectrophotometric titration, six out of seventeen phenolic groups in papain have been found to exhibit 'anomalous' high p*K* values.[26]

Before comparing the data of Tables 1 and 2 one point should be noted. Though the consideration of electrostatic interaction is not required for analysis of the data, this does not mean that the p*K* parameters for the two proteins discussed here are not influenced by factors such as proximal charge effects, local dielectric environments, hydrogen bonding, ionic strength, *etc.* In fact, by analogy with chymotrypsinogen and its acetylated derivative,[22] it is expected that succinylation of papaya peptidase A results in an increase of the p*K* values because the absence of positively charged groups makes it more difficult (more energy requiring) for proton dissociation to occur.

In accordance with this expectation, group sets 1 and 2 of Table 1 are refound as group sets 1 and 3 in Table 2, both having their p*K* values increased by nearly one p*K*-unit as a result of succinylation. It has previously been reported[8] that about three hydroxy amino acids (serine and threonine) and about twenty amino groups are succinylated under the conditions employed. The 'recovery' of 18 of these groups as group set 2 in Table 2 is very satisfactory, especially when considering the fact that titration data for succinyl-papaya peptidase A was obtained only above pH 4. Thus it is evident from the initial steepness of the titration curve of the modified enzyme (*cf.* Fig. 2) that additional acidic groups are present. The four groups of group set 4 in succinyl-papaya peptidase A may be assigned to imidazole groups, the p*K* value of which are increased by about one p*K*-unit relative to those of the unmodified enzyme due to the absence of positively charged groups. Finally, ε-amino and phenolic titrations of the succinylated enzyme are represented by the seven groups of group sets 5 and 6 in Table 2.

By comparing Tables 1 and 2, it appears that papaya peptidase A exhibits an excess of 28 'basic' ionizations relative to the modified enzyme, *i.e.* two out of six groups of set 3 in Table 1, the four groups of set 4, and a total of 22 groups in sets 5–6. This should be compared with the above-mentioned number of about 20 amino groups which are succinylated under the conditions employed. With respect to the difference of two groups between set 3 of Table 1 and set 4 of Table 2, one of these probably reflects the succinylation of the *N*-terminal amino groups of papaya peptidase A. However, the determination of groups which are present in much lower concentration than others (*i.e.* *C*-terminal carboxyl, *N*-terminal amino and thiol groups) is subject to relatively large error. Thus it is not possible on the basis of the present experiments to decide whether *e.g.* group set 3 of Table 1 contains 6 or 5.5 groups and/or the actual content of group set 4 of Table 2 is *e.g.* 4.5 instead of 4. On the other hand, it is significant that the four groups of group set 4 in Table 1 disappear as a result of succinylation. This observation supports the assumption that these groups, despite their low p*K*, represent ionizations of amino groups. The fact that about 8 basic ionizations are apparently 'missing' from the titration curve of succinyl-papaya peptidase A is not surprising for two reasons: first, by analogy with the upscale shift of the p*K* values of the carboxyl and imidazole ionizations upon succinylation, the introduction of a massive negative charge on the enzyme is expected to shift the p*K* value of some of the remaining basic groups out of the observ-

able range. Second, as was previously suggested,[8] the succinylation may produce a conformational change, thus making additional tyrosine residues inaccessible to the solvent.

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