Short Communication

Active Site Ionizations of Papaya Proteinase A

Niels C. Kaarsholm*

Fysisk-Kemisk Institut, DTH 206, The Technical University of Denmark, DK-2800 Lyngby, Denmark

Kaarsholm, N. C., 1987. Active Site Ionizations of Papaya Proteinase A. – Acta Chem. Scand., Ser. B 41: 629–632.

The cysteine proteinases papain (EC 3.4.22.2) and papaya proteinase A (ppA, formerly called peptidase A)[1,2] are examples of enzymes that display extensive overall similarity and yet exhibit prominent differences, notably concerning isoelectric points,[3] apparent specificity requirements[4-7] and the number of acidic ionizing groups that modulate their catalytic action.[5,8] Since the latter differences must originate in structural variations in the catalytically relevant regions of the enzymes, it is of interest to compare directly the ionization characteristics of the active center thiolate-imidazolium ion pair in papain (Cys-25, His-159) with those of the corresponding groups in ppA.

In this communication the fluorescence titration curves for papain, ppA and their corresponding active site methylthio derivatives are reported and compared. The results show that, in close qualitative analogy with papain, the predominant species of the ppA active center is a thiolate-imidazolium ion pair. The ratio of percentage of the thiolate-imidazolium ion pair to that of the thiol-imidazole tautomer in the active site, however, seems to differ significantly for the two enzymes.

Materials and methods

Papain and ppA were purified to the level of 1.0 mol SH per mol protein and assayed as previously described.[3,4] The active site cysteine-S-SCH_3 derivative of both enzymes was prepared according to the published procedure for papain.[9,10] The inactivated enzymes thus prepared were found to regain full activity upon treatment with a 30-fold molar excess of DL-dithiothreitol at pH 6.

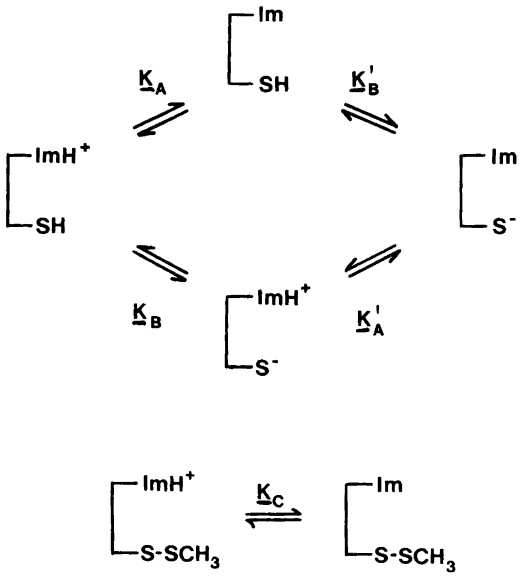
The fluorescence emission spectra (excitation wavelength 280 nm) of papain, papain-S-SCH_3, ppA and ppA-S-SCH_3 were recorded at 25 °C with a Perkin-Elmer-Hitachi spectrophotofluorimeter (model MPF-3). The samples (2.5 ml) contained 3 μM protein in the following buffers: acetic acid-sodium acetate (pH 3.2–5.5), sodium monophosphate-potassium diphosphate (pH 6.0–8.0), Tris-HCl (pH 8.0–9.0) and sodium bicarbonate-sodium carbonate (pH 9.2–9.9). All buffers contained 1 mM EDTA and KCl to produce an ionic strength of 0.3 M. For each sample, the intensity of emission was read at its λ_{max} (approx. 345 nm) and that of a tryptophan standard solution was subsequently rechecked to ensure that the fluorimeter output had not drifted; the pH of the protein solution was then measured. This procedure was repeated to obtain a set of fluorescence intensities between pH 3.2 and 9.9. Since both enzymes undergo time-dependent irreversible denaturation at low pH values, control experiments were run to ensure that the pH dependences reported here reflect reversible acid-base equilibria of undenatured protein. Back-titrations of samples at the lowest and highest pH values to neutral pH resulted in recovery of the expected fluorescence. Similarly, samples at the lowest and highest pH values of the fluorescence titrations exhibited catalytic activities upon activation which are at least 90 % of those of the fully active enzymes, provided they were transferred

*Present address: NOVO Research Institute, DK-2880 Bagsværd, Denmark.

to neutral buffer immediately after measurement of fluorescence.

Results and discussion

The catalytic activity of papain is modulated by three ionizable groups of which two are deprotonated and one is protonated at around neutral pH where the enzyme exhibits maximum activity.[5,11] Two of the groups have been identified as the side-chains of the Cys-25 and His-159 residues, respectively,[12,13] and the carboxylate moiety of the neighboring Asp-158 residue is an obvious candidate for the third group. While in the active form of the enzyme the latter group is almost certainly anionic, the relationship between the ionization behavior of the Cys-25/His-159 system and the catalytic activity of the enzyme is somewhat complicated because the two ionizations are thermodynamically linked. Thus, for the unmodified native enzyme, Cys-25 has a pK of about 3–4 and His-159 has a pK of about 8.5, whereas the pK of His-159 is about 4 in the derivative of papain wherein the negative charge on the sulfur atom of Cys-25 is neutralized by methylthiolation.[10,14] The model for these coupled ionizations is shown in Scheme 1.



Scheme 1. Ionization pattern for two interacting groups at the active sites of ppA and papain. For papain, SH represents Cys-25 and ImH^+ represents His-159.

The observed pH-dependent fluorescence of papain is due to quenching of the fluorescence of Trp-177 by the protonated form of the nearby His-159.[15] Therefore, the interactive system (Scheme 1) may be characterized by fluorescence titrations.[16] In Fig. 1, the fluorescence titration curves for ppA-S-SCH_3, active ppA, papain-S-SCH_3 and active papain are compared. The close qualitative analogy between the observed pH dependences for the two enzymes indicates that an arrangement similar to that of the papain Cys-25/His-159/Trp-177 system is responsible for the pH-dependent fluorescence of ppA. Thus, removal of the methylthio group from the active site cysteine residue of ppA results in an increase of more than 4 pK units in the fluorometrically determined pK value.

The macroscopic ionization constants K_I and K_{II} for removal of a proton from the doubly protonated form (SH/ImH^+) and the singly protonated forms [(S^-/ImH^+) + (SH/Im)] of ppA, respectively, are related to the microscopic constants of Scheme 1 by eqns. (1) and (2). Further-

$$K_I = K_A + K_B \tag{1}$$

$$\frac{1}{K_{II}} = \frac{1}{K'_A} + \frac{1}{K'_B} \tag{2}$$

more, the pH dependence below pH 6 of k_{cat}/K_M for the ppA-catalyzed hydrolysis of small specific substrates (at 25 °C; I = 0.3 M) has been shown to obey eqn. (3), where k'_{cat} and K'_M are the intrinsic pH-independent constants.[5]

$$\frac{k_{cat}}{K_M} = \frac{\dfrac{k'_{cat}}{K'_M}}{1 + \dfrac{[H^+]}{K_I}} \tag{3}$$

If the assumptions are made (a) that the kinetically determined macroscopic ionization constant K_I is not perturbed by ratios of rate constants and (b) that the fluorometrically determined pK value for the methylthio derivative is a good approximation to pK_A (i.e. $K_C = K_A$ in Scheme 1),[17] then eqns. (1) and (2) together with the identity $K_A K'_B = K_B K'_A$ can be used to calculate the microscopic equilibrium constants of Scheme 1 from K_I and K_{II}. These constants are

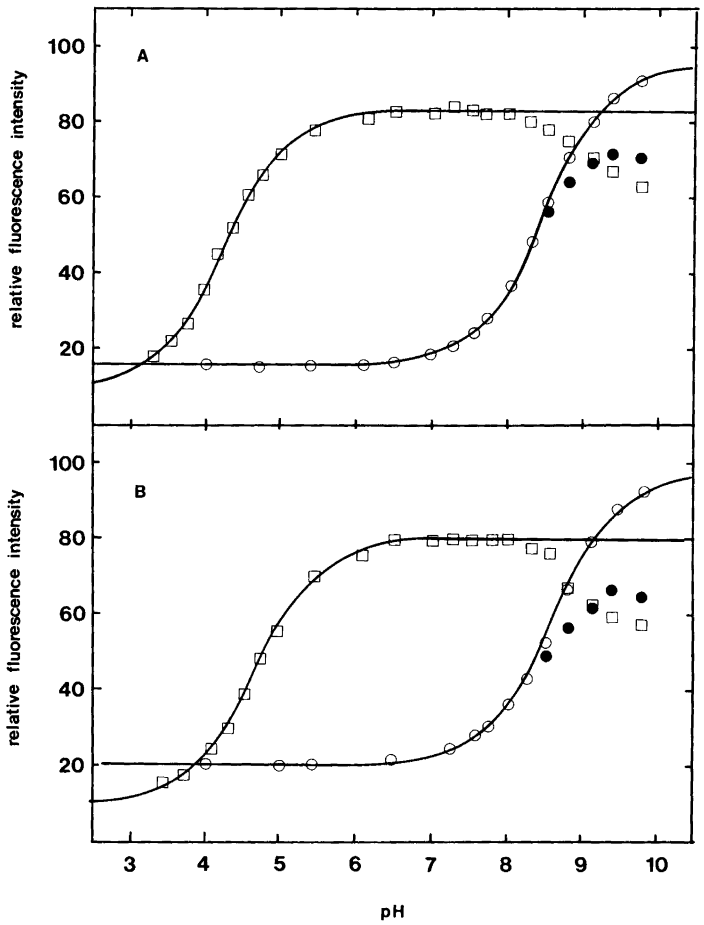


Fig. 1. Fluorescence titrations at 25 °C, I = 0.3 M. (A) ppA-S-SCH_3 (□) and active ppA (○). (B) papain-S-SCH_3 (□) and active papain (○). The decrease in fluorescence intensities for all four protein species above pH 8.5 is probably the result of quenching due to ionization of tyrosyl residues.[14] For the native proteins the corrected fluorescence values were obtained by multiplying the observed fluorescence intensity at high pH (●) by the ratio of the fluorescence intensity of the methylthio derivative in the plateau region (pH 6–8) to the fluorescence intensity of the methylthio derivative at the high pH of interest.[14] Apparent pK values were obtained from the pH dependences of the fluorescence intensities by curve-fitting to the data using the equation $F_{obs} = [F_{H^0} + 10^{(pK-pH)}F_{H^+}]/[1 + 10^{(pK-pH)}]$, where F_{H^0} and F_{H^+} are the fluorescence intensities at the high-pH and low-pH plateaus of the titration curves, respectively. The fit to the data yielded pK values for ppA-S-SCH_3 (pK = 4.25 ± 0.05), active ppA (pK = 8.48 ± 0.03), papain-S-SCH_3 (pK = 4.73 ± 0.04) and active papain (pK = 8.59 ± 0.08).

listed in Table 1 together with the constants obtained from a similar calculation based on the data for papain.

The values of pK_A and pK_B indicate that for ppA, the equilibrium constant (K_B/K_A) for the prototropic shift (SH/Im) $\rightleftarrows$ (S^-/ImH^+) is 3. Thus, in the physiological pH range, about 75 % of the ppA active centers contain a thiolate-imidazolium ion pair if the ionization pattern depicted in Scheme 1 obtains. For papain, the value of 13 obtained for K_B/K_A is in excellent agreement with the value of 9–12 reported on the basis of poten-

Table 1. Parameters for ionizations at the active sites of ppA and papain at 25 °C, I = 0.3 M.

	ppA	papain
pK_I[a]	3.65±0.14	3.58±0.26
pK_{II}	8.48±0.03	8.59±0.08
pK_C	4.25±0.05	4.73±0.04
pK_A[b]	4.25	4.73
pK_B[b]	3.78	3.61
pK'_A[b]	8.35	8.56
pK'_B[b]	7.88	7.44

[a]Kinetically determined values taken from Ref. 5.
[b]Microscopic ionization constants defined in Scheme 1 and calculated assuming $pK_C = pK_A$.

tiometric difference titrations under slightly different conditions.[10] The predominant form of the ppA active center at around neutral pH is the most attractive candidate for the catalytically competent form of the enzyme. In this form the thiol group exists in its nucleophilic anionic form and can efficiently attack the carbonyl carbon of the substrate; the protonated imidazolyl group is in a form which allows it to participate as an acidic catalyst by protonating the leaving group.

In conclusion, the active center of ppA has previously been shown to lack an ionizing group with properties analogous to those attributed to the Asp-158 residue in papain.[5,8] Nevertheless, the present work indicates that the predominant species of the ppA active site is a thiolate-imidazolium ion pair. The somewhat lower concentration of the charge-separated species in ppA than in papain (cf. Table 1) accords well with the apparent greater refinement of the latter molecule in terms of specificity and catalytic mechanism.

References

1. Schack, P. *C. R. Trav. Lab. Carlsberg 36* (1967) 67.
2. Polgar, L. *Biochem. J. 221* (1984) 555.
3. Kaarsholm, N. C. and Schack, P. *Acta Chem. Scand., Ser. B 37* (1983) 607.
4. Schack, P. and Kaarsholm, N. C. *Biochem. J. 219* (1984) 727.
5. Schack, P. and Kaarsholm, N. C. *Biochemistry 23* (1984) 631.
6. Johansen, J. T. and Ottesen, M. *C. R. Trav. Lab. Carlsberg 36* (1968) 265.
7. Brocklehurst, K., Kowlessur, D., O'Driscoll, M., Patel, G., Quenby, S., Salih, E., Templeton, W., Thomas, E. W. and Willenbrock, F. *Biochem. J. 244* (1987) 173.
8. Baines, B. S. and Brocklehurst, K. *Biochem. J. 205* (1982) 205.
9. Smith, D. J., Maggio, E. T. and Kenyon, G. L. *Biochemistry 14* (1975) 766.
10. Lewis, S. D., Johnson, F. A. and Shafer, J. A. *Biochemistry 15* (1976) 5009.
11. Lewis, S. D., Johnson, F. A., Ohno, A. K. and Shafer, J. A. *J. Biol. Chem. 253* (1978) 5080.
12. Johnson, F. A., Lewis, S. D. and Shafer, J. A. *Biochemistry 20* (1981) 44.
13. Lewis, S. D., Johnson, F. A. and Shafer, J. A. *Biochemistry 20* (1981) 48.
14. Sluyterman, L. A. Æ. and De Graaf, M. J. M. *Biochim. Biophys. Acta 200* (1970) 595.
15. Lowe, G. and Whitworth, A. S. *Biochem. J. 141* (1974) 503.
16. Johnson, F. A., Lewis, S. D. and Shafer, J. A. *Biochemistry 20* (1981) 52.
17. Migliorini, M. and Creighton, D. J. *Eur. J. Biochem. 156* (1986) 189.

Received August 3, 1987.