MERCAPTIDE–IMIDAZOLIUM ION-PAIR: THE REACTIVE NUCLEOPHILE IN PAPAIN CATALYSIS

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1. Introduction

The hydrolysis of amino acid derivatives catalyzed by papain (EC 3.4.4.10) proceeds through the formation of an acyl-thiolenzyme intermediate (cf. refs. [1,2]). The pH-rate profile of the formation of this intermediate displays a bell-shaped curve depending on the ionization of two groups with pK_a values of about 4 and 8 [1,2]. In the light of the steric structure of the active site of papain [3,4] these pK_a values can be assigned to His-159 and Cys-25, respectively [5–7], although the pK_a of 4 has earlier been attributed to the dissociation of a carboxyl group (cf. refs. [1,2]). Despite the extensive studies on the mechanism of action, the exact catalytic role of Cys-25 has not yet been established. It is widely accepted in the literature that during acyl-enzyme formation the thiol group reacts in its non-dissociated form assisted by general base catalysis [2, 7–13]. On the other hand, we have recently found [6] that alkylation of papain by haloacetamides displays double sigmoid pH-rate profiles with similar pK_a values (4.0 and 8.4) as found in acylation reactions. Since D_2O effects could not be observed in these reactions [6], the double sigmoid curves were interpreted to indicate the existence of a mercaptide–imidazolium ion-pair in the catalytically active papain rather than in terms of a general base-catalyzed attack of the non-dissociated thiol group on the substrate. Starting from low pH values the pK_a of 4.0 is characteristic of the formation of the ion-pair, whereas the pK_a of 8.4 reflects decomposition of the ion-pair to free mercaptide ion and imidazole.

The aim of this work was to test *directly* the presence of mercaptide ion in the catalytically active form of papain. To this end we employed our spectrophotometric method described recently, which is based on measuring the disappearence of the absorption band of the mercaptide ion during alkylation [14]. By this method we demonstrated the presence of mercaptide ion in catalytically active papain and confirmed our previous assumption [6] that ion-pair formation is responsible for the high reactivity of Cys-25 in the acide pH-range.

2. Experimental

Twice-crystallized papain (Sigma) was purified by the method of Sluyterman and Wijdenes [15]. The pure mercuri-papain was activated with 0.02 M cysteine and passed through a Sephadex G-25 column equilibrated with 0.05 M acetate buffer, pH 5.2., containing 1 mM EDTA. Titration of the purified papain with 5,5'-dithio-bis(2-nitrobenzoate) according to Ellman's procedure [16] showed at least 0.9 moles of thiol group per mole of enzyme. This active papain concentration was considered in all calculations.

Iodoacetamide and chloroacetamide were recrystallized from carbon tetrachloride and water, respectively.

Determination of the dissociated thiol group in papain was performed by measuring the absorbacne change on alkylation at 250 nm as described previously [14]. Correction was made for the iodoacetamide reaction [14].

The theoretical curve for the double sigmoid pH-dependence of absorbance changes was calculated from eq. 1. The two terms of eq. 1 represent the contributions by the two reactive forms of the thiol

group. The first term gives the contribution of the ion-pair, whereas the second term accounts for the contribution of free mercaptide ion.

$$\epsilon^{obs} = \epsilon_{IP}\left[\frac{1}{1+[H^+]/K_1}\right]\left[\frac{1}{1+K_2/[H^+]}\right] + \\ + \epsilon_{S^-}\left[\frac{1}{1+[H^+]/K_2}\right] \qquad \text{eq. (1)}$$

where K_1 and K_2 are the apparent ionization constants on which the absorbance change depends. ϵ_{IP} and ϵ_{S^-} stand for the molar absorption coefficients for the mercaptide ion of the ion-pair and the free mercaptide ion, respectively.

3. Results and discussion

Alkylation of Cys-25 of papain with chloroacetamide or iodoacetamide displays double sigmoid pH-rate profile with pK_a values of 4.0 and 8.4 [6]. If this double sigmoid character is due to ion-pair formation as we assumed [6], then the mercaptide ion of the ion-pair should be detectable by the aid of our spectrophotometric method [14]. In fact, it is seen in fig. 1 that the pH-dependence of absorbance change on alkylation of papain substantially deviates from the simple dissociation curve of a thiol group, and the experimental points conform to the double sigmoid curve described by eq. 1. The pK_a values (3.7, 8.4) agree fairly well with those obtained in the pH-rate studies [6]. The molar absorption coefficients are compared to the corresponding values of thiolsubtilisin, D-glyceraldehyde-3-phosphate dehydrogenase and glutathione in table 1. It is seen that both the ϵ_{IP} and ϵ_{S^-}-values of different compounds are remarkably similar. Furthermore, fig. 1 shows, that the absorbance changes are independent of whether chloroacetamide, iodoacetamide or chloroacetate was used, although these alkylating agents display different reactivities towards papain.* This indicates that

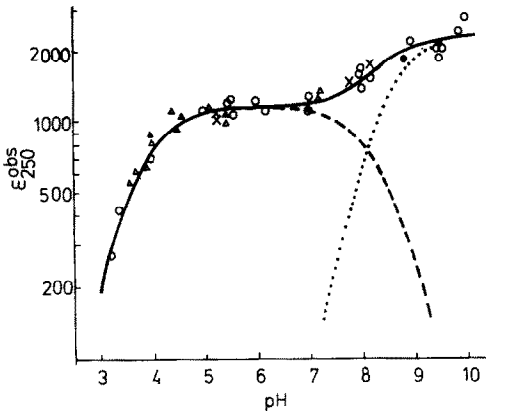

Fig. 1. The pH-dependence of absorbance changes on alkylation of papain. Alkylations were followed to completion at 250 nm under pseudo first-order conditions. 1.5–4.4 μM papain was alkylated with 2.4–100 mM chloroacetatmide (○), 0.39–1.86 mM iodoacetamide (△) and 2.7–9.6 mM chloroacetate (×) in the presence of 1 mM EDTA at 25°C. The reactions were performed in citrate, acetate, phosphate or carbonate buffers adjusted with KCl to $I \approx 0.07$ or in the case of the full symbols (●,▲) to $I = 0.38$. The continuous line was calculated by using eq. 1 ($pK_1 = 3.7$, $pK_2 = 8.4$ ϵ_{IP}= 1150 and $\epsilon_{S^-} = 2300$). The dashed line (bell-shaped curve) represents the contribution of the first term of eq. 1. The dotted line, a simple dissociation curve, shows the contribution of the second term of eq. 1.

the absorbance changes indeed reflect the ionization of Cys-25.

Deviation from normal dissociation of a functional group may also be due to changes in the ionization state of some nearby side chain(s) (cf. ref. [18]). For example, such electrostatic effects perturb the dissociation of a histidine residue in chymotrypsin [19].

Table 1
Molar absorbance changes at 250 nm on alkylation of thiolenzymes and glutathione

Thiol compound	ϵ_{IP}	ϵ_{S^-}
Papain	1150	2300
Glyceraldehyde-3-phosphate dehydrogenase	1050[a]	2400[a]
Thiolsubtilisin	1150[b]	
Glutathione		2300[b]

[a] Ref. [21]
[b] Ref. [14]

* Iodoacetamide reacts by more than two orders of magnitude faster than chloroacetamide [6]. Both haloacetamides display double sigmoid pH-rate profiles [6], whereas chloroacetate shows a bell-shaped pH-dependence curve [17].

However, this perturbation is abolished at enhanced ionic strength [19]. Therefore we tested the effect of ionic strength on the formation of ion-pair in papain. Fig. 1 demonstrates that high ionic strength ($I = 0.38$) does not affect the absorbance changes that occur on alkylation of papain. Consequently, the large devation from normal dissociation of Cys-25 cannot be due to the electrostatic effects of some solvent-separated side chain(s) but rather to the formation of an intimate ion-pair held together by the tertiary structure of the protein.

Our previous study on papain [6] has indicated that the ion-pair is the reactive nucleophile in the enzyme. However, the state of equilibrium between the thiolate–imidazolium and the thiol–imidazole systems shown in eq. 2, which includes all species existing in the pH-range studied, could not be established [6].

$$SH + HB^+ \underset{+H^+}{\overset{-H^+}{\rightleftharpoons}} [SH \ldots B] \underset{+H^+}{\overset{-H^+}{\rightleftharpoons}} S^- + B$$

$$\Updownarrow$$

$$S^- \cdot HB^+ \qquad \text{eq. (2)}$$

$$pH < pK_1 \qquad pK_1 < pH < pK_2 \qquad pK_2 < pH$$

where *SH* stands for the thiol group of Cys-25 and *B* for the imidazole base of His-159 (cf. ref. [6]).

The following consideration permit us to determine the state of equilibrium. According to eq. 1 the total amount of Cys-25 is equal to the sum of the amount of free mercaptide ion and of the ion-pair forms in the pH-range where these two forms are converted into each other. Since the experimental points fit fairly well to the curve of eq. 1, it can be excluded that the thiol–imidazole system (in brackets in eq. 2) would also be present in an appreciable amount, which implies that the equilibrium is strongly shifted towards ion-pair formation.

As for the catalytic reactions of papain, the activation of Cys-25 in the ion-pair confirms our previous suggestion that acylation and deacylation of thiol-proteases, in contrast to the same catalytic steps of serine proteases, proceed via a non-repetitive mechanism according to eq. 3 [6,20]:

$$E + (S \text{ or } P) \underset{\text{no catalysis}}{\overset{\text{no catalysis}}{\rightleftharpoons}} THI \underset{\text{general base}}{\overset{\text{general acid}}{\rightleftharpoons}} ES' \qquad \text{eq. (3)}$$

where *E*, *S*, *P*, *THI*, and *ES'* stand for enzyme, substrate, product, tetrahedral intermediate and acyl-enzyme, respectively. The forward and the reverse reactions represent acylation and deacylation, respectively.

Equation 3 implies that formation of the tetrahedral intermediate is not promoted by general base catalysis in acylation because the proton of Cys-25 is already on His-159 due to ion-pair formation. This mechanism is different from the repetitive mechanism of serine proteases where acylation, as well as deacylation, requires both general base and general acid catalyses.

It is important to point out that formation of the ion-pair is represented by a bell-shaped pH-dependence curve (dashed line in fig. 1) similarly to acyl-enzyme formation, i.e. there is correlation between ion-pair formation and catalytic activity. This implies that the imidazolium ion is also required for acyl-enzyme formation, in addition to the nucleophilic attack of free mercaptide ion on the substrate. The imidazolium ion assists the formation of acyl-enzyme by general acid catalysis probably in a one-encounter type reaction [6].

Summarizing one can conclude that demonstration of the existence of mercaptide ion far below the pK_a of Cys-25 provides compelling evidence in favor of ion-pair formation in the catalytically active papain. Since we have obtained similar results with thiol-subtilisin [14] and D-glyceraldehyde-3-phosphate dehydrogenase [21], it appears that ion-pair formation may be a general mode of activation of the SH-group of thiolenzymes.

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References

[1] Lowe, G. (1970) Philos. Trans. Roy. Soc. Lond. Ser. B. Biol. Sci. 257, 237–248.

[2] Glazer, A. N. and Smith, E. L. (1971) in: The Enzymes, 3rd edn (Boyer, P. D. ed) vol. 3, pp. 501–546, Academid Press, New York.
[3] Drenth, J., Jansonius, J. N., Koekoek, R. and Wolthers, B. G. (1971) Adv. Protein Chem. 25, 79–115.
[4] Drenth, J., Jansonius, J. N., Koekoek, R. and Wolthers, B. G. (1971) in: The Enzymes, 3rd edn (Boyer, P. D. ed) vol. 3 pp. 485–499, Academic Press, New York.
[5] Hussain, S. S. and Lowe, G. (1968) Biochem. J. 108, 855–859.
[6] Polgár, L. (1973) Eur. J. Biochem. 33, 104–109.
[7] Lucas, E. C. and Williams, A. (1969) Biochemistry 8, 5125–5135.
[8] Lowe, G. and Yuthavong, Y. (1971) Biochem. J. 124, 117–122.
[9] Williams, A., Lucas, E. C. and Rimmer, A. R. (1972) J. Chem. Soc. Perkin Trans, II. 621–627.
[10] Brocklehurst, K. and Little, G. (1972) Biochem. J. 128, 471–474.
[11] Campbell, P. and Kaiser, E. T. (1973) J. Am. Chem. Soc. 95, 3735–3741.
[12] Fersht, A. R. (1971) J. Am. Chem. Soc. 93, 3504–3515.
[13] Williams, A., Lucas, E. C. and Douglas, K. T. (1972) J. Chem. Soc. Perkin Trans. II. 1493–1499.
[14] Polgár, L. (1974) FEBS Letters 38, 187–190.
[15] Slyterman, L. A. AE, and Wijdenes, J. (1970) Biochim. Biophys. Acta 200, 593–595.
[16] Ellman, G. L. (1959) Arch. Biochem. Biophys. 82, 70–77.
[17] Chaikin, I. M. and Smith, E. L. (1969) J. Biol. Chem. 244, 5095–5099.
[18] Tanford, Ch. (1962) Adv. Protein Chem. 25, 69–165.
[19] Kézdy, F. J., Clement, G. E. and Bender, M. L. (1964) J. Am. Chem. Soc. 86, 3690–3696.
[20] Polgár, L. (1972) Acta Biochim. Biophys. Acad. Sci. Hung. 7, 319–334.
[21] Polgár, L. (1974) Eur. J. Biochem. in press.