THE pK_a VALUE OF THE ACTIVE SITE HISTIDINE IN PHOTO-OXIDISED PAPAIN

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1. Introduction

It has recently been suggested that the active site histidine residue in papain and stem-bromelain have pK_a values of 6.7 and 6.4 respectively and since these enzymes are essentially pH-independent for substrate hydrolysis at these pH values, histidine is not directly involved in their catalytic mechanism [1–3]. This conclusion is based on photo-oxidation experiments during which the active site cysteine residue was oxidised, but this could be reversed by dithiothreitol and the thiol titre of the enzyme completely restored. What was not clear from these experiments, however, was the relative rates of photo-oxidation of the various residues. If, as appeared likely, the thiol group was photo-oxidised more rapidly than the imidazole group, the pK_a derived from the pH-dependence curve for the loss of histidine would clearly not be that of the native enzyme. In order to test this possibility, papain was photo-oxidised under conditions described by Okumara and Murachi [3] and the pH-dependence of the fluorescence emission intensity of the product determined. Tryptophan-177 in papain is responsible for almost half the fluorescence intensity of the enzyme at high pH, and when the thiol group is blocked, the pH-dependence of its fluorescence intensity reflects the state of ionisation of the active site histidine residue [4].

2. Materials and methods

Twice-crystallised papain prepared from dried papaya latex [5] was activated with 2-mercaptoethanol and purified to 100% activity by affinity chromatography [6].

Tetrathionate inhibited papain was prepared from 100% active papain (19 μM) by incubation with a 50-fold molar excess of sodium tetrathionate under N_2 at pH 8.0 [7]. Inactivation was complete in 30 min. The excess sodium tetrathionate was removed by dialysis against deionised water and the enzyme concentrated by ultrafiltration (Amicon) to approx. 70 μM.

Methylene blue sensitised photo-oxidation of papain was conducted in sodium acetate buffer (50 ml, 0.1 M, pH 5.0) made 15 μM in papain and 16 μM in methylene blue. The stirred solution was kept at 15°C and illuminated by a Philips tungsten incandescent lamp (150 W) placed 25 cm above the surface of the solution [1–3]. Aliquots were withdrawn and assayed for enzymic activity as described below. After 2 h the solution was treated with Dowex 50X4 (H^+ form) to remove methylene blue, filtered and dialysed against deionised water. The solution was concentrated to approx. 70 μM by ultrafiltration. A sample was incubated with a 100-fold excess of 2-mercaptoethanol for 20 min and assayed for enzymic activity.

Enzymic activity was assayed by adding, with rapid mixing, an aliquot of enzyme solution (10 μl, approx. 15 μM) and *N*-benzyloxycarbonyl glycine *p*-nitrophenyl ester in acetonitrile (50 μl) to a thermally equilibrated (25°C) buffer solution (3 ml, 0.1 M NaH_2PO_4, 1 mM EDTA, pH 6.0) in a cuvette in a Unicam SP 1800 spectrophotometer. The release of *p*-nitrophenol was followed by the change in absorption at 340 nm [4].

Enzyme samples were prepared for amino acid analysis by dialysis against deionised water and lyophilisation. After hydrolysing for 20 h in 6 N HCl, the acid was removed in vacuo and the residue taken up in 0.01 M HCl. The sample was analysed on a Jeol JLC-5AH amino-acid analyser.

Fluorescence emission spectra were recorded on a Perkin-Elmer-Hitachi spectrofluorimeter (HPF 2A) in the ratio mode with excitation at 288 nm. Buffer solutions in the pH 3–8 range were prepared by mixing solutions of sodium citrate (20 mM) and sodium phosphate (20 mM) both 1 mM in EDTA and 0.3 M in NaCl. Buffer solutions in the pH 8–8.5 range contained 20 mM glycine, 0.3 M NaCl, and 1 mM EDTA. Buffer (2.7 ml) was thermally equilibrated at 25°C in a cuvette and the enzyme solution (100 μl, approx. 70 μM) added and mixed. The fluorescence intensity at the $\lambda_{max.}$ (approx. 340 nm) was recorded. The pH of the solution was measured within 10 min of the spectrum being recorded.

3. Results and discussion

Papain was photo-oxidised in the presence of methylene blue at pH 5.0 and the loss of enzymic activity followed. The photo-oxidation was stopped after 2 h when the activity had dropped to 5% of the original value; on treatment with 2-mercaptoethanol 75% of the original activity was regained. The product was hydrolysed and the amino acid analysis was compared with that for the native enzyme. No loss of histidine had occurred, as expected, when the photo-oxidation was performed at pH 5.0 [1,3]. The pH-dependence of the relative fluorescence intensity of the photo-oxidised papain is shown in fig.1. The theoretical curve fitted to the experimental data is for a single ionisation constant of pK_a 6.4, i.e. a value close to that derived from the pH-dependence of the photo-oxidation of the histidine residue [1,3].

When the active site thiol group of papain is blocked by an uncharged group, the pK_a derived from the pH-dependent fluorescence intensity is near 4 and can be assigned to the imidazole group of the active site histidine residue [4]. Although the nature of the photo-oxidised thiol group of papain has not been established, the fact that in can be reduced back to a thiol with dithiothreitol or 2-mercaptoethanol makes it highly probable that it is a sulphenic acid. Irreversibly oxidised papain has been shown by X-ray analysis to be at the next level of oxidation, i.e. the thiol group has been oxidised to a sulphinic acid [8]. Papain which has been inactivated with hydrogen peroxide can be reduced back to the active enzyme

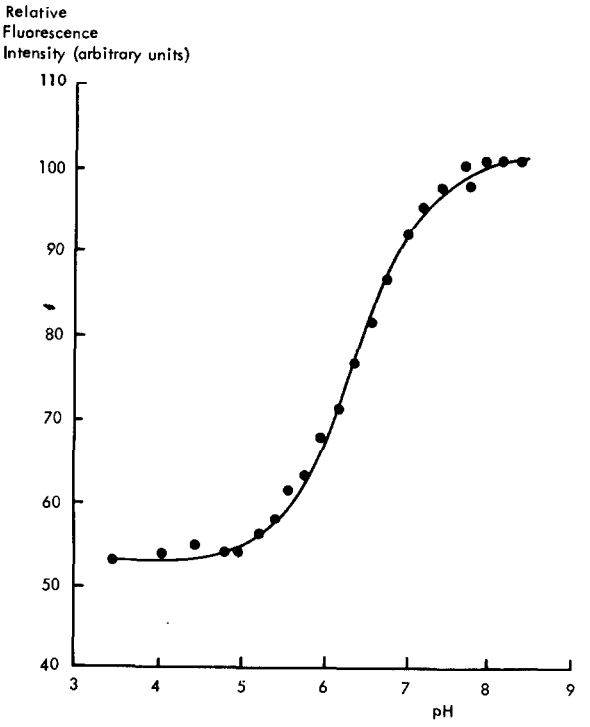

Fig.1. The pH-dependence of the relative intensity of the fluorescence emission at the $\lambda_{max.}$ (approx. 340 nm, excitation at 288 nm) for photo-oxidised papain (2.72 μM). The theoretical curve is for a single ionisation constant, pK_a 6.4.

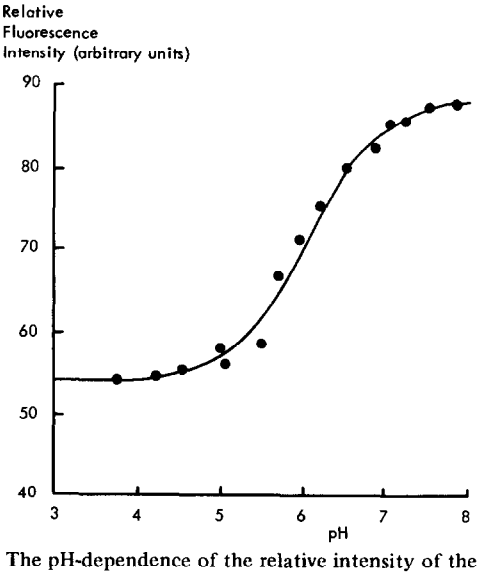

Fig.2. The pH-dependence of the relative intensity of the fluorescence emission at the $\lambda_{max.}$ (approx. 340 nm, excitation at 288 nm) for sodium tetrathionate inhibited papain (2.63 μM). The theoretical curve is for a single ionisation constant, pK_a 6.05.

with cysteine and good evidence has been provided that the thiol group of the active site cysteine residue has been oxidised to a sulphenic acid [9].

It would be expected that replacement of an uncharged blocked thiol group by the sulphenate ion would raise the pK_a of the active site histidine residue. The pK_a derived from the pH-dependent loss of histidine (and irreversible loss of enzymic activity) [1,3] is not therefore that of the active site histidine in the native enzyme, but that for the photo-oxidised enzyme in which the active site cysteine has been oxidised. The results of Murachi et al. [1–3] do not therefore invalidate mechanisms involving histidine-159 as a catalytically functional group in papain, nor presumably mechanisms involving the active site histidine in stem-bromelain.

Support for the postulate that the sulphenate ion derived from the active site cysteine residue of papain is responsible for raising the pK_a of the active site histidine residue in photo-oxidised papain is provided by the observation that the active site histidine in irreversibly oxidised papain has pK_a 6.7 [10]. It is known that the cysteine thiol has been oxidised to a sulphinate ion [8]. The pH-dependent fluorescence intensity of papain in which the thiol has been protected by treatment with sodium tetrathionate (to give $R\text{-}S\text{-}SSO_3^-$) is shown in fig.2. Here a pK_a 6.05 is observed, again showing that the presence of a negatively charged group in close proximity to the active site histidine raises its pK_a. Unfortunately these observations do not help to interpret the pH-dependent fluorescence intensity of the native enzyme which has a pK_a of about 8.6 [8,10], since the active site cysteine and histidine residues form an interactive system to which microscopic pK_a values cannot be assigned without making unjustifiable assumptions.

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