The Role of Papain in the Association Process of a 1-Pyrenoyl Pendant attached to its Active Site

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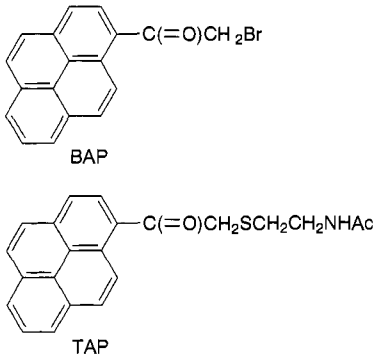
An analysis of the spectroscopic behaviour of papain modified by 1-bromoacetylpyrene demonstrates that the active site of this enzyme remarkably promotes the formation of the ground-state dimer derived from the pyrenoyl pendant covalently bound to the cysteine-25 residue.

Great effort has been devoted to the photochemical control of enzyme activity by the covalent linkage of a photoisomerizable probe molecule to the enzyme surface.[1] However, there are only a few studies concerning the interaction between a probe molecule and an enzyme active-site amino acid residue[2] and/or between reporter groups bound to the enzyme active site. Since pyrenyl groups are good probes for clarifying the behaviour of many biological systems,[3] 1-bromoacetylpyrene (BAP) was allowed to react with papain in order to introduce a pyrenoyl chromophore into the active site of this enzyme. Herein we report the novel observation that a pyrenoyl chromophore attached to the cysteine-25 residue of papain has a strong tendency to form an associated dimer in the ground state.

When papain (4.0×10^{-5} mol dm^{-3}; Sigma, Type IV; M_w 23 000) activated by treatment with L-cysteine and EDTA, was allowed to react with BAP (2.4×10^{-4} mol dm^{-3}) in an N_2-purged phosphate buffer (0.1 mol dm^{-3}; pH 7.0) containing 3% (*v*/*v*) Me_2SO at room temperature, the papain activity against *N*-benzoyl-L-arginine ethyl ester[4] was completely lost within 2 h. The removal of excess BAP by centrifugation and dialysis followed by purification of the dialysed enzyme solution by membrane filtration gave a UV absorption spectrum as shown in Fig. 1(*a*). The modified papain obtained from the reaction between papain and iodoacetamide, which alkylates the cysteine-25 residue selectively,[5] was treated with BAP under the same reaction conditions but showed no indication of the incorporation of a pyrenoyl chromophore into the modified enzyme. This finding provides definitive evidence for the selective alkylation of cysteine-25 with BAP.

We prepared the reference compound 1-[2-(*N*-acetyl)aminoethylthioacetyl]pyrene (TAP) and measured its absorption spectrum in MeOH owing to its negligible solubility towards a phosphate buffer solution [Fig. 1(*b*)]. A comparison of curves (*a*) and (*b*) in Fig. 1 establishes that the absorption maximum of the pendant attached to cysteine-25 is red-shifted by *ca.* 20 nm accompanied by an increase in the absorbance near 400 nm, compared with that of TAP, although the observed red shift could be ascribed in part to the difference in solvent employed. This finding suggests the occurrence of interactions between the pendants and/or between the pendant and amino acid residue(s) located at the papain active site. Interestingly, the BAP-derived reporter group on the enzyme exhibited dual fluorescence depending on the excitation wavelength as shown in Fig. 1 (*c*) and (*d*). Because the excitation spectrum (maximum wavelength *ca.* 390 nm) for the long-wavelength emission (465 nm; lifetime, $\tau_f = 2.6$ ns) is distinct from that (340 nm) for the short-wavelength one (380 nm; $\tau_f = 20$ ns), there should be two ground-state species. If an associated dimer or an aggregate is formed in the ground state giving the 465 nm fluorescence, we expect that the emission intensity ratio I_f(380 nm)/I_f(465 nm) should decrease with increasing the modified papain concentration. The result obtained for the enzyme (Fig. 2) is compatible with our expectation, indicating that the association of the pendant attached to the cysteine-25 residue takes place.

TAP in MeOH also showed weak dual fluorescence depending on the excitation wavelength. The long-wavelength emission (456 nm; $\tau_f < 0.6$ ns) is much stronger than the short-wavelength one (390 nm; $\tau_f = 10$ ns) at [TAP] = 1.0×10^{-4} mol dm^{-3}, whereas the decreased concentration of TAP rapidly increases the emission intensity ratio I_f(390 nm)/I_f(456 nm) as demonstrated in Fig. 2. Additionally, the short-wavelength fluorescence was preferentially observed in MeCN even at [TAP] = 1.0×10^{-4} mol dm^{-3}. Taking into account that pyrene gives a monomer fluorescence at 390 nm and an excimer one at 470 nm[6] and that the 1-pyrenoyl chromophore has a tendency to form a ground-state dimer,[7] it is suggested from these observations that the protic polar solvent promotes the associated dimer formation of a TAP molecule to a great extent mainly through hydrogen bonding between the TAP carbonyl oxygen and the solvent hydroxy hydrogen. Since the ground-state associated dimer may show its absorption around 390 nm as judged by the long-wavelength fluorescence excitation spectrum of the reference compound in MeOH, the gradual



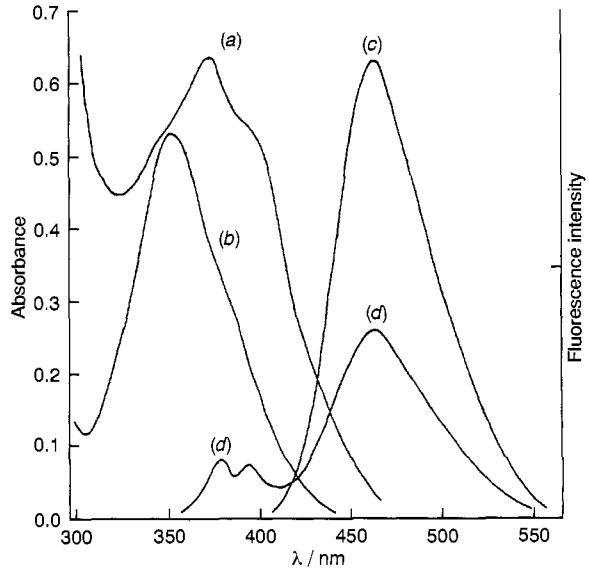


Fig. 1 UV absorption [(*a*) and (*b*)] and fluorescence [(*c*) and (*d*)] spectra of BAP-modified papain [3×10^{-5} mol dm^{-3}; curves (*a*), (*c*) and (*d*)] in 0.1 mol dm^{-3} phosphate buffer pH 7.0 and TAP [3.0×10^{-5} mol dm^{-3}; curve (*b*)] in MeOH at room temperature. Excitation wavelength: (*c*) 400; (*d*) 340 nm.

increase in molar extinction coefficient (ε) at 390 nm with the TAP concentration (ε = 6500 dm^3 mol^{-1} cm^{-1} at [TAP] = 1.0 × 10^{-6} mol dm^{-3} and 7600 dm^{-3} mol^{-1} cm^{-1} at [TAP] = 1.0 × 10^{-4} mol dm^{-3}) provides supporting evidence for the suggestion described above.

These results obtained for TAP, therefore, allow us to conclude that the short- and long-wavelength fluorescences exhibited by BAP-modified papain are attributable to the pendant monomer and dimer species, respectively, formed in the ground state. What are the roles played by the enzyme? One is to significantly enhance dimer emission intensity and lifetime compared with those of TAP, reflecting an increase in stability of the associated dimer in the active site. Solvent effects on the fluorescence behaviour of TAP, as already mentioned, make it possible to speculate that hydrogen bonding between the pyrenoyl carbonyl oxygen and the neighbouring amino acid residue increases the dimer stability. An inspection of the UV spectra of TAP and the modified enzyme indicates that an increase in the absorbance near 400 nm for the enzyme is more significant than that at absorption maximum wavelengths, being consistent with the enhanced pendant dimer stability at the active site. The other is to suppress appreciably dissociation into the monomer pendant, as revealed by a comparison of the concentration dependence of the intensity ratio, I_f(short-wavelength)/I_f(long-wavelength), for the BAP-derived reporter group with that for TAP (Fig. 2). The finding that upon increasing the ionic strength of a papain solution (pH 7.0) from 2.0 × 10^{-3} to 1.0 using KCl, the emission intensity ratio I_f(380 nm)/I_f(465 nm) is increased by a factor of *ca.* 3, allows us to interpret the prevention of the pendant dimer from dissociation into the monomer in terms of the high association ability of papain.[8] The relatively small dependence of the intensity ratio on the ionic strength implies that an electrostatic force and a hydrophobic one both become major factors determining the stability of the papain dimer.

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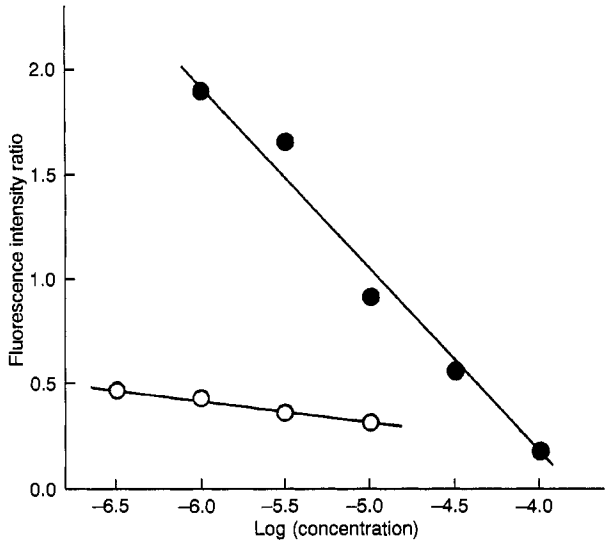

Fig. 2 Effects of BAP-modified papain (○) and TAP (●) concentrations on the fluorescence intensity ratios: (○), I_f(380 nm)/I_f(465 nm) in 0.1 mol dm^{-3} phosphate buffer (pH 7.0); and (●), I_f(390 nm)/I_f(456 nm) in MeOH. Excitation wavelength: (○) 340; (●) 337 nm.

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