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Evidence for Association–Activation Effects in Reactions of Papain from Studies on its Reactivity towards Isomeric Two-Protonic-State Reactivity Probes

By Keith BROCKLEHURST,* John A. L. HERBERT,† Roger NORRIS* and Hans SUSCHITZKY†
**Department of Biochemistry and Chemistry, St. Bartholomew's Hospital Medical College, University of London, Charterhouse Square, London EC1M 6BQ, U.K., and †The Ramage Laboratories, Department of Chemistry and Applied Chemistry, University of Salford, Salford M5 4WT, U.K.*

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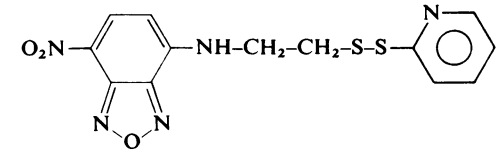
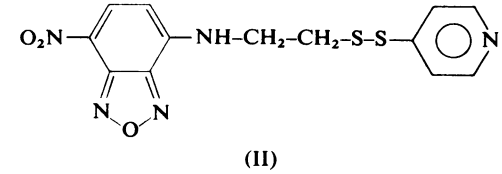
4-(*N*-Aminoethyl 4-pyridyl disulphide)-7-nitrobenzo-2-oxa-1,3-diazole was synthesized and evaluted as a two-protonic-state reactivity probe by kinetic study of its reactions with papain (EC 3.4.22.2) and with benzimidazol-2-ylmethanethiol. Evidence is presented to suggest that: (i) both this probe molecule and its 2-pyridyl isomer bind to papain; (ii) the binding is followed by a change in the environment of the thiol group of cysteine-25; (iii) the striking rate maximum in neutral media observed in the reaction of papain with the 2-pyridyl isomer but not with the 4-pyridyl isomer arises from association of the 2-pyridyl leaving group with the imidazolium ion of histidine-159.

Evidence that binding of a hydrophobic ligand to papain (EC 3.4.22.2) is followed by repositioning of the ionizing groups in its active centre, as may be required in the catalytic act, was obtained from kinetic study of its reactions with 2-pyridyl disulphide reactivity probes (Brocklehurst *et al.*, 1979). The shape of the profile of pH versus apparent second-order rate constant (k) for the reaction of the papain thiol group with 4-(*N*-aminoethyl 2-pyridyl disulphide)-7-nitrobenzo-2-oxa-1,3-diazole [compound (I)] was shown to be similar to that of the pH–$k_{cat.}/K_m$ profiles obtained for substrate hydrolysis, i.e. bell-shaped with pK_a values approx. 4 and 9. This striking rate maximum in neutral media, which is not exhibited by reactions of papain with 2-pyridyl disulphides containing short side chains (2,2'-dipyridyl disulphide and n-propyl 2-pyridyl disulphide) was postulated to arise from interaction of the 2-pyridyl nitrogen atom with the imidazolium ion of histidine-159 repositioned after binding of the hydrophobic nitrobenzo-oxadiazole side chain in or near to the primary binding site (S_2) of papain.

The present paper reports the synthesis of 4-(*N*-aminoethyl 4-pyridyl disulphide)-7-nitrobenzo-2-oxa-1,3-diazole [compound (II)], the 4-pyridyl isomer of compound (I), and a kinetic study of its reactions with papain designed to test the postulates proposed by Brocklehurst *et al.* (1979). Evidence is presented to suggest (i) that compounds (I) and (II) do bind to papain before the covalent modification reaction occurs, (ii) that the binding is followed by an increase in the hydrophobicity of the environment around the thiol group of cysteine-25 and (iii) that the rate maximum in neutral media observed in the reaction of papain with compound (I) but not in its reaction with compound (II) does arise from association of the 2-pyridyl leaving group with a proton-donating group in papain, probably the imidazolium ion of histidine-159.



(I)



(II)

Materials and Methods

Synthesis of 4-(*N-aminoethyl* 4-*pyridyl disulphide*)-7-*nitrobenzo*-2-*oxa*-1,3-*diazole* [*compound* (*II*)]

2-Aminoethyl 2-aminoethanethiolsulphonate (Stuchbury *et al.*, 1975) was allowed to react with pyridine-4-thione to provide 2-aminoethyl 4-pyridyl disulphide. This was isolated as the dihydrochloride and subsequently allowed to react with 4-chloro-7-nitrobenzofurazan (Nbf-Cl) to provide compound (II).

(i) *Preparation of* 2-*aminoethyl* 4-*pyridyl disulphide dihydrochloride.* A solution of pyridine-4-thione (4.43g, 40mmol) in hot ethanol (40ml) was added dropwise during 1h to a stirred solution of 2-aminoethyl 2-aminoethanethiolsulphonate (10.3g, 40.7mmol) in a mixture of water (12ml) and conc. HCl (4.8ml). It is necessary to keep the ethanolic solution of pyridine-4-thione warm during the addition to prevent crystallization. In addition, ethanol (200ml) and water (50ml) were added to the reaction mixture as required to maintain solution of all components. Addition of ethanol is required to keep the pyridine-4-thione in solution, but the increase in ethanol content results in precipitation of the hydrochloride of the thiolsulphonate. This can be redissolved by addition of water. When the additions were complete, the solution was stirred at room temperature (approx. 22°C) overnight. Rotary evaporation of the reaction mixture produced a white powder, which was dissolved in water (20ml) and extracted with chloroform (3×25ml), and both phases were kept. The combined chloroform extracts were dried over anhydrous $MgSO_4$, which was subsequently removed by filtration. Evaporation of the solvent from the filtrate provided 4,4'-dipyridyl disulphide (0.4g; m.p. 74–75°C) as a by-product.

The aqueous layer from the chloroform extraction was cooled to 5–10°C, and an ice-cold solution of KOH (11.2g) in water (12ml) was added. The resulting emulsion was extracted immediately with chloroform (2×30ml), and the combined chloroform layers were extracted immediately with conc. HCl (3×20ml). Rotary evaporation of the combined HCl extract produced a tar, which was triturated with a little ethanol. The slurry was kept at 4°C for 3h and then filtered. The residue was mixed with ethanol (50ml), and methanol was added to the boiling mixture until solution was effected. After treatment with decolorizing charcoal and filtration, the filtrate was allowed to evaporate to 50% of its volume, and ethanol (20ml) was then added. The solution was boiled until crystals began to form, and was then allowed to cool to room temperature. The colourless needles (5.65g; m.p. 176–177°C) were isolated by filtration and identified as 2-aminoethyl 4-pyridyl disulphide dihydrochloride by elemental and spectroscopic analysis. Elemental analysis: found, C, 32.0; H, 4.2; N, 10.9%; calculated for $C_7H_{12}Cl_2N_2S_2$, C, 32.4; 4; 4.7; N, 10.8%. P.m.r. (60MHz) in ^2H_2O showed a doublet centred at 1.35τ, $J = 8$Hz (two aromatic protons), a doublet centred at 1.80τ, $J = 8$Hz (two aromatic protons) and a multiplet centred at 6.70τ (four aliphatic protons). The u.v.-absorption spectrum (aqueous solution, pH7.0) was characterized by two absorption bands with $\lambda_{max.}$ 212nm and 247nm. On thiolysis with 2-mercaptoethanol the long-wavelength band was replaced by one with $\lambda_{max.}$ 324nm that is characteristic of pyridine-4-thione.

(ii) *Reaction of* 2-*aminoethyl* 4-*pyridyl disulphide with Nbf-Cl.* 2-Aminoethyl 4-pyridyl disulphide dihydrochloride (2.6g, 10mmol) was dissolved in water (20ml). Ethanol (40ml) was then added, and the apparent pH (glass electrode) was adjusted from 2.5 to 7.0 by addition of dilute NaOH solution. A solution of Nbf-Cl (2g, 10mmol) in a mixture of ethanol (80ml) and water (40ml) was added to the above solution with stirring during 3–4h, the pH of the mixture being maintained between 8 and 9 by the occasional addition of 4M-NaOH. $NaHCO_3$ (2g) was then added, and the reaction mixture was stirred overnight. The solid product (2.4g of a brown powder) was collected by filtration and washed first with water and then with diethyl ether. A sample of the brown powder (1g) was added to boiling acetone (50ml), and the mixture was filtered on to the top of a column (25cm×2cm) packed with alumina type H in a chloroform slurry (0.5g remained undissolved). Elution with ethanol provided 4-(*N*-aminoethyl 4-pyridyl disulphide)-7-nitrobenzo-2-oxa-1,3-diazole [compound (II)] (200mg; m.p. 156–158°C) as an orange–brown solid from the second band on the column. Elemental analysis: found, C, 45.0; H, 3.9; N, 19.5%; $C_{13}H_{11}N_5O_3S_2$ requires C, 44.7; H, 3.2; N, 20.1%. Mass spectrum shows M^+ *m/e* 349 (mol.wt. 349) and *m/e* [M^+–(pyridine-4-thione)]. The u.v.-absorption spectrum in aqueous solution, pH7.0, shows $\lambda_{max.}$ 343nm (ε_{343} 6400$M^{-1}\cdot cm^{-1}$), $\lambda_{min.}$ 388nm and $\lambda_{max.}$ 478nm (ε_{478} 16000$M^{-1}\cdot cm^{-1}$). Thiolysis by L-cysteine produced the following absorption bands: $\lambda_{max.}$ 326nm (ε_{326} 19000$M^{-1}\cdot cm^{-1}$) and $\lambda_{max.}$ 475nm (ε_{475} 17800$M^{-1}\cdot cm^{-1}$) (pyridine-4-thione is characterized by $\lambda_{max.}$ 324nm (ε_{324} 19800$M^{-1}\cdot cm^{-1}$).

Other materials and methods

These have been described previously, notably papain, benzimidazol-2-ylmethanethiol, buffers and determination of second-order rate constants (k) by stopped-flow spectral analysis (Brocklehurst & Little, 1973; Stuchbury *et al.*, 1975; Shipton & Brocklehurst, 1978; Brocklehurst *et al.*, 1979). Kinetic runs were performed at 13.4% (v/v) acetonitrile at 25°C at I 0.1 in a Durrum D110 stopped-flow spectrophoto-

meter linked to a Tektronix 5103N storage oscilloscope. All solutions were degassed as a routine before stopped-flow experiments were performed. One syringe contained an aqueous solution of papain or benzimidazol-2-ylmethanethiol and the other a solution of compound (II) in double-strength buffer containing 26.8% (v/v) acetonitrile. The initial concentrations of the reactants in the mixing chamber of the stopped-flow apparatus were [papain] or [benzimidazol-2-ylmethanethiol] 6–9 μM and [compound (II)] 12.5–23 μM. Release of pyridone-4-thione, which occurred stoicheiometrically with the thiol, was monitored at 324nm. Absorbance changes were converted into concentration changes by using:

$$\Delta\varepsilon_{324} = 1.98\times10^4/[1+(K_a/[H^+])]$$

where $pK_a = 8.6$ (see Brocklehurst & Little, 1973; Stuchbury *et al.*, 1975).

Results and Discussion

The possibility of using site-specific reactivity probes that exhibit different reactivities in two ionization states (two-protonic-state reagents; see Brocklehurst, 1979*a*) to detect association–activation phenomena (see Schultz *et al.*, 1977) that involve repositioning of acid–base groups in enzyme active centres was discussed by Brocklehurst *et al.* (1979). The finding that, whereas the pH–*k* profiles for the reactions of papain with 2,2′-dipyridyl disulphide, n-propyl 2-pyridyl disulphide and 4,4′-dipyridyl disulphide are each characterized by a rate minimum in the pH region 6–7, the profile for the reaction of papain with compound (I) is characterized by a striking rate maximum in this pH region (see also Table 1), is the basis of the argument in the case of papain. It was suggested that, when the probe molecule contains a hydrophobic side chain that may be able to mimic the part of a specific substrate that binds to the primary binding site of papain (the S_2 subsite; see Berger & Schechter, 1970), the rotation of the side chain of histidine-159 proposed as part of the catalytic act (Brocklehurst & Malthouse, 1978; Angelides & Finks, 1978) is detected by the ability of the pyridyl nitrogen atom to engage the imidazolium ion of histidine-159 in hydrogen-bonding (Scheme 1*b*). This interaction, which is analogous to some extent to the acid-catalysed expulsion of a leaving group of a substrate in acylation (Scheme 1*a*), would account for the rate maximum at pH

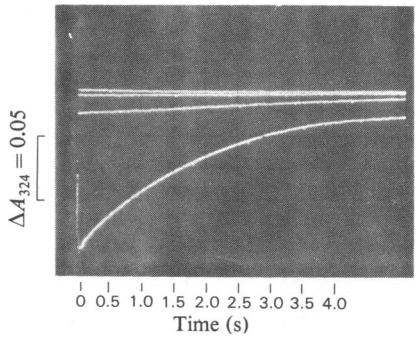


Fig. 1. *Stopped-flow record of the release of pyridone-4-thione during the reaction of papain* (6.68 μM) *with compound* (*II*) (22.3 μM) *at* 25°C *in formic acid/NaOH buffer at* *I* 0.1 *containing* 13.4% (*v*/*v*) *acetonitrile*
For further details see the text.

Table 1. *Second-order rate constant* (*k*) *for the reactions of the papain thiol group and of a low-molecular-weight model compound with disulphide reactivity probes in* 13.4% (*v*/*v*) *acetonitrile at* 25°C *at I* 0.1

Thiol	Disulphide	pH	$10^{-4}k$ ($M^{-1}\cdot s^{-1}$)
Benzimidazol-2-yl-methanethiol	Compound (II) (4-pyridyl isomer)	3.0	0.11
		4.4	0.76
		4.8	0.85
		5.1	0.80
		5.4	0.68
		6.1	0.47
		6.6	0.46
		7.6	1.76
		7.8	2.57
		8.3	3.95*
Papain	Compound (II) (4-pyridyl isomer)	2.8	0.40
		3.1	0.81
		3.8	2.01
		4.5	2.29
		4.8	2.08
		5.2	1.66
		5.5	1.37
		5.9	0.99
		6.5	0.94
		7.1	1.17
		7.6	1.74
		8.0	2.14
		8.9	3.02†
Papain	Compound (I) (2-pyridyl isomer)	3.2	1.3
		6.0	5.2
		8.3	3.3‡

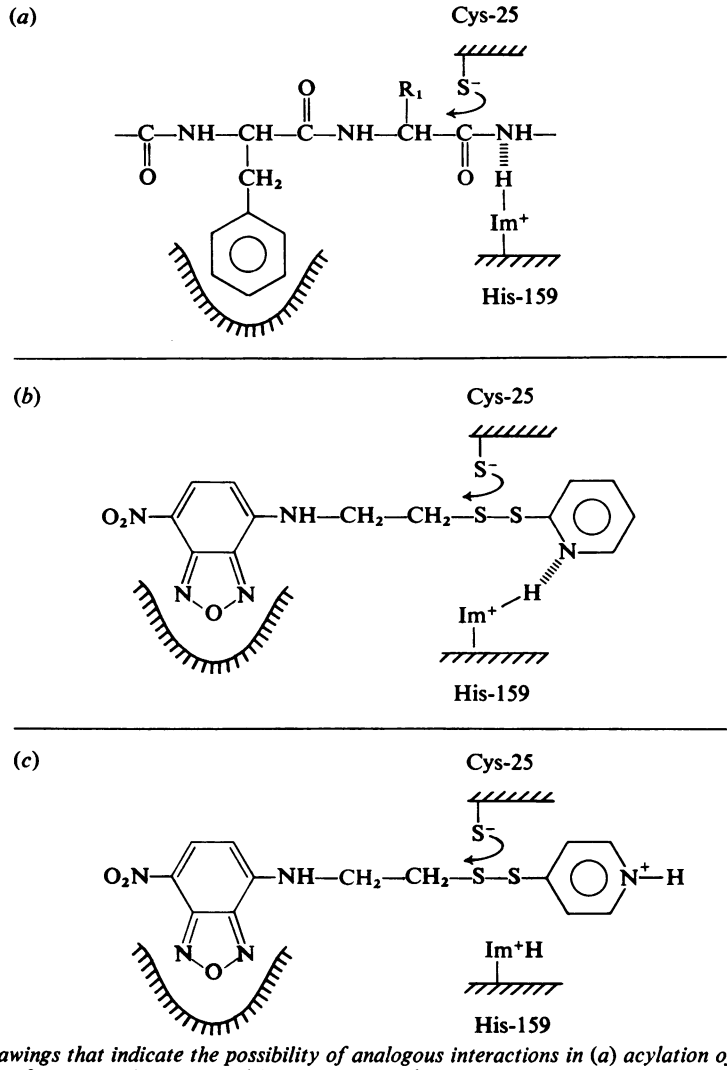
* The value of *k* at pH 8.3 (the pK_a value of benzimidazol-2-ylmethanethiol zwitterion) predicts a value of $\tilde{k}$, the pH-independent rate constant in alkaline media, of approx. $8\times10^4\,M^{-1}\cdot s^{-1}$.

† The value of *k* at pH 8.9 (the value of pK_{aII} of the imidazolium-thiol diad of the papain active centre) predicts a value of $\tilde{k}$ approx. $6\times10^4\,M^{-1}\cdot s^{-1}$.

‡ Data from Brocklehurst *et al.* (1979); the pH-dependence of *k* for this reaction is given by:

$$k = \tilde{k}/[1+([H^+]/K_I)+(K_{II}/[H^+])]$$

where the pH-independent rate constant, $\tilde{k} = 5.2\times10^4\,M^{-1}\cdot s^{-1}$, $pK_I = 3.5$ and $pK_{II} = 8.8$.



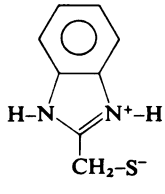
Scheme 1. *Schematic drawings that indicate the possibility of analogous interactions in (a) acylation of papain by a specific substrate and (b) reaction of papain with compound (I), and in (c) the lack of this possibility of hydrogen-bindong between the imidazolium ion of histidine-159 and the pyridyl nitrogen atom in compound (II), where reagent activation must be provided by protonation*

approx. 6. Both the reaction with compound (I) (Scheme 1*b*) and the acylation step of the catalytic act (Scheme 1*a*) require both the thiolate anion of cysteine-25 and the imidazolium ion of histidine-159. The other pyridyl disulphides studied previously (2,2′-dipyridyl disulphide, n-propyl 2-pyridyl disulphide and 4,4′-dipyridyl disulphide) would not be capable of both binding in or near to the S_2 subsite and engaging the imidazolium ion of histidine-159 in hydrogen-bonding.

This interpretation of the pH–*k* profile of the reaction of papain with compound (I) predicts a strikingly different shape for the pH–*k* profile of the reaction of papain with compound (II), the 4-pyridyl isomer of compound (I). By changing the position of the pyridyl nitrogen atom to the 4-position, its association with the imidazolium ion of histidine-159 should be prevented and activation of the probe reagent would be expected to rely on direct protonation (Scheme 1*c*), as is the case with the other pyridyl disulphides listed above. The consequence of this would be a rate minimum in the pH region 6–7 rather than a rate maximum, a rate maximum at pH approx. 4–5 consequent on reagent protonation

(see Brocklehurst & Little, 1973; Stuchbury *et al.*, 1975) and a rate plateau at pH values above 9. That this predicted shape of the pH–k profile is observed experimentally is suggested by the data in Table 1. A stopped-flow record of a typical progress curve is shown in Fig. 1. A similar shape of profile is observed also for the reaction of benzimidazol-2-ylmethanethiol with compound (II). This low-molecular-weight thiol possesses nucleophilic reactivity in two ionization states (compounds (III) and (IV); see Stuchbury *et al.* (1975)] and serves as a useful model for the interactive imidazole–thiol system of the papain active centre.

Apparent second-order rate constants (k) for protein-modification reactions studied under conditions in which second-order kinetics are obeyed are probably best regarded as ratios of true first-order rate constants for reactions within adsorptive complexes and dissociation constants of the enzyme–reagent complexes (see Brocklehurst, 1979*b*). Despite the inability of compound (II) to engage the imidazolium ion of histidine-159, the postulated binding of its benzo-oxadiazole moiety in or near to the S_2 subsite and subsequent conformational change would be predicted to affect the reactivity of the papain thiol group towards this reagent. The limited solubility of compound (II) precluded the possibility of demonstrating binding by conventional saturation kinetics. The fact that the values of k for the reaction of papain with compound (II) are substantially greater (see Table 1) than those for its reaction with the smaller reagent 4,4′-dipyridyl disulphide (Brocklehurst & Little, 1972; e.g. at pH5 $k = 360\,M^{-1}\cdot s^{-1}$, at pH6 $k = 240\,M^{-1}\cdot s^{-1}$ and at pH8.1 $k = 4000\,M^{-1}\cdot s^{-1}$), however, suggests better binding of the larger more hydrophobic reagent. The fact that the rate maximum at pH approx. 5 is very much more highly developed relative to the values of k in alkaline media in the reaction of papain with compound (II) than it is in either the reaction of papain with 4,4′-dipyridyl disulphide or the reaction of benzimidazol-2-ylmethanethiol with compound (II) suggests that the binding of compound (II) to papain produces a different reaction environment. That this might be a more hydrophobic environment is suggested by the known increase in the rate of reactions between oppositely charged ions with decrease in the dielectric constant of the medium (see Reichardt, 1965).



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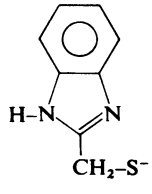

(IV)

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