Investigation of the Active Site of Papain with Fluorescent Probes

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7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD chloride) and 7-(2'-hydroxyethylthio)-NBD (obtained from NBD chloride and mercaptoethanol) undergo a reversible spectral change in alkaline solution that depends respectively on a single apparent pK_a 9.76 (at 25°C) and 8.81 (at 32°C). In acid solution however no spectral change was observed. NBD chloride reacts slowly with papain at pH 7, but the rate of inhibition increases at lower pH and depends on an apparent pK_a of 3.7 (at 35°C), which has been tentatively assigned to the carboxyl group of aspartic acid-158. The spectral properties of NBD-papain indicate that the thiol group of cysteine-25 is the site of reaction. The intensity of the fluorescence-emission spectrum of NBD-papain depends on a single pK_a of 4.2 (at 26.7°C). The intensity of the fluorescence-emission spectrum of the mixed disulphide formed from papain and 7-(2'-mercaptoethylamino)-NBD (obtained from NBD chloride and cysteamine) depended on a single pK_a of 3.94 in water and 3.89 in aq. 19.2% (v/v) dioxan (at 27°C). This small change to lower pK_a value in a medium of lower dielectric constant is characteristic of a cationic acid. The only acid of this type in the active-site region is the conjugate acid of histidine-159.

The reaction pathway for papain-catalysed hydrolyses of $N\alpha$ -acyl-L-amino acid derivatives involves an acyl-enzyme intermediate formed through the thiol group of the enzyme (Lowe, 1970; Glazer & Smith, 1971). The bell-shaped pH-dependence of the acylation rate constant suggests that two groups are involved with apparent pK_a values of about 4.2 and 8.2 (Lowe & Yuthavong, 1971; Williams *et al.*, 1972). The deacylation rate constant also shows pH-dependence on a single basic group with apparent pK_a of 3.3–4.7, depending on the structure of the acyl residue (Glazer & Smith, 1971; Lowe, 1970). The apparent pK_a near 8.2 has generally been ascribed to the thiol group of the active-site cysteine-25, and this is supported by the pH-dependence on a single basic group with an apparent pK_a near 8.2 for the irreversible inhibition of papain by uncharged inhibitors such as chloroacetamide (Chaiken & Smith, 1969a) and 1-chloro-4-phenyl-3-L-toluene-*p*-sulphamidobutan-2-one (TPCK) (Bender & Brubacher, 1966), which are known to alkylate the thiol group of cysteine-25. It has been suggested that a carboxyl group is responsible for the apparent pK_a near 4 in the acylation and deacylation steps, since this value and its low heat of ionization were consistent with those of normal carboxyl groups (Stockell & Smith, 1957). Model studies, however, suggested that the group involved was much more probably the imidazole group of a histidine residue (Lowe & Williams, 1965a), and subsequent chemical studies (Husain & Lowe, 1968a,b) and X-ray analysis (Drenth *et al.*, 1968) confirmed the presence

of such a residue in close proximity to the thiol group of the active-site cysteine-25. Inspection of the molecular environment of the active-site histidine as revealed by X-ray analysis led, however, to quite divergent views as to its probable pK_a . Sluyterman & Wolthers (1968) and their Dutch colleagues (Drenth *et al.*, 1970) suggested that it should be assigned a pK_a of 9.5–10, because of hydrogen-bonding to asparagine-175 and the electrostatic field of aspartic acid-158, which led them to propose a mechanism in which histidine-159 participated in the catalytic process as its conjugate acid. The pH-dependence of the acylation and deacylation rate-constants on an apparent pK_a near 4 was considered to be due to a carboxyl group, which in its undissociated state led to an inactive conformation of the enzyme. It seemed to us, however, that a more significant feature of the micro-environment of histidine-159, at least as far as its dissociation constant is concerned, is the fact that it is enclosed by a hemisphere of hydrophobic residues and in particular that it is partially shielded from solvent by the indole group of tryptophan-177. Such an environment would be expected to markedly lower the pK_a of an imidazole group, since the charged conjugate acid would be thermodynamically less stable compared with one in which it may be more easily solvated. Dissociation therefore would be strongly favoured, since the charge would be released as a proton into the bulk solvent. The effect of lowering the dielectric constant in the microenvironment of an enzyme would be much larger than that for a

cationic acid in free solution where the solvation of the proton and the imidazolium ion would be rather similar.

In an attempt to determine independently the pK_a values of the groups in the active site of papain, two fluorescent probes containing the 7-substituted 4-nitrobenzo-2-oxa-1,3-diazole (NBD) residue (I) were attached to cysteine-25. The pH-dependence of their fluorescence-emission spectra and the rate of reaction of NBD chloride (I, $R = Cl$) with papain have been studied. The properties of some simple NBD derivatives were also investigated.

Materials and Methods

7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD chloride; I, $R = Cl$) (Boulton *et al.*, 1966)

This was a gift from Dr. G. K. Radda.

7-(2'-Hydroxyethylthio)-NBD
(I, $R = S-CH_2-CH_2OH$)

A solution of NBD chloride (13.2 mm) in aq. ethanol (50%, v/v; 0.95 ml) was treated with aq. sodium acetate solution (0.11 M, 0.1 ml) and aq. 2-mercaptoethanol solution (1 M, 0.1 ml). After 2.5 h the solution had λ_{max} 424 nm and λ_{min} 338 nm, indicating that no NBD chloride remained. The solution was evaporated (at $<50^\circ C$) to remove solvent and excess of mercaptoethanol and the product dissolved in the aq. sodium acetate buffer (5 ml, pH 3.5).

7-(2'-Mercaptoethylamino)-NBD
(I, $R = NH-CH_2-CH_2SH$)

This was prepared from cysteamine hydrochloride (50 mg) and NBD chloride (3 mg) in the presence of trisodium citrate (300 mg) in aq. 50% acetonitrile (3 ml). After 5 h, the reaction mixture was diluted with water, acidified to pH 4–5 with acetic acid and the NBD derivative was extracted into ethyl acetate. The extract was dried ($MgSO_4$), and the solvent was evaporated. The product had λ_{max} 344 nm (E 0.48) and 477 nm (E 1.53) in acetonitrile–water (4%, v/v).

Papain

This was prepared by the method of Kimmel & Smith (1954) from dried papaya latex, and further purified from oxidized enzyme by the method of Sluyterman & Wijdenes (1970) on an agarose-mercurial column, thus giving 100%-activatable enzyme. Papain solutions were assayed against methyl hippurate, by the pH-stat method, or spectrophotometrically with *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester (Lowe & Williams, 1965*b*). Occasionally, papain was assayed by the caseinolytic method of Kunitz (1947). All assay solutions contained EDTA (1 mM). All assays were related to that with methyl hippurate, for which $k_{cat.} = 8.0 s^{-1}$ at $35^\circ C$, pH 6.0, with fully active papain. The specific activity of papain solutions was calculated by using an ϵ_{280} value of 57 500 litre $\cdot$ mol $^{-1} \cdot$ cm $^{-1}$ based on the molecular weight 23 406 (Mitchel *et al.*, 1970) and $E_{280}^{1\%}$ of 25 (Kimmel & Smith, 1957).

Fluorescence-emission spectra

These were determined by using an Aminco-Bowman or Hitachi-Perkin-Elmer spectrofluorimeter. U.v.- and visible-absorption spectra were determined with a Unicam SP.800 spectrophotometer pH measurements were determined with a Radiometer type TTT1c meter with glass and calomel electrodes. The meter was standardized at $20^\circ C$ with BDH standard buffer solutions (BDH Chemicals Ltd., Poole, Dorset, U.K.). The meter had automatic temperature compensation and no correction was necessary for measurements in aqueous solutions containing 20% dioxan (Purlee & Grunwald, 1957). All reagents used for protein work were of AnalaR grade, and the water was deionized.

The pH-dependence of the u.v.-absorption spectra of NBD chloride (I, $R = Cl$) and 7-(2'-hydroxyethylthio)-NBD (I, $R = S-CH_2-CH_2OH$) in aqueous solution were determined in buffers prepared from Na_2HPO_4 (50 mM) solution and either NaOH (0.1 M) or citric acid (0.1 M). A solution of NBD chloride in ethanol (0.1 ml) was added to each of the buffer solutions (3.0 ml) to give a concentration of 0.154 mM, and the spectrum was determined after 2 min at $25^\circ C$. The spectra of 7-(2'-hydroxyethylthio)-NBD (0.079 mM) in the same buffer solutions were determined at $32^\circ C$.

NBD-papain

Papain was activated with 2-mercaptoethanol, and separated from excess of thiol by gel filtration on a column of Sephadex G-25, in EDTA (1 mM) at pH 5. The reaction of papain with NBD chloride was followed spectroscopically at 405 nm. The pH-dependence of the reaction was studied at $26^\circ C$ and $35^\circ C$ with the following concentrations of reagents: papain (0.0275 mM), NBD chloride (0.115 mM), ethanol

(3.8%, v/v), sodium citrate buffers (total citrate 0.023M) and EDTA (1mM). Second-order rate constants were calculated from the initial concentration of papain and the observed total change in E_{405} for the reaction at pH 4.0. The pH was measured immediately after completion of the reaction, at the same temperature. At pH 5–9 the rate of reaction between NBD chloride and papain was also studied by determination of the rate of loss of enzymic activity in the absence of added thiol.

Stock solutions of NBD-papain were prepared by the reaction of active papain with excess of NBD chloride in a sodium acetate buffer, pH 3.9. The NBD-papain was purified on a column of Sephadex G-25 in EDTA solution (1mM), pH 5, and stored at 0°C. For fluorescence measurements, sodium citrate buffers (2.5 ml, 0.04M total citrate) in water, and aq. dioxan were prepared. The dioxan was freshly purified (see below), and these buffers were stored under N_2 and used within 1 day. NBD-papain solution (1 ml) was added to each of the buffer solutions, and, after mixing, a portion was transferred to a thermally equilibrated (26.7°C) 1-cm-light-path cuvette. The fluorescence spectra were determined after about 10 min. At pH 3.5, measurements were taken as a function of time and extrapolated to the time of mixing. The pH was determined immediately after the spectral measurements, at 25°C.

Mixed disulphide of papain and 7-(2'-mercaptoethylamino)-NBD (I, R = NH-CH₂-CH₂SH)

Active papain (0.030 mm) in EDTA solution (5 ml, 1 mM) was treated with trisodium citrate (100 mg) and a solution of 7-(2'-mercaptoethylamino)-NBD (approx. 2.5 mM) in acetonitrile (0.2 ml); the mixture was kept in air for 3 days at 0°C, and the protein was purified by gel filtration at pH 5. Fluorescence titrations were performed in aqueous buffers and aq. dioxan buffers as above, with excitation at 464 nm. The absorption of the solutions at 478 nm was 0.021 unit, so that correction for self-absorption was unnecessary.

Purification of dioxan

Dioxan (250 ml), 12M-HCl (3.5 ml) and water (25 ml) were refluxed for 15 h. The solution was cooled to 0°C and KOH pellets were added until no more dissolved. The upper layer of dioxan was decanted and kept over KOH pellets for 1.5 h. The dioxan was decanted, sodium wire (1 g) added and the mixture refluxed for 18 h under N_2 . The dioxan was distilled from the residual sodium, b.p. 100.5–101°C, and was stored at 0°C under N_2 . Immediately before use, the dioxan was passed through a column of neutral alumina (Camag; Hopkin and Williams, Chadwell Heath, Essex, U.K.), Brockmann activity 1, to remove traces of peroxides.

Results and Discussion

Properties of NBD chloride (I, R = Cl) and 7(2'-hydroxyethylthio)-NBD (I, R = S-CH₂-CH₂OH)

The u.v. absorption spectra of NBD chloride and 7(2'-hydroxyethylthio)-NBD were pH-dependent at alkaline pH. The spectrum of NBD chloride was, however, unchanged between pH –0.3 and pH 7, but in 1M-NaOH a steady, irreversible change occurred. However, addition of HCl to a solution of NBD chloride that had been kept at pH 11 for only a few minutes at 25°C resulted in a rapid reversal of this spectral change. The pH-dependence of the absorption spectra (Fig. 1) fits closely a titration curve for a single group with pK_a 9.76 (Fig. 2). The absorption spectrum of 7(2'-hydroxyethylthio)-NBD was also pH-dependent (Fig. 3), fitting a titration curve with pK_a 8.81 at 32°C (Fig. 4). Addition of an excess of mercaptoethanol to 7(2'-hydroxyethylthio)-NBD or to NBD chloride in aqueous solution at pH > 5 also resulted in striking spectral changes. At pH 6.3, the absorbance at 425 nm of 7(2'-hydroxyethylthio)-NBD (approx. 0.1 mM) in the presence of mercaptoethanol (40 mM) decreased to less than one-half the value observed in the absence of thiol. The use of labels based on the NBD fluorophore should be restricted to the acid pH region therefore, unless corrections for the alkaline pH-dependence of the group itself are made. Similarly, the use of this fluorescent probe in proteins where more than 1 thiol group may be juxtaposed may lead to a subsequent intramolecular reaction.

Reaction of papain with NBD chloride (I, R = Cl)

NBD chloride reacts rapidly in aqueous solution with thiols at pH 7.0, and more slowly with amines, to give fluorescent adducts (Ghosh & Whitehouse, 1968). The thiol group of papain, which shows unusually high reactivity towards some alkylating reagents, reacted with NBD chloride at pH 7 rather slowly, and hydrolysis of the reagent was a competing reaction. At pH 5.0, the reaction was faster and after gel filtration the papain derivative had λ_{max} 402 nm. The extent of reaction, measured by absorption at 402 nm, was well correlated with the loss of enzymic activity (Fig. 5). Mercaptoethanol re-activated the enzyme, presumably by thiolysis. The initial rate of reaction of papain with NBD chloride was pH-dependent, reaching a maximum at pH 3.2. The results are consistent with catalysis by an acidic group with pK_a 3.7, with deviations below pH 3.5 (Fig. 6). The rate of reaction at pH 5–9, where hydrolysis of the reagent and reaction with amino groups interfere with the spectral measurements, was determined by measurement of the loss of papain activity. The rate of reaction in this pH range was less than that below pH 5.

Fig. 1. U.v.-absorption spectra of NBD chloride (I, $R = Cl$) at approx. $25^{\circ}C$ and (1) pH 11.61, (2) pH 10.77, (3) pH 9.85, (4) pH 9.36, (5) pH 5.71 to -0.3

Fig. 2. Spectroscopic titration of NBD chloride (I, $R = Cl$) at approx. $25^{\circ}C$

The theoretical curve is for a single group with pK_a 9.76, as shown by the arrow.

Complete loss of enzymic activity was observed when 1.1 mol of NBD chloride had reacted with 1 mol of active papain, calculated from the loss of absorption at 343 nm owing to NBD chloride. The u.v.- and visible-absorption spectrum of NBD-papain (Fig. 7) had λ_{max} 402 nm (ϵ 12 100 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$), whereas 7-(2'-hydroxyethylthio)-NBD (I, $R = S-CH_2-CH_2OH$) had λ_{max} 425 (ϵ 12 100 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$). Denaturation of the NBD-papain, however, either with HCl or with 6M-guanidinium chloride, gave a spectrum with λ_{max} 422 nm (Fig. 7). The reac-

tion had occurred therefore at the active-site thiol group.

The irreversible inhibition of papain by α -halo acids also depends on a group with an apparent pK_a near 4 (Chaiken & Smith, 1969b; Wallenfels & Eisele, 1968). The pH-dependence of the rate of inhibition by NBD chloride is, however, strikingly similar to the observation made by Brocklehurst & Little (1970, 1972) that the pH-dependence of the inhibition of papain by 2,2'-dipyridyl disulphide depends on the acidic form of a group with pK_a 3.75, but whether this was due to an acidic residue on the enzyme or to the protonated pyridyl residue of the reagent itself perturbed from its normal value in the enzyme inhibitor complex was not clear. As NBD chloride has no ionization constant between pH -0.3 and 7 the pK_a of 3.7 observed for the rate of inhibition of papain must be associated with the enzyme. The tertiary structure of papain suggests that in the transition state for the attack by the thiol group of cysteine-25 on NBD chloride, the oxadiazole ring could be in close proximity to the carboxyl group of aspartic acid-158. Since the NBD residue must accept electrons in the transition state, a neighbouring undissociated carboxyl group could stabilize the transition state by hydrogen bonding, but a dissociated carboxyl group would destabilize the transition state and so lead to a slower rate of inhibition. The pK_a of 3.7 could therefore most reasonably be assigned to aspartic acid-158. The possibility that a conformational change, due to the ionization of a single group, leads to the increase in reactivity towards NBD chloride appears much less likely. The deviation of the pH-dependence

Fig. 3. U.v.-absorption spectra of 7-(2'-hydroxyethylthio)-NBD ($I, R = S-CH_2-CH_2OH$) at $32^\circ C$ and (1) $pH 4.73$, (2) $pH 8.09$, (3) $pH 8.83$, (4) $pH 9.62$, (5) $pH 10.95$

Fig. 4. Spectroscopic titration of 7-(2'-hydroxyethylthio)-NBD ($I, R = S-CH_2-CH_2OH$) at $32^\circ C$

The theoretical curve is for a single group with pK_a 8.81 as shown by the arrow.

Fig. 5. Enzymic activity of papain (initial concentration approx. $0.016 mM$) during the reaction with NBD chloride ($0.067 mM$ initial concentration) as a function of E_{400} at $pH 5.0$ and $20^\circ C$

of the rate of inhibition of papain by NBD chloride from the theoretical titration curve below $pH 3.5$ could be due to the ionization of histidine-159, but the limited stability of the system precluded an extension of this profile to lower pH .

Fluorescence titration of NBD-papain

A typical fluorescence-emission spectrum of NBD-papain is shown in Fig. 8. The low quantum yield made obligatory a high protein concentration and high instrumental sensitivity, resulting in relatively

intense scattering peaks. The pH -dependence of the fluorescence-emission intensity at $500 nm$ (excitation at $400 nm$) is shown in Fig. 9. In water the results fit a theoretical titration curve for a single group, pK_a 4.2, but the fluorescence-intensity measurements in aq.

Fig. 6. *pH*-dependence of the initial rates (s^{-1}) of reaction of NBD chloride with papain in citrate buffers at $35^{\circ}C$

The theoretical curve is for a single group with pK_a 3.7.

Fig. 7. *U.v.- and visible-absorption spectrum of NBD-papain in 1 mM-EDTA at pH 5.0 (—) and in 1 mM-EDTA at pH 5.0 containing 6M-guanidinium chloride (----)*

dioxan (20%, v/v) were unsatisfactory owing to time-dependent changes in the fluorescence-emission spectra below pH 4. Sluyterman & De Graaf (1970) observed that the fluorescence-emission spectrum of

Fig. 8. *Fluorescence spectrum of NBD-papain, pH 4.53, in citrate buffer (20 mM), with EDTA (0.29 mM), at $26.7^{\circ}C$*

E_{400} (1 cm) = 0.062 and excitation was at 400 nm. Relative fluorescence intensity is in arbitrary units.

Fig. 9. *Fluorescence titration of NBD-papain in citrate buffers (0.029 M) at $26.7^{\circ}C$*

The theoretical curve is for a single group with pK_a 4.20. Relative fluorescence intensity is in arbitrary units.

the mixed disulphide formed from papain and 2-mercaptoethanol depended on a group with pK_a 4.1, but it was not conclusively demonstrated that this group was in the active-site region of the enzyme. The fluorescence-emission spectrum of papain has, however, been subsequently shown to be dominated by a

Fig. 10. U.v.- and visible-absorption spectrum of the mixed disulphide of papain and 7-(2'-mercaptoethylamino)-NBD (*I*, $R = \text{NH}-\text{CH}_2-\text{CH}_2\text{SH}$), at pH 5, in 1 mM-EDTA

The spectrum (curves A and B) is shown on two scales (E_A and E_B respectively) differing in value by a factor of 10.

Fig. 11. Fluorescence spectrum of mixed disulphide of papain and 7-(2'-mercaptoethylamino)-NBD (*I*, $R = \text{NH}-\text{CH}_2-\text{CH}_2\text{SH}$) in water at 27°C

Excitation was at 464 nm. Relative fluorescence intensity is in arbitrary units.

single tryptophan residue (Steiner, 1971) and, since this is quenched in active papain (below pH 8) and in mercuripapain, this residue is almost certainly tryptophan-177. The only ionizable group close enough to

Fig. 12. Fluorescence titration of the mixed disulphide of papain and 7-(2'-mercaptoethylamino)-NBD (*I*, $R = \text{NH}-\text{CH}_2-\text{CH}_2\text{SH}$) in water (●) and aq. 19.2% (v/v) dioxan (▲) at 27°C in citrate buffers

Excitation was at 464 nm and emission at 530 nm. The theoretical curves are for single ionization groups with $\text{p}K_a$ values of 3.94 and 3.89 respectively. Relative fluorescence intensity is in arbitrary units.

this residue to affect the fluorescence-emission spectrum is the imidazole group of histidine-159. It seems likely therefore that the same group was influencing the fluorescence-emission spectrum in both these observations and that this was histidine-159.

*Fluorescence titrations of the mixed disulphide formed between papain and 7-(2'-mercaptoethylamino)-NBD (*I*, $R = \text{NH}-\text{CH}_2-\text{CH}_2\text{SH}$)*

Cysteine reacts rapidly at pH 7 with NBD chloride to form the *S*-NBD derivative, which rapidly rearranges to *N*-NBD-cysteine (Birkett *et al.*, 1970). 7-(2'-Mercaptoethylamino)-NBD was prepared from NBD chloride by using an excess of cysteamine, so that the major product was the mono-substituted

compound, which was separated from excess of cysteamine by solvent extraction. Aerial oxidation of papain with 7-(2'-mercaptoethylamino)-NBD gave the mixed disulphide. The protein was separated from low-molecular-weight material by gel filtration. The u.v.- and visible-absorption spectrum of the mixed disulphide is shown in Fig. 10. The spectrum was very similar to that of 7-(2'-mercaptoethylamino)-NBD.

A typical fluorescence-emission spectrum of the mixed disulphide is shown in Fig. 11, and the pH-dependence of the fluorescence-emission intensity is shown in Fig. 12. The results fit closely to a theoretical titration curve for a single group, pK_a 3.94, in water at 27°C. In aq. 19.2% (v/v) dioxan the points below pH 3.2 deviate from the curve for pK_a 3.89, which provides a good fit to the results at higher pH. Denaturation of the enzyme is probably responsible for this deviation at low pH. The slight decrease in the pK_a of the fluorescence titration curve on addition of dioxan indicates that the group that is titrated is not a carboxylate group, since the pK_a would be expected to increase, but is consistent with titration of a cationic acid, which can most reasonably be assigned to the imidazole group of histidine-159.

The pK_a values observed in NBD-papain and in the mixed disulphide of papain with 7-(2'-mercaptoethylamino)-NBD are most likely to be due to the same group on the protein, namely the imidazole group of histidine-159. It seems more likely, however, that the β -carboxyl group of aspartic acid-158 is responsible for the increase in the rate of inhibition of papain by NBD chloride at low pH. Since the orientation of the NBD chloride in the transition state leading to inhibition will be rather different from the NBD residue in the NBD-papain or the mixed disulphide, it is quite possible that the rate of inhibition by NBD chloride and the fluorescence-emission spectra will be influenced by different ionizable groups.

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