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4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole as a Reactivity Probe for the Investigation of the Thiol Proteinases

EVIDENCE THAT FICIN AND BROMELAIN MAY LACK CARBOXYL GROUPS CONFORMATIONALLY EQUIVALENT TO THAT OF ASPARTIC ACID-158 OF PAPAIN

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1. 4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (Nbd chloride) was used as a reactivity probe to characterize the active centres of papain (EC 3.4.22.2), ficin (EC 3.4.22.3) and bromelain (EC 3.4.22.4). 2. In the pH range 0–8 Nbd chloride probably exists mainly as a monocation, possibly with the proton located on N-1 of the oxadiazole ring. 3. Spectroscopic evidence is presented for the intermediacy of Meisenheimer-type adducts in the reaction of Nbd chloride with nucleophiles. 4. The pH-dependence of the second-order rate constants (k) of the reactions of the three enzymes with Nbd chloride was determined at 25°C, $I = 0.1$ mol/litre in 6.7% (v/v) ethanol in the pH range 2.5–5, where, at least for papain and ficin, the reactions occur specifically with their active-centre thiol groups. The pH-k profile for the papain reaction is bell-shaped ($pK_{a_I} = 3.24$, $pK_{a_{II}} = 3.44$ and $k = 86 M^{-1} \cdot s^{-1}$), whereas that for ficin is sigmoidal ($pK_a = 3.6$, $k = 0.36 M^{-1} \cdot s^{-1}$), the rate increasing with increasing pH. The profile for the bromelain reaction appears to resemble that for the ficin reaction, but is complicated by amino-group labelling. 5. The bell-shaped profile of the papain reaction is considered to arise from the reaction of the thiolate ion of cysteine-25, maintained in acidic media by interaction with the side chain of histidine-159, with the Nbd chloride monocation hydrogen-bonded at its nitro group to the un-ionized form of the carboxyl group of aspartic acid-158. The lack of acid catalysis in the corresponding reactions of ficin and probably of bromelain suggests that these enzymes may lack carboxyl groups conformationally equivalent to that of aspartic acid-158 of papain. The possible consequences of this for the catalytic sites of these enzymes is discussed.

4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (4-chloro-7-nitrobenzofurazan, Nbd chloride; I, Scheme 1) was first synthesized by Boulton *et al.* (1966) as an intermediate in a synthetic route to 4-aminobenzofurazans. This compound was shown to alkylate thiols, alcohols, amines and azide ion by Ghosh & Whitehouse (1968*a*), to provide products such as compound III (Scheme 1), probably through the intermediacy of Meisenheimer (1902) adducts (II, Scheme 1). Ghosh & Whitehouse (1968*b*) proposed the use of Nbd chloride as a fluorescent labelling reagent for amino groups, and pointed out the very much lower fluorescence intensity of the products of its reactions with other nucleophiles, notably thiols. Despite this drawback and problems with group specificity, Nbd chloride was used by Birkett *et al.* (1970) as a fluorescent thiol-labelling reagent for the investigation of phosphorylase *b* and glyceraldehyde 3-phosphate dehydrogenase, and it has been used subsequently in this way for other investigations (see, e.g., Price *et al.*, 1975). A mixed-disulphide derivative of Nbd chloride containing a 2-pyridyl moiety was shown by Stuchbury *et al.* (1975) to overcome both the specificity and low-fluorescence-intensity problems that detract from the use of Nbd chloride as a fluorescent labelling reagent for thiol groups.

The reactivity of Nbd chloride as an alkylating agent has been used to characterize conformational states of phosphorylase *b* (Birkett *et al.*, 1971; Radda, 1971), and its potential as a particularly useful reactivity probe became evident from the observations of Allen & Lowe (1973). These authors showed that

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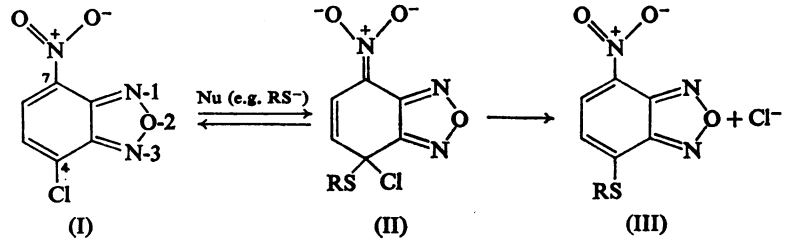


(I) (II) (III)

Scheme 1. *Action of Nbd chloride (I) in the alkylation of a nucleophile (Nu), typified by a thiolate ion (RS^-), to provide the product (III) through the intermediacy of a Meisenheimer-type adduct (II)*

the rate of the reaction of the essential thiol group of papain (EC 3.4.22.2) with Nbd chloride increased as the pH was lowered and appeared to depend on the acidic form of a group of $pK_a = 3.7$. They suggested that this group might be the un-ionized carboxyl group of aspartic acid-158.

In the preceding paper (Malthouse & Brocklehurst, 1976) it is pointed out that data obtained by using 2,2′-dipyridyl disulphide as a reactivity probe could be interpreted as evidence that ficin (EC 3.4.22.3) does not possess a carboxyl group conformationally equivalent to that of aspartic acid-158 in papain. It seemed appropriate, therefore, to study the kinetics of the reaction of Nbd chloride with ficin. The lack of acid catalysis in the ficin–Nbd chloride reaction found in the present work is in marked contrast with the catalysis found in the papain–Nbd chloride reaction and appears to confirm the structural difference in the active centres of these enzymes suggested in the preceding paper (Malthouse & Brocklehurst, 1976). A limited study of the reactions of bromelain (EC 3.4.22.4) with Nbd chloride suggests that the active centre of this enzyme may resemble that of ficin rather than that of papain.

Materials and Methods

Nbd chloride was obtained from Aldrich Chemical Co., Wembley, Middx. HA0 1PY, U.K., and recrystallized from aqueous ethanol (1:1, v/v) to give a product with m.p. 96–97°C. For most purposes, stock solutions (up to 20mM) were prepared in ethanol, protected from the light with aluminium foil and used within 12h. For experiments in strongly alkaline media, stock solutions (200μM) were prepared in water and diluted sixfold into the alkaline reaction mixtures.

Other reagents and some methods are described in the preceding paper (Malthouse & Brocklehurst, 1976) and by Stuchbury *et al.* (1975).

Enzymes

Fully active preparations of all three enzymes, containing 1 mol of thiol with high reactivity towards 2-Py–S–S–2-Py (2,2′-dipyridyl disulphide) at pH 4/mol of protein, were prepared by covalent chromatography: ficin as described in the preceding paper (Malthouse & Brocklehurst, 1976), papain as described by Stuchbury *et al.* (1975) and bromelain by a procedure similar to that used for papain (M. P. J. Kierstan & K. Brocklehurst, unpublished work). [Crude bromelain powder (BDH, Poole, Dorset, U.K.) is dissolved in 1mM-EDTA solution, pH 6.0, containing 0.3M-KCl. The solution is freed from insoluble material by filtration and then subjected to covalent chromatography as described for papain.]

Electronic-absorption spectra

These were recorded by using Aminco DW2, Cary 15 and Cary 118 spectrophotometers.

Kinetics of the reactions of Nbd chloride with papain, ficin and bromelain

Initial rates, and, for some of the papain reactions, complete progress curves, were recorded by using a Morrow stopped-flow attachment coupled to an Aminco dual-wavelength (DW2) spectrophotometer with chart-recorder output. This technique minimizes complications in reactions at low pH due to 'irreversible' acid denaturation. This is because the enzyme is kept in one syringe in aqueous solution (pH 6–7) and is subjected to the pH of the reaction mixture and to the reagent simultaneously. The reagent-syringe reservoir contained 10ml of triple-strength buffer (see Stuchbury *et al.*, 1975), 3ml of water (2–x)ml of ethanol and $x$ml of ethanolic Nbd chloride solution (20mM; $x \leqslant 2$ml). All solvents were de-gassed before use.

Determination of the characterizing parameters of pH–k profiles

(*i*) *The bell-shaped profile of Fig.* 2. The parameters $\tilde{k}$, pK_I and pK_{II} were evaluated by using an optimization procedure (see Swann, 1969). The method used was that of Nelder & Mead (1965) and the computer program used was essentially program AS47 described by O'Neill (1971).

(*ii*) *The sigmoidal profile of Fig.* 5. The parameters $\tilde{k}$ and pK_a were determined by linear regression of $1/k$ on $[H^+]$.

Results and Discussion

Structure of Nbd chloride in neutral and weakly acidic media

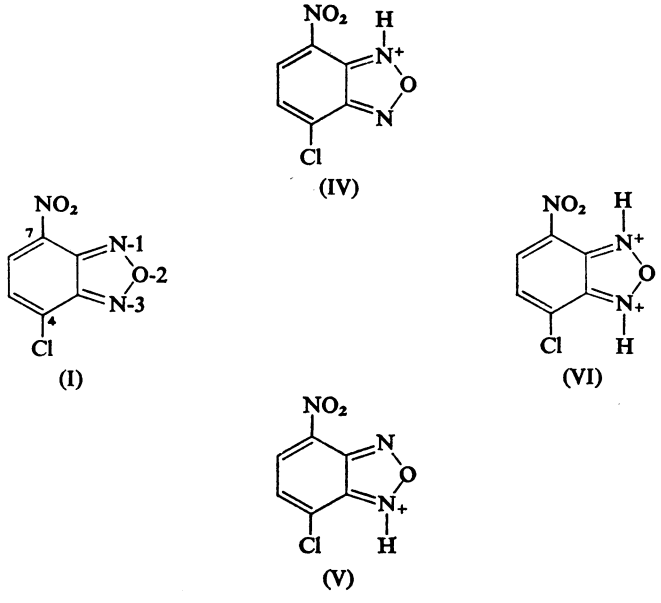
To interpret the acid catalysis of the reaction of papain with Nbd chloride described by Allen & Lowe (1973) (see below) it is important to know whether at pH2–5 this reagent exists predominantly as the neutral molecule (I, Scheme 1) or as a cation. This may be important also for reactions of Nbd chloride with other enzymes, and for other aspects of the chemistry of this compound.

Allen & Lowe (1973) reported that the electronic-absorption spectrum of Nbd chloride does not change between pH−0.3 and pH7 and that the change in alkaline media appears to conform to the ionization of a single group characterized by a pK_a value of 9.76. Although strictly this is subject to the usual ambiguity in spectroscopic titrations when extinction coefficients of individual ionic forms are not known (see, e.g., Brocklehurst & Little, 1973), the assumption that one group is titrated is probably valid in this case. If the spectral titration curve did represent the ionization of two groups, these would have to be non-interactive, and this is unlikely, bearing in mind that the probable proton-binding sites are N-1 and N-3 of the oxadiazole ring (see I, Scheme 1), which are linked by a conjugated system.

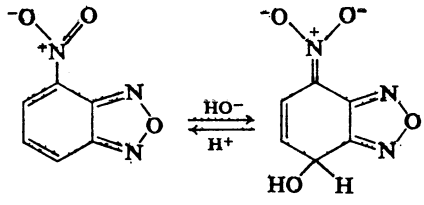
Potentiometric titration up to pH9.26 suggests that crossing the pK_a of 9.76 is accompanied by consumption of 1 mol of OH^- ions/mol of Nbd chloride, and also that no ionizations occur in the pH range approx. 3–8.

The most obvious interpretation of these data is that, in neutral and weakly acidic media, Nbd chloride exists as a cation [protonated presumably on one (IV or V) or perhaps, less likely, on both (VI) of the nitrogen atoms of the oxadiazole ring] and the pK_a of 9.76 is the molecular pK_a value that characterizes loss of one mol of protons/mol to provide either the neutral molecule (I) from monocations IV or V or the monocations IV and V from the dication (VI) (see Scheme 2).

Ghosh & Whitehouse (1968*b*) reported that a close structural analogue of Nbd chloride, 4-nitrobenzofurazan (VII, Scheme 3), undergoes reversible spectral changes in alkali. These spectral



Scheme 2. *Some protonic forms of Nbd chloride*



Scheme 3. *Adduct formation by 4-nitrobenzofurazan in alkaline media proposed by Ghosh & Whitehouse* (1968*b*)

changes are somewhat similar to those found with Nbd chloride (an absorption band at 320–330nm increases in intensity when the pH is increased from 6 to 12, although there is little if any shift to shorter wavelengths) and this spectral change is 50% complete at pH10.65. Ghosh & Whitehouse (1968*b*) do not appear to have considered deprotonation as an interpretation of these spectral changes and instead interpret them in terms of reversible formation of Meisenheimer-type adducts of the benzofurazan and OH^- ion (see Scheme 3). Their study made it necessary to consider carefully whether the spectral changes in solutions of Nbd chloride associated with an apparent pK_a of 9.76 are due to protonic dissociation from a cation or could instead be due to Meisenheimer adduct formation by the neutral molecule. In addition to their spectral studies, Ghosh & Whitehouse (1968*b*) investigated the preparation of this type of adduct from several 4-nitrobenzofurazans by using several nucleophiles. They reported that whereas adducts are isolatable from 4-nitrobenzofurazan itself by using nucleophiles such as methoxide ion, in the reactions of Nbd chloride the intermediate adduct 'rapidly passes to the substituted product'. This makes it unlikely, therefore, that the reversible spectral changes associated with the pK_a of 9.76 are due to Meisenheimer-adduct formation, with Nbd chloride at least. Further support that the 'pK_a' of 9.76 does characterize a protonic change was obtained by a spectroscopic study of Nbd chloride in 0.83M-NaOH. When a portion (0.5ml) of an aqueous solution of Nbd chloride (pH6.3) is mixed with 2.5ml of 1M-NaOH in a spectrophotometer cell, the spectrum, recorded immediately after mixing, contains an absorption band with $\lambda_{max.}$ 320nm, i.e. at slightly shorter wavelengths than the stable absorption band at 325nm found at pH values about 11. Repetitive scanning of the spectrum (500–275nm) showed that the absorption band at 320nm was collapsing and a shoulder was growing in the region around 375nm. This shoulder continued to grow and became a shoulder at approx. 350nm on an absorption band with $\lambda_{max.}$ 307nm. The production of this band, which is of higher intensity than the

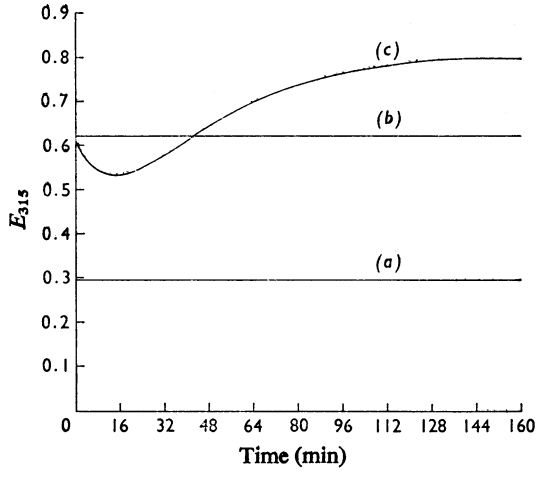


Fig. 1. *Demonstration of the intermediacy of a Meisenheimer adduct in the reaction of Nbd chloride with OH^- ion at 25°C*

The value of E_{315} was recorded continuously as a function of time by using a Cary 118C spectrophotometer, after addition of 0.5ml of Nbd chloride solution (approx. 0.25mM, in water, pH6.3) to 2.5ml of (*a*) water, (*b*) pH10.5 buffer (0.1M-$NaHCO_3$/35.6mM-NaOH) and (*c*) 1M-NaOH.

original band at 320nm, involved an increase in absorbance around 320nm after the collapse of the 320nm band. The various changes in E_{315} as a function of time are shown in Fig. 1. After an initial instantaneous increase in E_{315}, which corresponds to changing the pH of an Nbd chloride solution from 6.3 to 10.5, E_{315} decreases, to reach a minimum value after approx. 15min, and then increases more slowly to reach a steady value. The half-reaction time ($t_{\frac{1}{2}}$) for the decrease in E_{315} to the minimum is 3–4min and the $t_{\frac{1}{2}}$ for the increase from the minimum to the 'infinity' value is 56min.

Time-courses such as that shown in Fig. 1 are characteristic of the formation and decay of intermediates in reactions (see, e.g., Chance, 1951). It seems reasonable therefore to assign the instantaneous increase in E_{315} in Fig. 1 to the protonic dissociation from the Nbd chloride cation, the relatively rapid subsequent fall in E_{315} to Meisenheimer-adduct formation and the eventual slow increase in E_{315} (and E_{350}) to product formed from the adduct. In view of these observations, the spectral changes observed by Ghosh & Whitehouse (1968*b*) when the pH of a solution of 4-nitrobenzofurazan is changed from 6 to 12 could be due to deprotonation or to a combination of deprotonation and Meisenheimer-adduct formation.

If, as seems likely, Nbd chloride exists between pH0 and pH7 as a cation, the questions of proton stoicheiometry and location arise. The most likely proton-binding sites are N-1 and N-3 of the oxadiazole ring. If Nbd chloride exists as a dication below pH7, both of these sites therefore would probably be protonated. The pK_a of 9.76 is, of course, a molecular pK_a value, but consideration of the possibilities of conjugation and intramolecular hydrogen-bonding suggests that proton dissociation from N-3 might be expected to predominate over proton dissociation from N-1. Thus if Nbd chloride is a monocation below pH7, most of it might be predicted to be of form (IV) (Scheme 2), with the proton located on N-1. Whether Nbd chloride exists as form (IV) or as the dication (VI), therefore, N-1 might be expected to be protonated. Subsequent discussion is given in terms of Nbd chloride as the monocation (IV). The assignment as a monocation is supported by the further changes in the spectrum of Nbd chloride observed in conc. H_2SO_4 (an instantaneous shift of the band at 343nm to 353nm) and by preliminary results from a paper-electrophoretic study at pH6.9 (movement towards the cathode) and 10.6 (movement towards the anode). Electrophoretic movement is accompanied by colour changes in the band, indicating reaction of Nbd chloride with solvent. The anion produced at high pH could derive from deprotonation of the postulated cationic centre, together with ionization of a hydroxy derivative.

Implications of the cationic structure of Nbd chloride for its reaction with papain

Allen & Lowe (1973) pointed out that the three-dimensional structure of papain suggests that in the transition state for the attack of the thiol group of cysteine-25 on C-4 of Nbd chloride, the oxadiazole ring could be near to the carboxyl group of aspartic acid-158. They also argued that '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'. This argument applied to the oxadiazole ring, although valid if Nbd chloride is unprotonated on N-1, cannot account for the acid catalysis if, as seems possible, Nbd chloride is a cation, protonated on N-1. Allen & Lowe's (1973) interpretation in fact needs only minor modification to make it apply also to the Nbd chloride cation (IV). This modification is to change the suggested site of interaction with the carboxyl group of aspartic acid-158 from N-1 of the oxadiazole ring to the nitro group on C-7. The nitro group would be expected to accept electrons in the transition state leading to the formation of a Meisenheimer adduct analogous to structure (II) (Scheme 1).

Spectroscopic and kinetic studies of the reaction of papain with Nbd chloride

In agreement with the observations of Allen & Lowe (1973) the present work confirmed that (*a*) at pH values below 5, Nbd chloride reacts specifically with the papain thiol group, (*b*) at pH values above 5 the reaction is not specific for the thiol group and considerable amino-group labelling also occurs, and (*c*) the initial rate of reaction around pH3.5 is faster than the initial rate around pH5.

The reactions of papain (approx. 20μM) with Nbd chloride (approx. 1mM) at pH3.6 and 4.4 resulted in increases in E_{405}. The values of ΔE_{405} for these reactions became stable after approx. 4min and 12min respectively and were equal. In each case, the protein (*S*-Nbd–papain) was separated from low-molecular-weight material by chromatography on a column (15cm×3cm) of Sephadex G-25 (0.1M-KCl/1mM-EDTA as eluent) and subjected to spectral analysis. At each pH the spectrum of *S*-Nbd–papain was similar in shape to that reported for a pH5 solution by Allen & Lowe (1973) and the long-wavelength absorption band was characterized by $\lambda_{max.} = 405$nm [Allen & Lowe (1973) give 402nm] and $\varepsilon_{max.} = 10700 litre \cdot mol^{-1} \cdot cm^{-1}$. The latter parameter was calculated by using for ε_{280} of the labelled protein the value of ε_{280} for native papain $56000 litre \cdot mol^{-1} \cdot cm^{-1}$; see the Materials and Methods section. The value of ε_{405} thus calculated for *S*-Nbd–papain corresponds closely to the value of $\Delta\varepsilon_{405}$ ($10800 litre \cdot mol^{-1} \cdot cm^{-1}$) calculated from complete progress curves (E_{405} versus time) of the reactions of papain and Nbd chloride at pH3.6 and at pH4.4.

We determined the pH-dependence of the initial rate of increase in E_{405} for the reaction of papain with Nbd chloride in the pH range 2.6–4.3. It was possible to extend the pH range of the investigation down to 2.6 without apparent interference from denaturation by using a stopped-flow spectrophotometer with chart-recorder output as described in the Materials and Methods section. The initial rate of reaction was found to be linear in initial concentrations of Nbd chloride up to 600μM at pH2.79 and at pH4.01. The pH-dependence of the second-order rate constant (k) for this reaction calculated from initial rates and the relevant concentrations is given in Fig. 2. This shows a bell-shaped profile characterized [at 25°C, $I = 0.1$mol/litre, in 6.7% (v/v) ethanol] by $pK_{a_I} = 3.24$, $pK_{a_{II}} = 3.44$, and a pH-independent rate constant, $k = 86 M^{-1} \cdot s^{-1}$. The sigmoid curve characterized apparently by $pK_a = 3.7$ (35°C) reported by Allen & Lowe (1973) is in reasonable accord with part of the bell-shaped curve of Fig. 2. The two molecular pK_a values of this profile

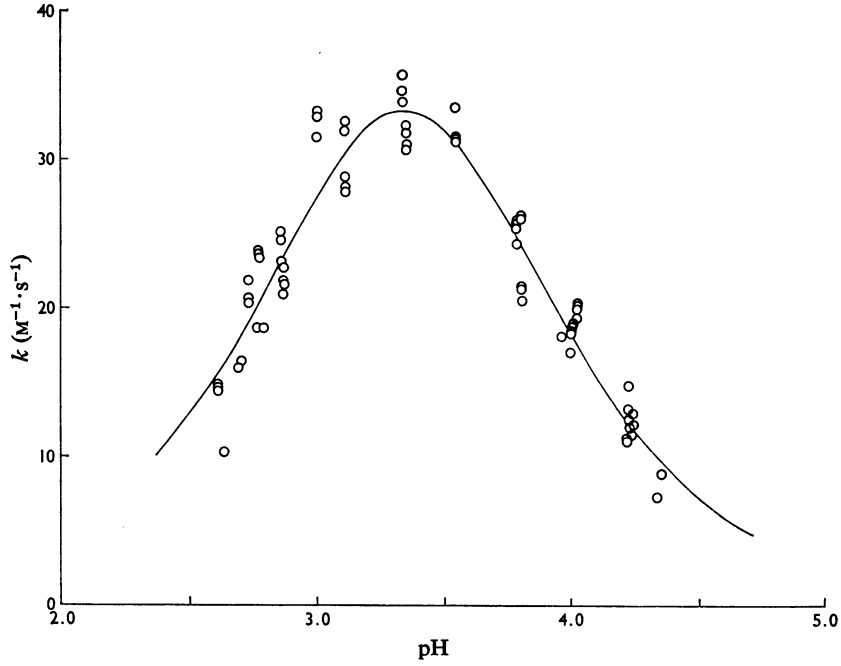


Fig. 2. *Dependence on pH of the apparent second-order rate constant, k, for the reaction of papain* (21 μM) *with Nbd chloride* (123.4 μM) *in* 6.7% *ethanol at* 25.0°C, $I = 0.1$ *mol/litre*

The line is theoretical for

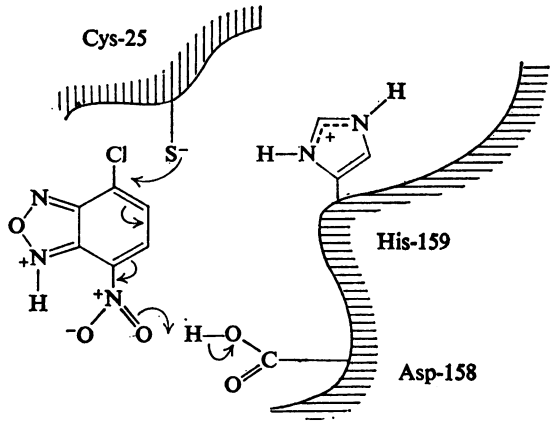
$$k = \frac{\tilde{k}}{1 + \frac{[H^+]}{K_I} + \frac{K_{II}}{[H^+]}}$$

where $\tilde{k} = 86 M^{-1} \cdot s^{-1}$, $pK_I = 3.24$ and $pK_{II} = 3.45$. For further details, see the text.

are in reasonable agreement with those that characterize the reaction of papain with 2-Py–S–S–2-Py in 6.7% (v/v) ethanol ($pK_{a_I} = 3.6$, $pK_{a_{II}} = 3.6$; M. Shipton & K. Brocklehurst, unpublished work). It is difficult to be sure whether the apparent small discrepancies in the pK_a values for the two reactions are real, in view of the scatter in the profile of Fig. 2. If, in both reactions, these pK_a values characterize ionizations of the free enzyme, both sets should, of course, be identical. Perturbations of free-reactant-state pK_a values arising from non-equilibrium steady state in a two-step reaction have been discussed by Brocklehurst & Dixon (1976), but since for a simple model this would cause symmetrical displacements of the limbs of a bell-shaped curve, it would be unlikely to be responsible for the possible discrepancies discussed above unless other factors also contributed.

One possible complication in the Nbd chloride reaction that would not obtain in the 2-Py–S–S–2-Py reaction is that of covalent-intermediate (Meisenheimer-adduct) formation in the former case. However, this would not be a complicating factor, if, as seems likely, the rate-limiting step under the conditions of concentration used to obtain the data in Fig. 2 is formation of the Meisenheimer adduct (see Bunnet, 1958). Under these conditions, adduct formation was not detected by spectral analysis, in accord with the postulate that its formation is rate-limiting. We demonstrated above (Fig. 1) that if a very high concentration of nucleophile (HO^-) is used, it is possible to increase the rate of adduct formation so that it becomes comparable with the rate of adduct breakdown.

One feature that the pH–k profiles of the reactions of 2-Py–S–S–2-Py and Nbd chloride with papain appear to have in common is a width at half-height less than 1.53 units (1.41 units in the Nbd chloride profile and 1.36 units in the 2-Py–S–S–2-Py profile; see also Shipton *et al.*, 1975). Normally, the width at half-height of a bell-shaped curve is $\geqslant 1.53$ units (i.e. $pK_{a_{II}} - pK_{a_I} \geqslant 0.6$). Widths in the range 1.17–1.53 units can be obtained if the bell shape arises from the ionization of two separate groups (see Brocklehurst & Dixon, 1976), if these ionizations are subject to positive co-operativity (Dixon, 1973). It is suggested that the two molecular pK_a values of



Scheme 4. *Schematic drawing of the postulated transition state for the acid-catalysed formation of a Meisenheimer adduct by papain and Nbd chloride*

This transition state could also be depicted such that the oxadiazole ring lies in the cleft which separates cysteine-25 from histidine-159. This might permit N-3 of Nbd chloride to hydrogen-bond with the imidazolium ion of histidine-159.

approx. 3.5 (in 6.7% ethanol) that characterize both the Nbd chloride and 2-Py-S-S-2-Py profiles are associated with pK_{a_1} of the thiol–imidazolium interactive pair and the pK_a value of the carboxyl group of aspartic acid-158. It is therefore noteworthy that the histidine residue (159) that contributes the imidazolium ion and the aspartic acid residue (158) are adjacent residues in the papain sequence.

In summary, the bell-shaped profile in Fig. 2 is considered to arise from the reaction of the thiolate ion of cysteine-25 of papain, which is maintained in acidic media by its interaction with the side chain of histidine-159, with the $NbdH^+$ chloride monocation hydrogen-bonded at its nitro group to the un-ionized form of the carboxyl group of aspartic acid-158 (see Scheme 4). When all three active-centre residues are proton-rich the thiol group has no nucleophilic character and when the aspartic acid carboxyl group is ionized the transition state for Meinsenheimer-adduct formation is destabilized.

Reaction of ficin with Nbd chloride

The reaction of ficin (49.1 μM) with Nbd chloride at pH3.84 (25°C, $I = 0.1$ mol/litre, in 6.7% ethanol) produced a slow increase in E_{425}, which had not reached a stable value even after 1.5h. A spectral scan of this reaction mixture is given in Fig. 3(*a*) and is compared with the spectrum of *S*-Nbd–papain (Fig. 3*b*) and the spectrum of a reaction mixture

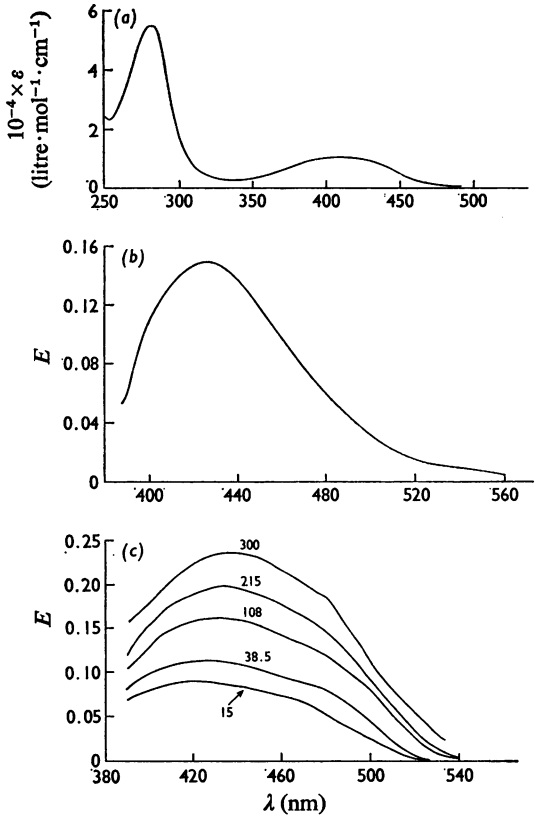


Fig. 3. *Electronic-absorption spectra at 25.0°C and $I = 0.1$ mol/litre of (a) S-Nbd–papain at pH2.4; an identical spectrum is obtained at pH6.0; (b) a mixture of ficin (49.1 μM) and Nbd chloride (1.24 mM) at pH3.84 approx. 1.5h after mixing; the reference cell contained Nbd chloride (1.24 mM) in pH3.84 buffer; (c) a mixture of bromelain (60.6 μM) and Nbd chloride (1.33 mM) at pH3.79; the reference cell contained Nbd chloride (1.33 mM) in pH3.79 buffer*

Reactions were allowed to proceed for the times (in min) shown before spectral scanning was carried out. The buffers were those given in Stuchbury *et al.* (1975).

containing bromelain and Nbd chloride (Fig. 3*c*, see below).

The value of $\lambda_{max.}$ in the ficin–Nbd chloride reaction is consistent with the spectra of the products of the reactions of Nbd chloride with simple thiols (Birkett *et al.*, 1970) and with bovine serum albumin at low pH (J. P. G. Malthouse & K. Brocklehurst, unpublished work). The assignment of the product as *S*-Nbd–ficin is supported by the correlation of ΔE_{405} with loss of amidase activity (see Fig. 4).

The rate of reaction of ficin with Nbd chloride at pH approx. 4 could not be increased significantly because of the limited solubility of the reagent in

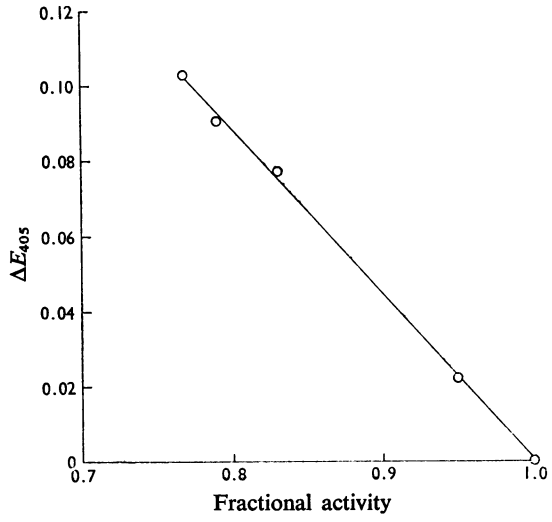


Fig. 4. *Relationship between ΔE_{405} produced by, and fractional amidase activity remaining after, reaction of ficin with Nbd chloride in 6.7% ethanol, pH3.84, $I = 0.1$ mol/litre at 25.0°C*

The initial concentrations were for ficin, 42.5 μM and for Nbd chloride, 1.24 mM. Amidase activity was determined by using α-*N*-benzoyl-DL-arginine *p*-nitroanilide as described in the preceding paper (Malthouse & Brocklehurst, 1976) and was plotted on a fractional basis with activity in the absence of Nbd chloride set equal to 1.0. The negative slope of the regression line divided by the initial concentration of ficin provides a value for $\Delta\varepsilon_{405} = 10500$ litre·mol^{-1}·cm^{-1}.

6.7% ethanol. The reaction rate increased when the reaction was carried out at pH values above 5, but the spectrum of the product contained an absorption band at approx. 470 nm additional to that at 425 nm, indicating that, as in the papain reaction, substantial amino-group labelling was occurring.

Kinetic study of the ficin–Nbd chloride reaction was limited to the pH range 2.6–5.3. The lower limit was set by the difficulties caused by acid denaturation even when using the stopped-flow technique (see the Materials and Methods section) and the upper limit by the incursion of amino-group labelling at pH values above 5. All reactions were monitored at 405 nm, where the absorption of *S*-Nbd–ficin is still considerable ($\Delta\varepsilon_{405}/\Delta\varepsilon_{425} = 0.83$; see Fig. 3*a*), but the contribution to ΔE from the small amount of amino-group labelling at pH approx. 5 is negligible. Since ε_{405} for Nbd chloride is negligible (500 litre·mol^{-1}·cm^{-1}), the value of ε_{405} for *S*-Nbd–ficin should provide an accurate estimate of $\Delta\varepsilon_{405}$ for the ficin–Nbd reactions. Similarly, the value of ε_{425} for *S*-Nbd–ficin should provide an accurate estimate of $\Delta\varepsilon_{425}$ for the reaction at pH 3.8, where no amino-group labelling is apparent. Assuming that ε_{425} for *S*-Nbd–ficin = 13000 litre·mol^{-1}·cm^{-1}, the value that obtains with some other *S*-Nbd compounds (Birkett *et al.*, 1970), the value of $\Delta\varepsilon_{405}$ for the ficin–Nbd chloride reaction (i.e. ε_{405} for *S*-Nbd–ficin) is calculated to be 10800 litre·mol^{-1}·cm^{-1}. This value is in good agreement with that (10500 litre·mol^{-1}·cm^{-1}) obtained from the slope of the plot of fractional activity versus ΔE_{405} (Fig. 4).

Control experiments showed that even though the rates of the ficin–Nbd chloride reactions in the pH range 2.6–5.3 are considerably slower than those of the corresponding papain reactions, decomposition of Nbd chloride by processes not involving the protein did not contribute to the spectral changes.

Kinetics of the reaction of ficin with Nbd chloride

Initial rates were determined at 25.0°C, I = 0.1 mol/litre, in 6.7% ethanol, by using the stopped-flow method described in the Materials and Methods section. In most experiments the initial concentration of ficin was 18.1 μM and Nbd chloride was 1.32 mM. At pH 4.08 the reaction was studied by using an initial ficin concentration of 23.35 μM and initial concentrations of Nbd chloride in the range 325 μM–1.34 mM. The initial rates increased linearly with the initial Nbd chloride concentration. Initial rates obtained at pH 2.6–5.3 were used to calculate second-order rate constants (k) and the pH–k profile is shown in Fig. 5.

Comparison of the pH–k profiles for the reactions of Nbd chloride with papain and ficin

The profile for the ficin–Nbd chloride reaction (Fig. 5) is markedly different from that for the papain–Nbd chloride reaction (Fig. 2). In the profile for the ficin–Nbd chloride reaction up to pH approx. 5 the rate increases with increasing pH according to a pK_a value of 3.6 and a limiting (pH-independent) rate constant of 0.36 M^{-1}·s^{-1}. Above pH 5, the rate increases more sharply, but in this region amino-group labelling is also occurring. At pH 3.3 (the rate maximum of the papain profile) the ficin reaction is 330 times slower than the papain reaction and at around pH 4.5–5 it is still approx. 30 times slower.

The marked differences in the two pH–k profiles point to a structural difference in the two enzymes that greatly influences the reactions of the two active-centre thiol groups with this reactivity probe. The difference in the $\lambda_{max.}$ values of *S*-Nbd–papain (405 nm) and *S*-Nbd–ficin (425 nm) also indicates a structural difference. The active-centre region of papain is characterized by three molecular pK_a values, two approx. 3.5–4 and one approx. 9, which can be demonstrated by using 2-Py–*S*–*S*–2-Py as a reactivity probe and reasonably associated with three active-centre ionizing groups (Shipton *et al.*, 1975). One

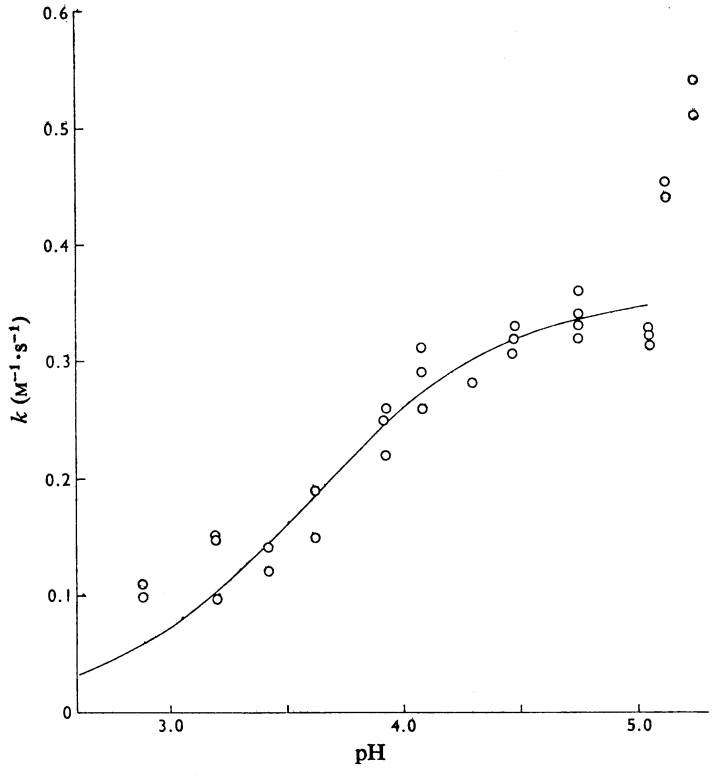


Fig. 5. *Dependence on pH of the apparent second-order rate constant, k, for the reaction of ficin* (18.1 μM) *with Nbd chloride* (1.32 mM) *in* 6.7% *ethanol at* 25.0°C *and* $I = 0.1$ *mol/litre*

The line is theoretical for

$$k = \frac{\tilde{k}}{1 + \frac{[H^+]}{K_a}}$$

where $\tilde{k} = 0.36 M^{-1} \cdot s^{-1}$ and $pK_a = 3.6$.

pK_a value of 3.5–4 is reasonably associated with the carboxyl group of aspartic acid-158 and the other value of 3.5–4 with pK_{a_1} of the thiol–imidazolium ion pair of cysteine-25 and histidine-159.

The bell-shaped profile (Fig. 2) of the papain–Nbd chloride reaction is considered to arise from assistance by the undissociated carboxyl group of aspartic acid-158 of the reaction of the thiolate ion of cysteine-25 generated by a pK_a of approx. 4 as described above. Since the thiol and imidazole groups of the ficin active centre are known to be within 0.5 nm of each other (Husain & Lowe, 1968), the lack of acid catalysis in the profile for the ficin–Nbd chloride reaction (Fig. 5) is most obviously suggested to be due to a lack in ficin of a carboxyl group equivalent to that of aspartic acid-158 in papain.

The simplest version of this interpretation is that the residue in ficin equivalent to aspartic acid-158 in papain is a residue with a non-ionizing side chain, e.g. asparagine. In papain the active-centre histidine and the aspartic acid residue implicated in the acid catalysis of the reaction with Nbd chloride are adjacent residues in the sequence (159 and 158 respectively). Husain & Lowe (1970*b*) isolated the peptide containing the active-centre histidine residue from ficin and reported the adjacent residue to be an aspartic acid residue, as in papain. Isolation of peptides containing intact asparagine-amine groups, however, is notoriously difficult. Indeed, residue 64 in papain, now known to be an asparagine residue (Husain & Lowe, 1970*a*), was originally assigned as an aspartic acid residue (see Drenth *et al.*, 1968).

If, however, this residue in ficin is an aspartic acid residue, its conformational disposition with respect to the rest of the active-centre region must be different from that of aspartic acid-158 of papain. In this event, the possibility must remain that the identity of pK_{a_1}

in the pH–k profile for the ficin–2-Py–S–S–2-Py reaction with $pK_{a_{II}}$ of the reagent [see the preceding paper, Malthouse & Brocklehurst (1976)] is coincidental, and the pK_a in the profile could be associated with an aspartic acid residue in ficin, although the symmetry of the bell in Fig. 4 of Malthouse & Brocklehurst (1976) makes this unlikely. The orientation of this residue in ficin would have to lead to an enhanced rate of reaction with 2-Py–S–S–2-Py relative to that of the papain reaction and an inability to catalyse the reaction of ficin with Nbd chloride.

Reaction of bromelain with Nbd chloride

The active centre of bromelain, like those of papain and ficin, contains a cysteine residue and a histidine residue whose side chains are interactive (Husain & Lowe, 1970*c*; Brocklehurst *et al.*, 1972; see also Shipton *et al.*, 1975).

The absorption spectrum of a reaction mixture containing 60.6μM-bromelain and 1.33mM-Nbd chloride at pH3.79, recorded after various time-intervals against an Nbd chloride/buffer blank, is shown in Fig. 3(*c*). The spectral changes, which were not complete even after 4h, show the development of two overlapping absorption bands with $\lambda_{max.}$ values of approx. 430 and 470nm. This result indicates that, even at pH3.79, appreciable amino-group labelling occurs, in addition to thiol-group labelling. This complication makes a detailed kinetic study difficult, but it was observed that the rate of increase in E_{430} is lower at pH<3.79 than at pH3.79. This, together with the apparently low reactivity of the bromelain thiol group towards Nbd chloride at pH3.79 (k = approx. $0.2 M^{-1} \cdot s^{-1}$) compared with that of the papain thiol group (k = $24 M^{-1} \cdot s^{-1}$) suggests that like ficin, and unlike papain, bromelain does not possess a carboxyl group capable of enhancing the rate of reaction of the active-centre thiol group with Nbd chloride.

Active centres of the thiol proteinases

There is continuing discussion as to whether the pK_a value near to 4 that characterizes the kinetics of catalysis by papain, ficin and bromelain should be associated with a thiol–imidazolium pair or a carboxyl group. Shipton *et al.* (1975) pointed out that in papain both of these 'groups' are associated with molecular pK_a values of 4. The results reported in the present and in the preceding paper (Malthouse & Brocklehurst, 1976) suggest that, whereas an interactive thiol–imidazole pair is a feature of the active-centre regions of all three thiol proteinases, a uniquely orientated carboxyl group is not. This suggests that a carboxyl group may not be part of the catalytic site of any of these enzymes, although where it exists, of course, it may affect substrate binding. Attempts to implicate a carboxyl group in the catalytic sites of papain and bromelain (Murachi & Okumura, 1974; Löffler & Schneider, 1974) have been criticized (Shipton *et al.*, 1975; Clark & Lowe, 1976).

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