A Reporter Group Delivery System with Both Absolute and Selective Specificity for Thiol Groups and an Improved Fluorescent Probe Containing the 7-Nitrobenzo-2-oxa-1,3-diazole Moiety

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1. 4-(N-2-Aminoethyl 2'-pyridyl disulphide)-7-nitrobenzo-2-oxa-1,3-diazole (compound I) was synthesized and evaluated as a fluorescent labelling reagent for thiol groups. 2. The design of compound (I) as one example of a general type of reporter group delivery reagent (2-pyridyl-S-S-X, where X contains an environmentally sensitive spectroscopic probe) is discussed. 3. The electronic absorption spectrum of compound (I) was determined over a wide range of pH and the spectral changes that accompany its reaction with low-molecular-weight thiols, e.g. L-cysteine, and with papain (EC 3.4.22.2) and bovine serum albumin are discussed. 4. A new value of ϵ_{343} for 2-thiopyridone (Py-2-SH) was determined as $8.08 \times 10^3 \pm 0.08 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$. 5. Spectral analysis of the reactions of compound (I) with L-cysteine and with papain (in the pH range 3.5-8.0) showed that even under equimolar conditions the reaction (thiol-disulphide interchange to release Py-2-SH) is essentially stoichiometric and probably proceeds by specific attack at the sulphur atom distal from the pyridyl ring of compound (I). 6. The fluorescence-emission spectra of compound (I) and of the products of its reaction with papain and with ficin (EC 3.4.22.3) were determined. Compound (I) is highly fluorescent in aqueous solution. Excitation within the intense visible absorption band (λ_{max} , 481 nm, ϵ_{max} , $2.52 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$) provides green fluorescence with an emission maximum at 540 nm. Both papain and ficin labelled by reaction with compound (I) are characterized by fluorescence-emission maxima (535 nm and 530 nm respectively) of even higher intensity. The fluorescence emission of the product of the reaction of papain with compound (I) was shown to be 25 times more intense than that of the product of the reaction of papain with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (Nbd chloride). 7. The second-order rate constants (k_2) for the reactions of compound (I) and of Nbd chloride with GSH, papain, albumin, ficin, 2-benzimidazolylmethanethiol and 2-benzimidazolylethanethiol were determined at 25.0°C and various pH values. At pH 4 the values of $k_{2(\text{compound I})}/k_{2(\text{Nbd chloride})}$ are: GSH, 288; albumin, 36; papain 3×10^3 ; ficin, 3×10^4 . 8. The pH- k_2 profiles for the reactions of compound (I) and of Nbd chloride with the two 2-benzimidazolylalkanethiols were determined. Of the four profiles only that for the reaction of compound (I) with 2-benzimidazolylmethanethiol is characterized by a striking rate maximum in acidic media. The three reactive protonic states implicit in this profile suggest that, like 2,2'-dipyridyl disulphide, compound (I) increases its electrophilicity by protonation on its pyridyl nitrogen atom and is thus a sensitive reagent for nucleophilic states of thiols in acidic media. 9. The ease with which active enzyme can be regenerated from enzyme labelled by reaction with compound (I) is demonstrated with papain. 10. The major disadvantages of Nbd chloride that are rectified by combination of the Nbd fluorophore with the 2-aminoethyl 2'-pyridyl disulphide delivery system of compound (I) are delineated. 11. A carboxylated analogue of compound (I), prepared from L-cysteine instead of from cysteamine, reacts one-tenth as fast as compound (I) with papain at pH 3.8 (k_2 = approx. $4 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$, compared with $5.5 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the reaction at 25.0°C, $I = 0.1 \text{ mol/l}$).

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Following the work of Koshland (Koshland *et al.*, 1964; Burr & Koshland, 1964; Conway & Koshland, 1967), substantial interest has developed in site-specific environmentally sensitive spectroscopic reporter groups or probes for the study of proteins and other macromolecular assemblies such as multienzyme complexes and cell organelles. One approach to probe studies is to carry out exploratory experiments with a reagent of broad specificity and hope to find macromolecules in which only one particular site is labelled in a given case. For other types of work the reagent that introduces the probe should react specifically with a single known site or at least with a single chemical type of group. The object of the present work was to produce a probe delivery reagent with (a) absolute specificity for thiol groups and (b) the ability, under some conditions, to discriminate in favour of thiol groups in a particular type of environment. The type of environment we chose is one that provides relatively high concentrations of a nucleophilic state of the thiol in acidic media (see the Results and Discussion Section).

The present paper describes the synthesis and some properties of one such reagent, 4-(*N*-2-aminoethyl 2'-pyridyl disulphide)-7-nitrobenzo-2-oxa-1,3-diazole (compound I).

Materials and Methods

GSH and α -*N*-benzoyl-L-arginine ethyl ester hydrochloride were purchased from Sigma (London) Chemical Co., London S.W.6, U.K. 2,2'-Dipyridyl disulphide (2-Py-S-S-2-P) (II) was the product of Aldrich Chemical Co., Wembley, Middx. HA0 1PY, U.K., twice recrystallized from light petroleum (b.p. 60–80°C). 2-Thiopyridone (Py-2-SH) was the 'purum' product of Fluka A.G. (Buchs S.G., Switzerland), twice recrystallized from benzene. Acetonitrile was the BDH (Poole, Dorset, U.K.) 'special for spectroscopy' product. Buffer salts were of the highest available commercial grade.

Papain (EC 3.4.22.2)

Fully active papain containing 1 mol of thiol with high reactivity towards 2-Py-S-S-2-Py at pH4/mol of protein (see Brocklehurst & Little, 1970, 1973) was prepared by the covalent-chromatography procedure described previously (Brocklehurst *et al.*, 1973, 1974) modified to provide a batchwise process. This was found to be more convenient than a column procedure when large amounts of papaya latex (100g or more) are processed.

The papain fraction of 100g of dried papaya latex (BDH) was isolated as described previously (Brocklehurst *et al.*, 1973, 1974) by extraction with L-cysteine solution, alkali treatment and two (NH₄)₂SO₄ precipitations. This was dissolved in 200ml of 0.1M-

sodium acetate buffer, pH4.5 (0.036M-sodium acetate and 0.064M-acetic acid), containing 0.3M-NaCl and 1mM-EDTA. This solution was mixed with 100ml of Sepharose-GSH 2-pyridyl disulphide gel (Brocklehurst *et al.*, 1973, 1974) that had been allowed to equilibrate in the sodium acetate buffer and the mixture was stirred gently for 20min at 22°C. The gel was isolated on a sintered-glass filter and washed with the acetate buffer (approx. 3 litres) until the values of *E*₂₈₀ and *E*₃₄₃ of the filtrate had fallen below 0.03. The gel was then allowed to equilibrate in 2 litres of 0.1M-Tris-HCl buffer, pH8.0 (0.045M-Tris and 0.055M-Tris-HCl), containing 0.3M-NaCl and 1mM-EDTA, and the covalently bound papain was released by treatment with L-cysteine. A solution of L-cysteine (300ml of 20mM) in the Tris-HCl buffer was added to the gel, and the mixture was stirred gently for 30min and then subjected to vacuum filtration. The gel was washed on the filter with two 150ml batches of the Tris-HCl buffer and these washings were added to the L-cysteine filtrate. The combined filtrate and washings were adjusted to 35% saturation with (NH₄)₂SO₄ (solid) and the mixture was left at 4°C overnight. The resulting precipitate was isolated by centrifugation at 14000*g* for 30min at 4°C, and stored at 4°C as a suspension in the 0.1M-sodium acetate buffer, pH4.5, containing 1mM-EDTA and (NH₄)₂SO₄ (35% saturation) until required. On storage under these conditions the papain becomes reversibly blocked by mixed-disulphide formation with the residual L-cysteine. When required for kinetic or labelling studies, samples of the suspension were activated by treatment with 5mM-dithiothreitol at pH8 for 15min and isolated by Sephadex G-25 chromatography (see Brocklehurst *et al.*, 1973, 1974).

Ficin (EC 3.4.22.3)

Ficin containing 1 mol of thiol with high reactivity towards 2-Py-S-S-2-Py at pH4/mol of protein was prepared from dried latex (Koch-Light Laboratories, Colnbrook, Bucks, U.K.) by covalent chromatography. The NaCl precipitate of the procedure of Englund *et al.* (1968) was prepared from 20g of latex with the omission of the sodium tetrathionate. The precipitate was dissolved in 400ml of the 0.1M-sodium acetate buffer, pH4.5, containing 0.3M-KCl and 1mM-EDTA and subjected to batchwise covalent chromatography as described above for papain.

2-Benzimidazolylmethanethiol (III) and 2-benzimidazolylethanethiol (IV)

These compounds were prepared by the method of Milner *et al.* (1964) for use in another investigation (E. Ager, T. Stuchbury, G. V. Garner, H. Suschitzky & K. Brocklehurst, unpublished work).

(I)

(II)

(III) $n = 1$ (IV) $n = 2$

(V)

(VI), R contains a spectroscopic probe, e.g. u.v., fluorescence, e.s.r. etc.

method of Field *et al.* (1964). The product was obtained in 70% yield and had m.p. 150°C (decomp.). Field *et al.* (1964) give 82% yield and m.p. 144–145°C.

4-(N-2-Aminoethyl 2'-pyridyl disulphide)-7-nitrobenzo-2-oxa-1,3-diazole (I)

Aqueous 4M-NaOH was added dropwise with stirring to a solution of 2-pyridyl 2'-aminoethyl disulphide dihydrochloride (1.3 g, 5mmol) in a mixture of ethanol (20ml) and water (8ml) to adjust the apparent pH (glass electrode) from 2.4 to 7.0. A solution of Nbd chloride (1 g, 5mmol) in a mixture of ethanol (70ml) and water (20ml) was added dropwise over 15min and the fluorescent yellow mixture was stirred overnight. After adjusting the pH to 9.0, the reaction mixture was stirred for a further 3 h and then the dark-brown solid (200mg, m.p. 150–160°C) was collected by filtration. Nbd chloride [0.5g, m.p. 96–98°C; Boulton *et al.* (1966) gave m.p. 97°C] was recovered from the filtrate by evaporation to small volume, addition of water (25ml), extraction with chloroform, carbon treatment of the chloroform layer and evaporation after removal of carbon by filtration. The dark-brown solid (100mg) was boiled in ethanol (50ml) and residual solid removed by filtration. The filtrate was applied to a column (25 cm × 2 cm) packed with alumina, type H (BDH Chemicals) in a chloroform slurry. The column was eluted with ethanol. The intensely fluorescent yellow fraction was eluted first and was collected. Evaporation of this solution provided an orange-brown solid (35mg, m.p. 166°C). This was identified as compound(I) by elemental analysis, mass spectrum and electronic absorption spectrum before and after treatment with L-cysteine (see the Results and Discussion Section). Elemental analysis: Found: C, 45.2; H, 3.5; N, 20.8%; compound (I), $C_{13}H_{11}N_5S_2O_3$ requires C, 44.7; H, 3.2; N, 20.1%. Mass-spectral analysis gave mol.wt. 349, which is the value for compound(I). For spectral analysis a sample was dried over P_2O_5 *in vacuo*.

Mass-spectral measurements

These were carried out in an AEI MS12 mass spectrometer.

Electronic absorption spectra

These were recorded in an Aminco DW2 spectrophotometer.

Kinetics of the reactions of compound (I) and of Nbd chloride with thiols

Kinetics of the various reactions were studied by following u.v. and visible spectral changes. For

2-Aminoethyl 2'-aminoethanethiolsulphonate dihydrochloride

This was prepared by the oxidation of cysteamine hydrochloride with aq. H_2O_2 by the method of Field *et al.* (1964). The product was obtained in 85% yield and had m.p. 165–166°C. Field *et al.* (1964) give 93% yield and m.p. 166°C. The same product was obtained by oxidation of cystamine dihydrochloride with aq. H_2O_2 by the method of Christiansen & Dolliver (1941). These authors refer to the product incorrectly as a disulphoxide.

4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (4-chloro-7-nitrobenzofurazan, Nbd chloride) (V)

This was prepared by the method of Boulton *et al.* (1966) and had m.p. 97–98°C [Boulton *et al.* (1966) give m.p. 97°C].

2-Aminoethyl-2'-pyridyl disulphide hydrochloride

This was prepared from 2 thiopyridone and 2-aminoethyl 2'-aminoethanethiolsulphonate by the

reactions with second-order rate constant (k_2) greater than approx. $500\text{M}^{-1}\cdot\text{s}^{-1}$, complete progress curves were recorded by using a Durrum stopped-flow spectrophotometer coupled to a Tektronix oscilloscope. Data were taken from photographs of the progress curves and rate constants computed from conventional first- or second-order plots. Progress curves of slower reactions and initial-rate data were obtained by using either a Cary 16K spectrophotometer or an Aminco-Morrow DW2 stopped-flow spectrophotometer. In both these cases progress curves were recorded on a chart. The Aminco-Morrow DW2 instrument with chart-recorder output is particularly useful in initial-rate studies.

(i) *Reactions of compound (I)*. These were all followed at 343 nm. Typical conditions of concentration are described. The buffer concentrations were such that reaction mixtures had $I = 0.1\text{mol/l}$ and contained 13.4% (v/v) acetonitrile. Rate constants were calculated by using $\Delta\epsilon_{343} = 8.08 \times 10^3\text{M}^{-1}\cdot\text{cm}^{-1}$. This value of $\Delta\epsilon_{343}$ is equal to ϵ_{343} for Py-2-SH. The value of ϵ_{343} is the same in 13.4% acetonitrile as in aqueous solution and $\Delta\epsilon_{343}$ for the reaction of compound (I) with L-cysteine is the same in 6.7% acetonitrile as in 13.4% acetonitrile.

(a) Reactions with papain. Complete progress curves were recorded by using a Durrum stopped-flow spectrophotometer. One syringe contained $30\mu\text{M}$ -papain in 0.1M-KCl and 1mM-EDTA. The other syringe contained $45\mu\text{M}$ -compound (I) in buffer containing 1mM-EDTA and 26.8% acetonitrile.

(b) Reactions with bovine serum albumin. Initial rates were determined by using an Aminco-Morrow DW2 stopped-flow spectrophotometer and a chart recorder (0–0.02E full scale). The reference wavelength was 600 nm. The contents of the syringes were (1) albumin ($22.9\mu\text{M}$ -SH) in 0.1M-KCl containing 1mM-EDTA, (2) compound (I) ($61.8\mu\text{M}$) in buffer containing 1mM-EDTA and 26.8% acetonitrile.

(c) Reactions with GSH. Initial rates were determined by using the 0–0.02E scale of a Cary 16K spectrophotometer. The extinction at 343 nm of a reference cell containing 2.6 ml of buffer and 0.2 ml of acetonitrile was balanced against that of a reaction cell containing $4\mu\text{M}$ -thiol in 2.6 ml of buffer and 0.2 ml of acetonitrile. The reaction was started by adding 0.2 ml of $815\mu\text{M}$ -compound (I) in acetonitrile to the reaction cell after the analogous addition to the reference cell.

(d) Reactions with 2-benzimidazolylalkanethiols. Initial rates were determined as described in (b) for bovine serum albumin. The contents of the syringes were (1) compound (I) ($54.5\mu\text{M}$) in buffer containing 1mM-EDTA and 26.8% acetonitrile, (2) $42.1\mu\text{M}$ -thiol for 2-benzimidazolylmethanethiol or $31.4\mu\text{M}$ -thiol for 2-benzimidazolethianethiol in 0.1M-KCl containing 1mM-EDTA.

(ii) *Reactions of Nbd chloride*. These were followed

at 405 nm for the reaction of papain and of ficin and at 420 nm in the other cases. The buffer concentrations were such that reaction mixtures had $I = 0.1\text{mol/l}$ and they contained 6.7% (v/v) ethanol. Rate constants were calculated from initial rates by using $\Delta\epsilon_{405} = 1.08 \times 10^4\text{M}^{-1}\cdot\text{cm}^{-1}$ for the papain and ficin reactions and $\Delta\epsilon_{420} = 1.3 \times 10^4\text{M}^{-1}\cdot\text{cm}^{-1}$ (see Birkett *et al.*, 1970) for the other reactions. The value of $\Delta\epsilon_{405}$ was determined from a complete progress curve of the reaction of papain and Nbd chloride at pH 4. The corresponding reaction of ficin is so slow that stoichiometric reaction is difficult to achieve. The spectrum of incompletely labelled ficin is sufficiently similar to that of labelled papain, however, to suggest that use of the same value of $\Delta\epsilon_{405}$ as for the papain reaction might be a reasonable approximation.

(a) Reactions with papain, ficin and 2-benzimidazolylalkanethiols. These were followed by using an Aminco-Morrow DW2 stopped-flow spectrophotometer and a chart recorder (0–0.02E full scale). The two syringes [(1) and (2)] contained the following solutions: papain, (1) $42\mu\text{M}$ -thiol in 0.1M-KCl and 1mM-EDTA, (2) $246.8\mu\text{M}$ -Nbd chloride in buffer containing 1mM-EDTA and 13.4% ethanol; ficin, (1) 25–30 μM -thiol in 0.1M-KCl and 1mM-EDTA, (2) as for papain; 2-benzimidazolylmethanethiol, (1) $42.1\mu\text{M}$ -thiol in 0.1M-KCl and 1mM-EDTA, (2) $116\mu\text{M}$ -Nbd chloride in buffer containing 1mM-EDTA and 13.4% ethanol; 2-benzimidazolethianethiol, (1) $31.4\mu\text{M}$ -thiol in 0.1M-KCl and 1mM-EDTA, (2) as for 2-benzimidazolylmethanethiol.

(b) Reactions with bovine serum albumin and GSH. These were followed by using the 0–0.02E scale of a Cary 16K spectrophotometer. The extinction at 420 nm of a reference cell containing 2.8 ml of buffer was balanced against that of a reaction cell containing 2.8 ml of thiol in buffer ($19.8\mu\text{M}$ -albumin thiol or $64\mu\text{M}$ -GSH thiol). The reactions were started by adding 0.2 ml of 1.73mM-Nbd chloride in ethanol to the reaction cell after the analogous addition to the reference cell.

Kinetics of the release of the fluorescent label from papain labelled with compound (I) by reaction with L-cysteine and 2-mercaptoethanol at 25.0°C and pH 7.0 and 8.2

These were studied by following the decrease in fluorescence intensity that accompanies release of the label from papain labelled with compound (I) by using a Durrum stopped-flow fluorescence instrument. The stock solutions of buffers were: phosphate, pH 7.0, 0.14M- KH_2PO_4 , 0.08M-NaOH and 1mM-EDTA; borate, pH 8.2, 0.15M- H_3BO_3 , 0.15M-KCl, 0.018M-NaOH and 1mM-EDTA. One syringe contained 10 ml of buffer and 5 ml of thiol in water

(237 μM -2-mercaptoethanol or 297 μM -L-cysteine). The other syringe contained 3.77 μM -papain labelled with compound (I) in 0.1 M-KCl and 1 mM-EDTA. The excitation wavelength was 475 nm and the emitted fluorescence was filtered through a Corning 3-69 filter to exclude light of wavelengths shorter than approx. 510 nm. Progress curves, recorded on a Tektronix oscilloscope and subsequently photographed, were analysed by using conventional first-order plots.

Fluorescence spectra and fluorescence polarization measurements

These were determined by using a Zeiss spectrofluorimeter and are uncorrected for instrumental response.

pH measurements

These were made with Radiometer equipment.

Thiol determination

Thiol contents were determined by reaction with 2-Py-S-S-2-Py (see Brocklehurst & Little, 1973), but by using a new ϵ_{343} for Py-2-SH ($8.08 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$; see the Results and Discussion Section).

Preparation of samples of papain, ficin and bovine serum albumin labelled by reaction with compound (I)

For papain and ficin a sample of enzyme (4 ml of 54.5 μM -papain or 30 μM -ficin) in 0.1 M-KCl and 1 mM-EDTA was mixed at room temperature (22°C) with a solution containing 0.6 ml of sodium acetate buffer pH 5.0 (0.438 M-acetic acid and 0.3 M-NaOH) and 0.7 ml of 750 μM -compound (I) in acetonitrile. Kinetic studies had shown that under these conditions the reactions should be complete within 1 min of mixing. Labelled protein was separated by chromatography on a Sephadex G-25 column (15 cm $\times$ 3 cm), with 0.1 M-KCl containing 1 mM-EDTA as eluent.

Albumin was labelled by a similar procedure except that the reaction was allowed to occur at pH 7.

Preparation of a sample of papain labelled on its thiol sulphur atom by reaction with Nbd chloride

Papain (4 ml of 54.5 μM -enzyme) in 0.1 M-KCl and 1 mM-EDTA was mixed at room temperature with a solution containing 0.4 ml of 5 mM-Nbd chloride in ethanol and 0.6 ml of sodium acetate buffer, pH 4.2 (1.17 M-acetic acid and 0.3 M-NaOH). The mixture was left for 30 min and the labelled protein was isolated by chromatography on a Sephadex G-25 column as described above.

Attempted preparation of ficin labelled on its essential thiol sulphur atom by reaction with Nbd chloride

An attempt to label ficin in a manner analogous to that described above for papain failed because of the very low rate of reaction. When the labelling was carried out under comparable conditions of concentration but at pH 6, reaction occurred but a mixture of products resulted. Spectral analysis (see Allen & Lowe, 1973) suggested that labelling had occurred both on the thiol group and on amino groups.

Results and Discussion

Reagent design

We have previously used 2,2'-dipyridyl disulphide (2-Py-S-S-2-Py) (II) as an active-site titrant and reactivity probe for thiol enzymes (see, e.g., Brocklehurst & Little, 1970, 1972, 1973; Brocklehurst *et al.*, 1972; Brocklehurst & Kierstan, 1973; Carlsson *et al.*, 1974a; Shipton *et al.*, 1975).

Disulphides possess, for all practical purposes, in reactions with proteins an absolute specificity for thiol groups. Reactions of 2-Py-S-S-2-Py with thiol groups are readily monitored spectrophotometrically and the equilibrium constants of these reactions are so high that they are essentially stoichiometric at pH values below 9, even with equimolar concentrations of thiol and 2-Py-S-S-2-Py. The ability of 2-Py-S-S-2-Py to increase markedly its electrophilicity consequent on protonation (or hydrogen-bonding) on one of its nitrogen atoms (see Brocklehurst, 1974) makes it a very sensitive reagent for the detection of nucleophilic reactivity of thiols in acidic media (see, e.g., Carlsson *et al.*, 1974a,b). These features of 2-Py-S-S-2-Py are retained in mixed disulphides containing the 2-pyridyl moiety, e.g. *n*-propyl 2-pyridyl disulphide (M. Shipton & K. Brocklehurst, unpublished work; see also Brocklehurst *et al.*, 1973, 1974).

A logical extension of this work is the synthesis of mixed disulphides of type (VI). These contain, in one half of the molecule, the 2-pyridyl moiety to provide the reactivity and selectivity discussed above, and in the other, a spectroscopic probe. We decided to attempt to synthesize compound (I) to permit comparison with an existing relatively non-specific fluorescent probe [Nbd chloride, (V)] that also contains the nitrobenzo-oxadiazole fluorophore. In view of the high fluorescence intensity of 4-amino-7-nitrobenzo-oxadiazoles (see Ghosh & Whitehouse, 1968; Birkett *et al.*, 1970; Allen & Lowe, 1973), we considered it desirable that our reagent should have an amino group in the 4-position of the nitrobenzo-ring.

Synthesis of compound (I)

Compound (I) was synthesized as described in the Materials and Methods section by the route shown in

Scheme 1. Synthesis of compound (I)

Fig. 1. Electronic absorption spectra of compound (I) in 6.7% acetonitrile at various pH values

The numbers on the spectra give the pH values. For absorption parameters see the text.

Scheme 1. Cysteamine (VII) or cystamine (VIII) is oxidized by H_2O_2 to the thiol-sulphonate (IX). Reaction of (IX) with Py-2-SH provides 2-aminoethyl 2'-pyridyl disulphide (X) and this is alkylated with Nbd chloride (V) to give compound (I).

Electronic absorption spectrum of compound (I) at 25.0°C

Neutral media. The u.v. spectra above 240 nm of compound (I) in solutions (6.7% acetonitrile) of various pH values between 1.31 and 11.52 are shown in Fig. 1. In approximately neutral solution (e.g.

pH 6.3) the spectrum is characterized by three absorption maxima: λ_{max} , 282 nm, ϵ_{max} , $5.90 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$; λ_{max} , 347 nm, ϵ_{max} , $8.49 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$; λ_{max} , 481 nm, ϵ_{max} , $2.52 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The spectrum of compound (I) is consistent with the value of λ_{max} (475 nm) of the cyclohexylamine derivative of Nbd reported by Birkett *et al.* (1970).

Alkaline media. In alkaline media, the longest-wavelength band (481 nm) is replaced by a new band at shorter wavelengths (λ_{max} , 402 nm, ϵ_{max} , $2.06 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$). This is the only marked spectral change and depends on an apparent pK_a value of 9.16, which probably characterizes proton loss from N-1 of the oxadiazole ring. Proton loss from the analogous position of the Nbd chloride molecule could account for the spectral changes (pK_a 9.76 at 25°C and pK_a 8.81 at 32°C) reported by Allen & Lowe (1973).

Acidic media. In acidic media the band with λ_{max} , 282 nm shifts to approx. 313 nm (ϵ_{max} , $\geq 8.97 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$). This spectral change would be predicted to arise from protonation of the pyridyl nitrogen atom (cf. the spectra of protonated and deprotonated 2-Py-S-S-2-Py reported by Grassetti & Murray, 1967). The change depends on a pK_a value of 1.93, which may be compared with the appropriate value for 2-Py-S-S-2-Py [pK_{a2} (group) = 2.15; pK_{a11} (molecular) = 2.45] (Brocklehurst & Little, 1973).

Isosbestic point at 343 nm (Fig. 1). The wavelength that would normally be used to follow the release of Py-2-SH from a disulphide such as compound (I) or 2-Py-S-S-2-Py is its absorption maximum, 343 nm (see, e.g., Brocklehurst & Little, 1973). It is

Fig. 2. Electronic absorption spectra of (1) compound (I) and (2) the products of its reaction with L-cysteine

The spectrum of compound (I) was recorded with 3.0 ml of a 23.2 μM solution in phosphate buffer, pH 6.6 (0.176 M- KH_2PO_4 and 0.62 M- NaOH), containing 6.7% acetonitrile; 20 μl of 100 mM-L-cysteine free base in water was then added to both the sample and reference cells and the spectrum was recorded again when the increase in E_{343} was complete (after approx. 5 min). For absorption parameters see the text.

noteworthy in this connexion that 343 nm is an isobestic point in the pH-dependent spectra of compound (I) (Fig. 1) and thus no pH-dependence in the difference extinction coefficient for such reactions will result from pH-dependent changes in the spectrum of compound (I).

Extinction coefficient of Py-2-SH at 343 nm

The literature value of ϵ_{343} for Py-2-SH in neutral media is $7.06 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Grassetti & Murray, 1967). When using this value to compute the thiol content of papain prepared by covalent chromatography (Brocklehurst *et al.*, 1973, 1974) and titrated by reaction with 2-Py-S-S-2-Py, we have on many occasions obtained thiol contents of 1.1–1.2 mol of thiol/mol of protein instead of the expected value of 1.0 mol of thiol/mol of protein. Since one explanation for these findings is that $7.06 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ is a slightly low estimate of the value of ϵ_{343} , we redetermined this parameter. Two methods were used, (a) direct spectral analysis of solutions of purified Py-2-SH, and (b) reaction of purified 2-Py-S-S-2-Py with excess of 2-mercaptoethanol which provides 2 mol of Py-2-SH/mol of 2-Py-S-S-2-Py. Three different stock solutions of each of the sources of Py-2-SH (i.e. Py-2-SH itself and 2-Py-S-S-2-Py) were prepared and analysed by the appropriate method, i.e. six determinations were made. This study yielded a value of ϵ_{343} for Py-2-SH of $8.08 \times 10^3 \pm 0.08 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$. This value is pH-independent in the range approx. 1–8 (see Brocklehurst & Little, 1973).

Changes in electronic absorption spectrum accompanying reaction of compound (I) with low-molecular-weight thiols, e.g. L-cysteine

When compound (I) was allowed to react with an excess of L-cysteine at pH 6.6, its absorption spectrum changed as shown in Fig. 2. The most marked changes are those that produce the absorption bands at 271 nm and 343 nm. Similar spectral changes characterize the reactions of compound (I) with 2-mercaptoethanol and with GSH. Absorption maxima at these wavelengths characterize the spectrum of Py-2-SH (Grassetti & Murray, 1967). In addition, the absorption band of compound (I) at 481 nm shifts a little to shorter wavelengths (479 nm) and increases slightly in intensity (ϵ_{max} , $2.73 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$). The increase in E_{343} corresponds to release of $96 \pm 2\%$ of the Py-2-SH that would be predicted from stoichiometric reaction of the thiol at the sulphur atom of compound (I) distal from the pyridyl ring (Scheme 2). That the release of Py-2-SH does not result from initial reaction at the sulphur atom of compound (I) proximal to the pyridyl ring followed by a subsequent reaction with another molecule of RSH (see Scheme 2) is demonstrated by the stoichiometric release of Py-2-SH from reaction mixtures containing initially equimolar (20 μM) concentrations of compound (I) and L-cysteine.

Changes in electronic absorption spectrum accompanying reaction of compound (I) with the thiol-containing proteins papain and bovine serum albumin

Reaction of compound (I) with papain. Reaction of papain (containing 1.0 mol of thiol/mol of protein as measured by titration with 2-Py-S-S-2-Py) with a 5–10-fold molar excess of compound (I) at pH values in the range 3.5–8.0 resulted in an extinction change at 343 nm corresponding to release of 0.97 ± 0.02 mol of Py-2-SH/mol of enzyme. The simplest interpretation of these data is that the enzyme reacts with compound (I) to provide stoichiometric release of Py-2-SH, and $\Delta\epsilon_{343}$ is essentially equal to ϵ_{343} of Py-2-SH. This implies that ϵ_{343} of compound (I) and ϵ_{343} of the enzyme derivative [(XI), Scheme 2, R = enzyme] are essentially the same.

This interpretation is strongly supported by experiments carried out under equimolar conditions. The same stoichiometric release of Py-2-SH was found when equimolar papain and compound (I) were allowed to react at pH values in the range 3.5–8.0. This demonstrates that the reaction is essentially stoichiometric and also that it does not occur to a significant extent by reaction at the S atom proximal to the pyridyl ring to provide compound (XII) (Scheme 2, R = enzyme) as a final product.

Further support was obtained from the electronic absorption spectrum of the protein derivative

Scheme 2. Reaction of compound (I) with a thiol

The simplest mechanism involves direct attack at the sulphur atom distal from the pyridyl ring of compound (I) to provide the mixed disulphide (XI); R = alkyl group in a low-molecular-weight molecule or in a protein. The usual ambiguity in thiol-disulphide interchanges involving three different thiols, i.e. reaction of compound (I) with RSH to provide compounds (XII) and (XIII) and their subsequent reaction to provide compound (XI), cannot be excluded. The subsequent reaction of compound (XI) with RS^- to provide R-S-S-R would not be expected to occur in many cases when R is a protein moiety, on steric grounds.

Fig. 3. Electronic absorption spectrum of papain-S-S-[CH₂]₂-NH-Nbd

The buffer was the phosphate buffer, pH 6.6, described in Fig. 2 legend but without acetonitrile. The [protein] was 13.4 μM and the [label] was 12.8 μM (see the text for further details).

obtained by reaction of papain with a twofold excess of compound (I) followed by separation from low-molecular-weight components by chromatography on Sephadex G-25. The spectrum, shown in Fig. 3, is in accord with the designation of the protein derivative as papain-S-S-[CH₂]₂-NH-Nbd [i.e. (XI),

Scheme 2, R = papain] if it is assumed that the values of ϵ_{481} for compound (I) and for papain-S-S-[CH₂]₂-NH-Nbd are identical. The calculation of the fraction of the original papain converted into papain-S-S-[CH₂]₂-NH-Nbd was carried out as follows. The protein concentration was calculated from E_{280} by using ϵ_{280} for labelled papain = $5.68 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The value of ϵ_{280} for native papain was taken as $5.60 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (R. Axen & J. Carlsson, unpublished work; see Brocklehurst *et al.*, 1973). The value of ϵ_{280} for the label was taken as ϵ_{280} for compound (I) - $\frac{1}{2}\epsilon_{280}$ for 2-Py-S-S-2-Py = $5.9 \times 10^3 - 5.1 \times 10^3 = 8 \times 10^2 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The concentration of label was determined from E_{481} by using $\epsilon_{481} = 2.52 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (the value of ϵ_{481} for compound I). These calculations gave a value of 0.96 mol of label/mol of papain. Allen & Lowe (1973) published a spectrum of a papain derivative that should be papain-S-S-[CH₂]₂-NH-Nbd prepared by another method (see below). If the spectral data are taken from their Fig. 10 and used in a calculation analogous to the one described above, they provide a value of 0.36 mol of label/mol of protein. Allen & Lowe (1973) prepared papain-S-S-[CH₂]₂-NH-Nbd by subjecting a solution of fully active papain and the thiol 7-(2'-mercaptoethylamino)-Nbd (XIII, Scheme 2) to aerial oxidation at 0°C for 3 days. The low incorporation of label may be due to incomplete reaction or possibly to a competing oxidation of papain to the sulphinic or sulphonic acid.

Fig. 4. Fluorescence emission spectra at 25.0°C of compound (I) (Δ), papain labelled by reaction with compound (I) (●) and ficin labelled by reaction with compound (I) (□)

The buffer was the phosphate buffer, pH 6.6, containing 6.7% acetonitrile described in Fig. 2 legend. All solutions had equal extinction (0.053) at the exciting wavelength (475 nm). The close similarity of the ϵ_{475} values, at least for compound (I) and papain labelled by reaction with compound (I) means that the concentrations of the fluorescence emitters are similar (approx. $2 \mu\text{M}$). The fluorescence intensity is given relative to papain labelled by reaction with compound (I) = 100.

Reaction of compound (I) with bovine serum albumin

In the reaction of papain with compound (I), stoichiometric reaction to provide 1 mol of Py-2-SH and 1 mol of papain-S-S-[CH₂]₂-NH-Nbd/mol of original papain is characterized by a value of $\Delta\epsilon_{343}$ essentially equal to ϵ_{343} for Py-2-SH. This would not be the case if the spectrum of the chromophore in the labelled protein had been significantly different from the spectrum of the same chromophore in compound (I). It would obviously be convenient if this situation obtains also when compound (I) reacts with other thiol-containing proteins, and with this in mind we prepared a sample of albumin-S-S-(CH₂)₂-NH-Nbd [(XI), Scheme 2, R = albumin] by reaction of albumin with compound (I). The electronic absorption spectrum of labelled albumin is similar to that of labelled papain. The values of E_{470}/E_{343} for compound (I), labelled papain and labelled albumin are 2.97, 2.98 and 2.88 respectively. This suggests that the extinction coefficient of the label at 343 nm might be rather insensitive to environmental perturbation, and in many cases the value of ϵ_{343} for Py-2-SH should provide a good estimate of the stoichiometry of the reaction. In any case the labelling reactions of compound (I) are sufficiently fast that the value of $\Delta\epsilon_{343}$ for reaction of a particular protein with compound (I) obtained with a particular spectrophotometer is readily determined.

Fluorescence spectra

Compound (I) is highly fluorescent in aqueous solution. Excitation within the intense visible absorption band (λ_{max} , 481 nm, ϵ_{max} , $2.52 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$) provides green fluorescence with an emission maximum at 540 nm. Both papain and ficin labelled by reaction with compound (I) are characterized by fluorescence emission of even higher intensity (3–4-fold) at slightly shorter wavelengths (by 5–10 nm). The fluorescence emission spectra of compound (I) and of the products of its reaction with papain and with ficin are compared in Fig. 4. There is a large increase in the fluorescence polarization of the fluorophore of compound (I), from 0.058 to 0.276, consequent on its incorporation into the active-centre region of papain. This is consistent with rotational restrictions imposed by the protein.

The highly fluorescent character of papain-S-S-[CH₂]₂-NH-Nbd is demonstrated clearly in Fig. 5, in which its fluorescence spectrum is compared with that of S-Nbd-papain, i.e. the product of reaction of the papain-thiol group with Nbd chloride [see (XIV), Scheme 3].

Kinetics of the reactions of compound (I) and of Nbd chloride with thiols

Some second-order rate constants for the reactions of compound (I) and of Nbd chloride with GSH, bovine serum albumin, papain, 2-benzimidazolylmethanethiol (III) and 2-benzimidazolylethanethiol (IV) at 25.0°C are collected in Table 1. The methods used to determine these rate constants are described in the Materials and Methods section. Reactions of the last two thiols were studied at several pH values in the range approx. 2–7 and the other reactions were studied mainly at pH 4.0. At this pH, even Nbd chloride would be expected to possess high specificity for thiol groups. That these reactions did occur at sulphur atoms was strongly suggested by u.v.-spectral analysis of the reaction products (see Allen & Lowe, 1973).

Reactions other than those of the two benzimidazolyl-alkane thiols

The data in Table 1 demonstrate the much higher reactivity of compound (I) towards thiols compared with that of Nbd chloride. This difference in reactivity is particularly striking for the papain and ficin reactions. High reactivity of papain towards compound (I) in acidic media was expected from our earlier work on papain-2-Py-S-S-2-Py reactions (see, e.g., Brocklehurst, 1974). The relatively low value of $k_{2(\text{compound I})}/k_{2(\text{Nbd chloride})}$ (= 36) at pH 4 for the albumin-thiol group derives from an abnormally high value of $k_{2(\text{Nbd chloride})}$ (see Table 1). Kinetic analysis of the reactions of albumin with Nbd chloride and with

2-Py-S-S-2-Py (J. P. G. Malthouse, R. Norris, M. Shipton, T. Stuchbury & K. Brocklehurst, unpublished work) has suggested a specific interaction of Nbd chloride with albumin similar to that postulated in the analogous papain reaction (see Allen & Lowe, 1973). This might involve one of the carboxyl groups (glutamate-38 or aspartate-39) that could be close to the albumin thiol group (cysteine-34) (King & Spencer, 1972). As pointed out by Allen & Lowe (1973) it is advisable when using Nbd chloride to label thiol groups to carry out the reactions in acid solution in an attempt to improve thiol specificity. No such restriction need be applied to the use of compound (I), and for compounds in which the reactive species is uncomplicated thiolate ion it may be preferable to carry out the reaction at pH values considerably higher than 4, where the reaction will be faster. For example, the second-order rate constants for the reactions of GSH and albumin with compound (I) at pH 7.8 under the conditions given in Table 1 are 476 and $620\text{ M}^{-1}\cdot\text{s}^{-1}$ respectively.

Blocking of the papain thiol group by reactions with compound (I) and with Nbd chloride and the subsequent unblocking by thiolysis

Fig. 6 shows the initial part of a progress curve for the hydrolysis of α -N-benzoyl-L-arginine ethyl ester at pH 6.0 catalysed by papain. When compound (I) is added (to give a concentration of $40\text{ }\mu\text{M}$ in the reaction mixture), catalytic activity is abolished essentially instantaneously. By contrast, when the same concentration of Nbd chloride is introduced into a similar assay mixture, catalytic activity is lost very much more slowly. Fig. 6 shows also that when 2-mercaptoethanol is added to the papain blocked by reaction with compound (I) (to give a concentration of 100 mM in the assay mixture) the original catalytic activity of the papain is fully regenerated essentially instantaneously. In the experiments illustrated in Fig. 6 the catalysis and the thiol-blocking and unblocking reactions were all allowed to proceed in the assay mixture at pH 6.0. Fig. 7 shows the results of another set of experiments in which the thiol-blocking reactions were allowed to proceed at pH 4.0 (to

accelerate the Nbd chloride reaction; see Allen & Lowe, 1973). Samples of the reaction mixtures were

Fig. 5. Fluorescence-emission spectra at 25.0°C of *S*-Nbd-papain (Δ) and papain labelled by reaction with compound (I) ($\bullet$)

The buffer was the phosphate buffer, pH 6.6, described in Fig. 2 legend but without organic solvent. Both solutions had equal extinction at the exciting wavelength (420 nm). This wavelength is between λ_{max} for *S*-Nbd-papain (405 nm) and λ_{max} for papain labelled by reaction with compound (I) (475 nm) and was chosen to provide equal extinction (0.07) for solutions of the two fluorescent papain derivatives of approximately equal concentration (approx. $10\text{ }\mu\text{M}$). The fluorescence intensity is given relative to papain labelled by reaction with compound (I) = 100 .

Scheme 3. Reaction of Nbd chloride with the papain thiol group

Table 1. *Second-order rate constants (k_2) for the reactions of some thiols with compound (I) (in 13.4% acetonitrile) and with Nbd chloride (in 6.7% ethanol) at 25.0°C and $I = 0.1 \text{ mol/l}$*

Buffers: pH 1.90, glycine-HCl; pH 2.88–4.53, formic acid-NaOH; pH 4.82–5.65, acetic acid-NaOH; pH 6.09–7.91, KH_2PO_4 -NaOH. These were all made up at three times the concentrations given for $I = 0.1 \text{ mol/l}$ and 25°C in *Biochemist's Handbook* (1961, Long, C., ed., Spon Ltd., London) and diluted 3-fold into the reaction mixtures.

Thiol	pH	k_2 (compound I) ($\text{M}^{-1} \cdot \text{s}^{-1}$)	k_2 (Nbd chloride) ($\text{M}^{-1} \cdot \text{s}^{-1}$)	$\frac{k_2 \text{ (compound I)}}{k_2 \text{ (Nbd chloride)}}$
GSH	4.0	7.5	0.026	288
	7.8	476	—	—
Bovine serum albumin	4.0	20	0.56	35.8
	7.8	620	—	—
Papain	4.0	55000	18.3	3000
Ficin	4.0	8680	0.284	30560
2-Benzimidazolylmethanethiol	1.9	290	—	—
	2.88	465*	0.18	2583
	3.11	478	0.2†	2390
	3.79	498*	0.89	560
	4.23	490*	2.45	200
	4.53	483	4.5†	107
	4.82	425*	6.98	61
	5.24	375*	11.90	32
	5.65	304	—	—
	6.09	278	—	—
	6.60	780	—	—
	7.09	1416	—	—
2-Benzimidazolyethanethiol	1.90	14.8	—	—
	2.88	15*	0.09	167
	3.11	16.0	0.1†	160
	4.23	18*	0.17	106
	4.53	20	0.25†	80
	4.82	20*	0.29	69
	5.24	21*	0.65	32
	5.65	21.7	—	—
	6.09	25.7	—	—
	7.09	74.1	—	—
	7.91	454	—	—

* Values interpolated from data in Fig. 8.

† Values interpolated from data in Fig. 9.

removed and assayed for catalytic activity at pH 6.0. Thiolytic reactivation was carried out at pH 7.8 by using 150 mM 2-mercaptoethanol. The results in Fig. 7 show that not only does compound (I) label the papain thiol group very much more rapidly than does Nbd chloride but that papain is regenerated by thiolysis very much more rapidly from papain blocked by reaction with compound (I) than from Nbd-blocked papain.

The thiolytic unblocking of papain labelled by reaction with compound (I) was followed by stopped-flow fluorescence measurements. The second-order rate constants for the unblocking at 25.0°C, $I = 0.1 \text{ mol/l}$, are: by reaction with L-cysteine, $486 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 7.0 and $1080 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 8.2, and by reaction with 2-mercaptoethanol, $109 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 7.0 and $914 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 8.2.

Reactions of the benzimidazolylalkanethiols (III and IV)

The kinetics of these four reactions were studied to demonstrate the selectivity of compound (I). Our earlier work with 2-Py-S-S-2-Py has demonstrated that it is sometimes possible to achieve reaction specifically with a particular thiol group even in the presence of other thiol groups. For example, it is possible to titrate papain active sites with 2-Py-S-S-2-Py even in the presence of up to 100-fold molar excess of 2-mercaptoethanol (Brocklehurst & Little, 1973). This type of selectivity is particularly marked with 2-Py-S-S-2-Py in cases such as papain, where the thiol group to be labelled is reactive in acidic media where 2-Py-S-S-2-Py is even more highly electrophilic than it is in neutral media.

Fig. 6. Progress curves for the hydrolysis of α -N-benzoyl-L-arginine ethyl ester catalysed by papain (a) before, and (b)–(d) after the addition of Nbd chloride (b), compound (I) (c) and compound (I) followed by 2-mercaptoethanol (d)

Three separate experiments were carried out at 25.0°C by using a Radiometer titrator as a pH-stat and 0.1 M-NaOH as titrant and the results are shown as lines (a)–(d). Expt. 1, line (a): 2.8 ml of 100 mM- α -N-benzoyl-L-arginine ethyl ester in 0.3 M-NaCl containing 2 mM-EDTA was adjusted to pH 6.0 with a negligible volume of NaOH solution. The reaction was started by adding 50 μ l of 46.6 μ M-papain (2.33 nmol) in 0.1 M-KCl containing 1 mM-EDTA, and the progress of the reaction was recorded (line a) until the pen had completed one traverse of the chart. This is equivalent to the addition of 0.25 ml of titrant (25 μ mol). Recording of the complete hydrolysis of the substrate would have required approx. 10 traverses of the chart. Expt. 2, lines (a) and (b): Expt. 1 was repeated except that at point X 0.2 ml of 500 μ M-Nbd chloride in ethanol was added to the stirred reaction mixture. After point X the progress curve (line b) falls under line (a) in a manner consistent with slow inactivation of the enzyme. Expt. 3, lines (a), (c) and (d): Expt. 1 was repeated again except that at point X 0.2 ml of 600 μ M-compound (I) in acetonitrile was added. Catalytic activity was abolished essentially instantaneously (line c). After approx. 2.7 min, 20 μ l of 2-mercaptoethanol was added to the reaction mixture to give a concentration of approx. 100 mM (point Y). Catalytic activity was regained essentially instantaneously (line d). Control experiments in which 0.2 ml of ethanol and 0.2 ml of acetonitrile respectively were added in place of the Nbd chloride and compound (I) solutions produced no significant deviation from line (a) when allowance was made for the 8% dilution involved.

High reactivity of thiol groups in acidic media (pH approx. 3–6) would be predicted in cases where a sizeable fraction of the thiol exists as thiolate ion,

Fig. 7. Comparison of compound (I) and Nbd chloride as inhibitors of papain activity and comparison of the rates of thiolysis of the two enzyme derivatives leading to regain of activity

The experiments were carried out at 25.0°C with a Radiometer titrator as a pH-stat and 0.1 M-NaOH as titrant. The following mixture was used to obtain the initial (control) papain activity: 1.5 ml of 27.6 μ M-papain in 0.1 M-KCl containing 1 mM-EDTA, 0.9 ml of water, 0.2 ml of 0.3 M-sodium acetate buffer, pH 3.8, and 0.4 ml of acetonitrile were mixed. A 100 μ l sample of this mixture was added to 2 ml of 100 mM- α -N-benzoyl-L-arginine ethyl ester in 0.3 M-NaCl containing 2 mM-EDTA at pH 6.0 in the pH-stat and the initial rate of catalysis was determined. Similar reaction mixtures were then made up to study inhibition of papain by Nbd chloride and by compound (I). In the compound (I) reaction the mixture was made up with 0.2 ml of acetonitrile instead of 0.4 ml, and the reaction with compound (I) was started by addition of 0.2 ml of 0.93 mM-compound (I) in acetonitrile; 100 μ l samples were removed at intervals of time and their activity towards α -N-benzoyl-L-arginine ethyl ester was determined as before. In the Nbd chloride reaction the reaction mixture contained no acetonitrile and 1.1 ml of water instead of 0.9 ml. The reaction was started by addition of 0.2 ml of 1.77 mM-Nbd chloride in ethanol and 100 μ l samples were removed for activity measurements as above. The essentially instantaneous abolition of papain activity by reaction with compound (I) (▲) and the slower inhibition by reaction with Nbd chloride (●) are demonstrated. After both reactions had been allowed to proceed for approx. 90 min the residual reaction mixtures were adjusted to pH 7.8 by addition of NaOH (30 μ l of 4 M + 1.4 ml of 0.1 M) and then 40 μ l portions of 2-mercaptoethanol were added. This provided a concentration of approx. 150 mM in the reaction mixture. Samples (100 μ l) were removed for activity measurement as above. The activities determined after addition of 2-mercaptoethanol were corrected for the dilution of the reaction mixture caused by the adjustment of the pH to 7.8 so that they could be compared with those determined before the addition. Reactivation of papain blocked by reaction with compound (I) (▲) was essentially instantaneous. Reactivation of Nbd-blocked papain (●) was much slower. The apparent biphasicity of this reaction profile and a preliminary spectroscopic study of the reactivation of Nbd-blocked papain suggests that this may not be a simple one-step process.

Scheme 4. Nucleophilic protonic states of two homologous 2-benzimidazolylalkanethiols

i.e. the effective pK_a of the thiol is low. This would be expected to arise in proteins from interactions through space, e.g. with other proton-binding sites (see Shipton *et al.*, 1975; and Dixon & Tipton, 1973). In an attempt to find a low-molecular-weight model of the papain catalytic site, we have studied the kinetics of the reactions of 2-Py-S-S-2-Py with a number of 2-benzimidazolylalkanethiols (T. Stuchbury, E. Ager, G. V. Garner, H. Suschitzky & K. Brocklehurst, unpublished work). This study demonstrated that, whereas 2-benzimidazoleethanethiol exhibits appreciable nucleophilicity only in one protonic state [that at high pH where both the thiol and the benzimidazole moieties are proton-deficient; (XV), Scheme 4], 2-benzimidazolemethanethiol clearly exhibits nucleophilicity in two protonic states. One of these would be analogous to (XV), i.e. (XVI). The second nucleophilic state [(XVII), Scheme 4] must arise from interaction of the benzimidazole and thiol moieties and it is probable that the interaction is through the molecule, i.e. the second nucleophilic state is composed mainly of the zwitterion (see Scheme 4). Reaction of a thiol that can exist in two nucleophilic protonic states with a reagent such as 2-Py-S-S-2-Py that can increase its electrophilicity by protonation can give rise to a striking maximum in acidic media in the pH-rate profile, i.e. three reactive protonic states (see Brocklehurst, 1974). Such a maximum was found in the pH-rate profile for the reaction of 2-Py-S-S-2-Py with compound (III) but not in its reaction with compound (IV).

This feature of 2-Py-S-S-2-Py was predicted to be retained in compound (I), and Fig. 8 demonstrates that this is indeed the case. In marked contrast, Fig. 9 shows that Nbd chloride reacts at low pH only very slowly and at comparable rates with both compounds (III) and (IV). The rates of both reactions increase with increasing pH. Even though these reactions are very much slower than those of compound (I), the lower pK_a value of the thiol group in compound (III) compared with that in compound (IV) (T. Stuchbury, unpublished work) provides selectivity at pH values approx. 5.

Specificity in Nbd chloride reactions

The difficulty with the reactions of Nbd chloride with thiol groups in proteins is that, in general, their rates under conditions where Nbd chloride is probably reliably specific for thiol groups (pH values ≤ 5 ; see Allen & Lowe, 1973) are very low. This means complete and specific labelling of the thiol may be difficult or impossible to achieve because of competition from oxidation, denaturation or amino group labelling. This difficulty does not exist in the papain-Nbd chloride reaction because of the specific binding of Nbd chloride to aspartic acid-158, which provides a relatively high rate of reaction at pH approx. 4 (Allen & Lowe, 1973).

Kinetic analysis of the reactions of papain and ficin with 2-Py-S-S-2-Py and with Nbd chloride over a wide range of pH (M. Shipton, J. P. G. Malthouse, T. Stuchbury & K. Brocklehurst, unpublished work) suggests that ficin may not possess a carboxyl group functionally equivalent to that of aspartic acid-158 in papain. This could explain why it was not possible to label specifically and completely the essential thiol group of ficin by reaction of Nbd chloride in a manner analogous to that used successfully with papain.

Although the difficulties encountered in the ficin-Nbd chloride reaction might be expected to exist in many protein-Nbd chloride reactions, it is possible that in some cases environmental factors could lead to unusually high intrinsic protein thiolate ion reactivities towards Nbd chloride. In such cases it may be possible to label protein thiol groups with Nbd specifically even in weakly alkaline media. An example of initial specific labelling of thiol groups by reaction with Nbd chloride at pH 7.9 has been reported for porcine muscle adenylate kinase (Price *et al.*, 1975), although the label does subsequently migrate to a neighbouring amino group.

Comparison of compound (I) and Nbd chloride as fluorescent probe reagents

The major drawbacks of Nbd chloride that are rectified by combination of the Nbd fluorophore with

Fig. 8. pH-dependences of the second-order rate constants for the reactions of compound (I) with (●) 2-benzimidazolylmethanethiol and (▲) 2-benzimidazoleethanethiol in 13.4% acetonitrile at 25.0°C and $I=0.1 \text{ mol/l}$

The buffers are given in Table 1. The solid line was calculated by using

$$k_2 = \frac{\tilde{k}_2}{1 + \frac{[\text{H}^+]}{K_{a1}} + \frac{K_{a11}}{[\text{H}^+]}}$$

and $\text{p}K_{a1} = 1.93$, $\text{p}K_{a11} = 5.21$ and $\tilde{k}_2 = 525 \text{ M}^{-1} \cdot \text{s}^{-1}$; $\text{p}K_{a1} = 1.93$ is the $\text{p}K_a$ value of compound (I) that probably characterizes proton loss from its pyridyl nitrogen atom (see the text) and $\text{p}K_{a11} = 5.21$ is the $\text{p}K_{a1}$ value of 2-benzimidazolylmethanethiol determined spectrophotometrically. The broken lines were drawn by eye.

the 2-aminoethyl 2'-pyridyl disulphide delivery system of compound (I) are listed below.

(1) *Specificity*. The reaction of Nbd chloride with proteins is, in general, not specific for thiol groups (Ghosh & Whitehouse, 1968; Allen & Lowe, 1973) whereas that of compound (I) is.

Fig. 9. pH-dependences of the second-order rate constants for the reactions of Nbd chloride with (●) 2-benzimidazolylmethanethiol and (Δ) 2-benzimidazoleethanethiol in 6.7% ethanol at 25.0°C and $I=0.1 \text{ mol/l}$

The buffers are given in Table 1. The lines were drawn by eye.

(2) *Reactivity*. The selectivity of Nbd chloride for thiol groups should be increased by carrying out the reactions in acidic media. Under such conditions, however, it may be necessary to expose the protein to Nbd chloride (and an acidic medium) for 1 h or more to achieve stoichiometric labelling. This is particularly serious for proteins that are easily denatured in acidic media. By contrast, stoichiometric labelling by compound (I) in general is achieved within a few minutes or even a few seconds.

(3) *Quantification of reaction*. The reactions of Nbd chloride with nucleophiles result in substantial u.v. and visible extinction changes. These changes arise from replacement of the chlorine atom of the reagent by the nucleophilic atom of the protein. Thus these extinction changes can be used for determination of labelling stoichiometries only if the difference extinction coefficient $\Delta\epsilon$ for the reaction has been determined in each individual case. This limitation will apply also to kinetic studies because the low reactivity of Nbd chloride will often necessitate measurement of initial rates for which $\Delta\epsilon$ must be known. For proteins that are easily denatured in acidic media, determination of $\Delta\epsilon$ will be particularly difficult.

By contrast, reactions of compound (I) can be followed by the increase in E_{343} that results mainly from release of Py-2-SH. Any small changes in $\Delta\epsilon$ that may arise from environmental perturbation of the spectrum of the part of the reagent bound to the protein can be readily determined because of the rapidity of the stoichiometric reactions (see above). In some kinetic studies, knowledge of an exact value of $\Delta\epsilon$ will not be obligatory because of the ease with which reactions can be carried out under pseudo-first-order conditions.

(4) *Fluorescence intensities of the thiol derivatives.* Another respect in which Nbd chloride is found wanting as a fluorescent probe for thiols is the low fluorescence intensity of the products of its reaction with thiols [see, e.g., Ghosh & Whitehouse (1968), Birkett *et al.* (1970), Allen & Lowe (1973)]. This drawback is not shared by the products of the reaction of compound (I) with thiols; the reaction that attaches the fluorescent label of compound (I) to a thiol occurs at a site in the reagent electronically remote from the fluorophore and a highly fluorescent product is obtained.

(5) *Ease of regeneration of native protein by thiolysis of the labelled protein.* The results from the experiments on unblocking of papain labelled by reaction with compound (I) and with Nbd chloride suggest that regeneration of native proteins from labelled proteins may be considerably easier for proteins labelled by reactions with compound (I).

Selective thiol specificity of compound (I)

The advantages of compound (I) as a general reagent for the rapid and specific fluorescent labelling of thiol groups discussed above are augmented by its ability to label selectively thiol groups that display enhanced nucleophilicity in acidic media.

Possible advantages of Nbd chloride

Nbd chloride seems to possess properties that make it useful as a reactivity probe for certain types of perturbed thiol group (Allen & Lowe, 1973; M. Shipton, J. P. G. Malthouse, T. Stuchbury & K. Brocklehurst, unpublished work). It may be interesting in some cases to use Nbd chloride as a fluorescent probe for comparison with compound (I). If the same thiol can be labelled separately with both reagents, there is the possibility that the labels will 'see' different environments.

One feature of Nbd chloride that might find useful application is the possibility of detecting spectrophotometrically $S \rightarrow N$ transfer of the fluorescent label if there is a suitably aligned amino group (Birkett *et al.*, 1970; Price *et al.*, 1975). Although this type of label mobility might be exploited for some types of investigation, in others it could serve only to

confuse the picture. Similar advantages and disadvantages may be predicted when compound (I) is used for reactions with systems containing two thiol groups in close juxtaposition.

One respect in which Nbd chloride is better endowed than compound (I) is solubility in aqueous organic solvents. Although reaction mixtures containing even 13.4% acetonitrile cannot hold more than approx. $100\mu\text{M}$ -compound (I), this is not a disadvantage for labelling of proteins except for those which are seriously and irreversibly perturbed by this concentration of acetonitrile. It is rarely necessary or indeed possible to use protein concentrations greater than $100\mu\text{M}$, and because compound (I) is absolutely specific for thiol groups, the labelling can be carried out in weakly alkaline media (cf. Nbd chloride) where efficient labelling occurs (second-order rate constant greater than approx. $100\text{M}^{-1}\cdot\text{s}^{-1}$) under equimolar conditions.

A highly water-soluble carboxylated analogue of compound (I)

It is not convenient to work with compound (I) in solutions containing less than approx. 10% acetonitrile, and for some purposes it may be better to use the carboxylated analogue of compound (I) prepared from L-cysteine instead of from cysteamine. This reagent is freely soluble in water. Our interest in protein thiol groups situated in reasonably close proximity to carboxyl groups, particularly thiol groups with an effective pK_a of approx. 4, however, led us to prefer to use compound (I). This is because the additional reagent pK_a value (that of the carboxyl group) in the acid region could complicate kinetic analysis of the pH-dependence of the labelling reaction, and the presence of a negative charge on the reagent in weakly acidic media could lead to unfavourable electrostatic interactions with the protein. Preliminary experiments using this carboxylated reagent showed that the second-order rate constant for its reaction with papain at pH 3.8 (25.0°C and $I = 0.1\text{ mol/l}$) is about one-tenth of the rate constant for the analogous reaction of compound (I) (i.e. approx. $4 \times 10^3\text{M}^{-1}\cdot\text{s}^{-1}$ compared with $5.5 \times 10^4\text{M}^{-1}\cdot\text{s}^{-1}$).

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