A Thiol-Labelling Reagent and Reactivity Probe containing Electrophilic Mercury and a Chromophoric Leaving Group

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2-(2'-Pyridylmercapto)mercuri-4-nitrophenol was synthesized and evaluated as a thiol-labelling reagent containing a chromophoric leaving group and as a reactivity probe by studies on its reactions with 2-mercaptoethanol and with papain (EC 3.4.22.2).

The technique of using site-specific reactivity probes in the delineation of molecular environments and details of enzyme mechanism will undoubtedly benefit from the availability of related reagents containing different electrophilic centres that provide for a variety of transition-state geometries (see Gutfreund & McMurray, 1970; Brocklehurst, 1979). A particular type of reactivity probe is the two-protonic-state electrophile, a reagent that possesses substantially different reactivities in different ionization states (Brocklehurst, 1974, 1979). The techniques of using this type of reactivity probe to detect and characterize thiol groups in interactive systems and to introduce selectivity into thiol-group modification in acidic media were developed by using pyridyl disulphides in which reaction occurs at electrophilic sulphur (see e.g. Stuchbury *et al.*, 1975; Malthouse & Brocklehurst, 1976; Shipton & Brocklehurst, 1978; Baines & Brocklehurst, 1978).

It seemed possible that 2-pyridylmercurials (Ia and Ib) might constitute another type of reactivity probe analogous to the 2-pyridyl disulphides. We decided to synthesize 2-(2'-pyridylmercapto)mercuri-4-nitrophenol (II) as a prototype, because it was thought that this compound might be useful also as a chromogenic (see compound III) reporter-group delivery reagent.

In using this particular reagent for detailed reactivity studies, however, it might be necessary to take account of the state of ionization of the phenolic hydroxy group, particularly when a given active centre contains additional charges.

McMurray & Trentham (1969) described the preparation and properties of a number of nitrophenol mercurials typified by 2-chloromercuri-4-nitrophenol (IV). These have been used to study thiol-enzymes (see e.g. Gutfreund & McMurray, 1970; Quijcho & Thomson, 1973; Quijcho & Olson, 1974; Laue & Quijcho, 1977) mainly as reporter groups in the sense proposed by Burr & Koshland (1964). It is possible to monitor the reaction of these probes with thiol compounds spectrophotometrically by making use of the change in pK_a of the phenolic hydroxy group that results from replacement of the leaving group by the thiol as follows:

The 2-pyridylmercurial (II) differs from the chloromercurial (IV) in that it contains a chromophoric leaving group, pyridine-2-thione (III) ($\lambda_{\max}$, 343 nm), which should permit quantification of reaction with thiol compounds to be achieved more easily over a wide range of pH.

The present paper reports the preparation of 2-(2'-pyridylmercaptomercuri-4-nitrophenol (II) and its characterization both as a thiol-labelling reagent with improved spectral properties and as a two-protonic-state reactivity probe containing electrophilic mercury.

Materials and Methods

Stopped-flow kinetic experiments and spectroscopic experiments were performed as described previously (Shipton & Brocklehurst, 1978). Buffers were prepared as described by Dawson *et al.* (1969) and by Long (1971).

Preparation of 2-chloromercuri-4-nitrophenol

This compound, prepared from 4-nitrophenol and mercuric acetate as described by McMurray & Trentham (1969), had m.p. 240°C (McMurray & Trentham, 1969, give m.p. 238°C for decomposition) and $\lambda_{\max}$, 405 nm in 0.1 M-KOH.

Synthesis of 2-(2'-pyridylmercaptomercuri-4-nitrophenol

2-Chloromercuri-4-nitrophenol (2 g; 0.027 mol) was dissolved in methanolic KOH (10 ml of 0.1 M-KOH + 10 ml of methanol) at room temperature (approx. 22°C). To this solution was slowly added a solution of pyridine-2-thione (0.7 g; 0.31 mol) in the same solvent with continuous stirring over 30 min.

The white solid that formed was redissolved by heating the mixture to 90°C. After hot filtration the solution was allowed to cool to room temperature. The crystalline product was collected by vacuum filtration, washed with 50 ml of the methanolic KOH and recrystallized from the methanolic KOH. The final product was a fine white powder (m.p. 120–123°C). The elemental analysis (for C, H, N and Hg) was as expected for 2-(2'-pyridylmercaptomercuri-4-nitrophenol ($C_{11}H_8N_2O_3SHg$).

In 0.1 M-KOH the long-wavelength absorption maximum is characterized by $\lambda_{\max}$, 405 nm and $\epsilon_{\max}$, $1.7 \times 10^4 M^{-1} \cdot cm^{-1}$. At pH 4 (sodium formate buffer) $\lambda_{\max}$, was 325 nm and $\epsilon_{\max}$, was $3.8 \times 10^3 M^{-1} \cdot cm^{-1}$, and thiolysis by 2-mercaptoethanol provided the increase in A_{343} predicted by stoichiometric liberation of pyridine-2-thione (ϵ_{343} $8.08 \times 10^3 M^{-1} \cdot cm^{-1}$) (Stuchbury *et al.*, 1975).

Results and Discussion

Preparation of 2-(2'-pyridylmercaptomercuri-4-nitrophenol

The pyridine-2-thione anion reacts rapidly (eqn. 1) with the 2-chloromercuri-4-nitrophenolate ion in methanolic KOH at room temperature, as was predicted from the known nucleophilic characteristics of this aminothione (Carlsson *et al.*, 1974)

Comparison of 2-(2'-pyridylmercaptomercuri-4-nitrophenol with existing mercuri-4-nitrophenol probes

The electronic absorption spectra of (a) the 2-pyridylmercurial and (b) the chloromercurial in KH_2PO_4/Na_2HPO_4 buffer, I 0.1, containing 13% (v/v) ethanol, pH 7.8, before and after addition (in negligible volume) of a large excess of 2-mercaptoethanol are shown in Fig. 1. Thiols of the chloro compound provides only a small spectral change, whereas thiolysis of the 2-pyridyl compound provides a larger change, not only in the absorption band ($\lambda_{\max}$, 405 nm) of the nitrophenolate reporter group, but also in the region around 340 nm consequent on release of the pyridine-2-thione leaving group. For probes like the chloromercurial the spectral change results from a change in the pK_a of the nitrophenol when the initial ligand or leaving group (e.g. the chloro group) is replaced by the mercapto group. Although it is possible to increase the magnitude of the spectral change at pH values around the pK_a of the nitrophenol by replacing the chloro ligand by EDTA, the spectral change resulting from reaction with a thiol group still varies markedly with pH as shown in Table 1. At pH 4.35 the value of $\Delta\epsilon_{405}$ is only 2% of the value at pH 8. In marked contrast with the characteristics of this type of mercurial, the change in A_{343} resulting from reaction of a thiol group with the 2-pyridylmercurial is insensitive to change in pH in acidic media. This is because the increase in A_{343} observed on thiolysis is due very largely to absorption by the pyridine-2-thione released and does not depend on a change in the state of ionization of the nitrophenol. Even at pH values near the pK_a of the nitrophenol, the value of $\Delta\epsilon_{343}$

Fig. 1. Electronic absorption spectra of (a) 2-(2'-pyridylmercapto)mercuri-4-nitrophenol and (b) 2-chloromercuri-4-nitrophenol (i) before and (ii) after the addition of a large excess of 2-mercaptoethanol

Solutions were in $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer (pH 7.8; I 0.1), containing 13% (v/v) ethanol. The spectra were scanned on a Cary 118C spectrophotometer before and 5 min after addition of 2-mercaptoethanol in negligible volume. Repeat scans demonstrated the completeness of reaction after this time interval. The concentration of the mercurial was approx. $10\ \mu\text{M}$ in each case and the concentration of 2-mercaptoethanol was approx. 45 mM.

Table 1. Effect of pH on the change in A_{405} that occurs consequent on the reaction of 2-mercaptoethanol with 2-chloromercuri-4-nitrophenol in the presence of sufficient excess of EDTA to displace the chloro ligand

Reaction mixtures (25°C ; I 0.1) contained 2-chloromercuri-4-nitrophenol ($5.64\ \mu\text{M}$) and the disodium salt of EDTA (10 mM). Reaction was effected by addition of 2-mercaptoethanol ($46\ \mu\text{M}$). All reactions were eventually complete within 15 s. The values of ΔA_{405} are expressed as percentages of the value observed for the reaction at pH 8.07 (ΔA_{405} 0.16). Inclusion of additional EDTA (up to a total concentration of 55 mM) at pH 5.72 and 8.07 did not result in increased values of A_{405} .

Buffer	pH	A_{405} (%)
Sodium formate	4.35	2
$\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$	5.72	29
$\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$	6.29	23
$\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$	6.84	42
$\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$	7.04	52
Tris/HCl	7.35	88
Tris/HCl	8.07	100
Na_2CO_3	9.47	63
Na_2CO_3	9.50	59

for the reaction is not much lower than the value predicted from the ϵ_{343} of pyridine-2-thione. At pH 7.8 $\Delta\epsilon_{343}$ was $7.3 \times 10^3\ \text{M}^{-1}\cdot\text{cm}^{-1}$, which is 91% of ϵ_{343} for pyridine-2-thione at this pH.

2-(2'-pyridylmercapto)mercuri-4-nitrophenol as a thiol-labelling reagent

In common with other mercuri-4-nitrophenols, the 2-pyridylmercurial possesses rather low solubility in aqueous media at pH values where the phenolic hydroxy group is unionized. Although even under these conditions the limited solubility of the reagent is sufficiently high [approx. $4\ \mu\text{M}$ in 6.7% (v/v) ethanol] to allow its use in kinetic studies (see below) it is sometimes desirable to produce labelled proteins in rather higher concentrations. That it is possible to do this by reaction in stirred suspension was illustrated by using papain (EC 3.4.22.2). Stoichiometric labelling of the papain thiol group was achieved by stirring a solution of the enzyme (5 ml of $25\ \mu\text{M}$ -enzyme in $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.8 and I 0.1) in which had been suspended the 2-pyridylmercurial (20 mg, which in solution would have provided a concentration of approx. 10 mM) at room temperature (approx. 22°C) for 15 min. Excess reagent was removed by filtration and the A_{343} of the filtrate showed that stoichiometric labelling ($23.6\ \mu\text{M}$ -pyridine-2-thione released) had occurred. Separation of the pyridine-2-thione by chromatography on a column ($2.5\ \text{cm} \times 25\ \text{cm}$) of Sephadex G-25 provided a solution of labelled papain (at pH 7.87; $\lambda_{\text{max.}(1)}$ 280 nm; ϵ_{280} $5.6 \times 10^4\ \text{M}^{-1}\cdot\text{cm}^{-1}$ $\lambda_{\text{max.}(2)}$ 415 nm; ϵ_{415} $4.4 \times 10^3\ \text{M}^{-1}\cdot\text{cm}^{-1}$).

2-Pyridylmercurials as reactivity probes

The reactions of the 2-pyridylmercurial with thiol compounds typified by 2-mercaptoethanol are readily followed over a wide range of pH by stopped-flow spectral analysis at 343 nm, even though ΔA_{343} is only approx. 0.03.

The relatively small value of the second-order rate constant for the reaction of the 2-pyridylmercurial with the thiolate ion of 2-mercaptoethanol (k approx. $3.5 \times 10^3\ \text{M}^{-1}\cdot\text{s}^{-1}$) may be an advantage in some studies because it increases the probability that pH-versus k data will provide $\text{p}K_a$ values characteristic of the free reactant molecules (see Brocklehurst & Dixon, 1977).

The fact that k for this reaction is constant in the pH range 4–7 at a value ($500\ \text{M}^{-1}\cdot\text{s}^{-1}$) that is a substantial fraction of the plateau value of k at high pH supports the postulate (see the Introduction section) that, like 2-pyridyldisulphides (see Brocklehurst 1974, 1979), 2-pyridylmercurials may be two-protonic-state electrophiles and thus valuable reactivity probes for the characterization of thiol groups in interactive systems (see Shipton & Brocklehurst, 1978) and for all thiol groups at low pH (see e.g. Malthouse & Brocklehurst, 1976).

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