Kinetics of the Reversible Reaction of Papain with 5,5'-Dithiobis-(2-nitrobenzoate) Dianion: Evidence for Nucleophilic Reactivity in the Un-ionized Thiol Group of Cysteine-25 and for General Acid Catalysis by Histidine-159 of the Reaction of the 5-Mercapto-2-nitrobenzoate Dianion with the Papain-5-Mercapto-2-nitrobenzoate Mixed Disulphide

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In the accompanying paper (Brocklehurst & Little, 1972) evidence is presented for the existence of nucleophilic reactivity in the un-ionized thiol group of cysteine-25 in papain (EC 3.4.4.10) occasioned by its interaction with the imidazole group of the histidine-159-asparagine-175 hydrogen-bonded system. This evidence arises from kinetic analysis of the reactions of the papain thiol group with 2,2'- and with 4,4'-dipyridyl disulphide.

These results, together with the principle of microscopic reversibility, suggest that the reaction of a thiolate ion with a mixed disulphide composed of papain and another thiol should be subject to general acid catalysis by the imidazolium ion of histidine-159. The reactions with the above disulphides are effectively irreversible because the 'thiol' products exist very largely in the tautomeric thione forms.

To investigate the reverse reaction of a thiol-disulphide interchange involving papain, we carried out a kinetic analysis of the reaction of papain with 5,5'-dithiobis-(2-nitrobenzoate) dianion and its reverse, the reaction of 5-mercapto-2-nitrobenzoate dianion with the papain-5-mercapto-2-nitrobenzoate disulphide. This demonstrates a pH-rate profile for the reverse reaction characteristic of the expected general acid catalysis by histidine-159. It demonstrates also a profile for the forward reaction characteristic of nucleophilic reactivity in the un-ionized thiol group of papain towards a disulphide that, by contrast with the pyridyl disulphides, cannot effectively increase its electrophilicity by protonation.

Materials and methods

Active enzyme was prepared as reported previously (Brocklehurst & Little, 1970). 5,5'-Dithiobis-(2-nitrobenzoic acid) (m.p. 238–240°C), recrystallized from acetic acid, was shown to contain less than 0.1% of 5-mercapto-2-nitrobenzoic acid by spectrophotometric analysis. Apparent dissociation constants at 25.0°C and *I* 0.1 determined spectrophotometrically are: 5,5'-dithiobis-(2-nitrobenzoic acid),

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$pK_{a1}=2.2$; 5-mercapto-2-nitrobenzoic acid, $pK_{a11}=4.4$.

The stoichiometries and kinetics of the reactions of 5,5'-dithiobis-(2-nitrobenzoate) dianion with papain, L-cysteine and 2-mercaptoethanol at 25.0°C and *I* 0.1 were determined spectrophotometrically at 412 nm in deoxygenated buffers containing 1 mM-EDTA. Stoichiometries were calculated by using $\epsilon_{412}=1.36 \times 10^4/(1+[H^+]/4 \times 10^{-5}) M^{-1} \cdot cm^{-1}$. In the papain reaction at pH values below 7 the infinity readings of preliminary experiments showed that progress curves represent reactions approaching a pH-dependent equilibrium position (eqn. 1) in which Pap-SH represents papain and Ar represents

The analysis in terms of eqn. (1) when $[\text{Ar-S-S-Ar}_0] \gg [\text{Pap-SH}_0]$ and $[\text{Ar-SH}_0]=[\text{Pap-S-S-Ar}_0]=0$ was carried out as described by Frost & Pearson (1963) with a computer program. The computer printed out values of k_{+2} , k_{-2} and K_{eq} . ($=k_{+2}/k_{-2}$).

Results and discussion

The reactions of 5,5'-dithiobis-(2-nitrobenzoate) dianion with L-cysteine and with 2-mercaptoethanol in the pH range 5–9 are first-order in each reactant. The pH-dependences of the second-order rate constants showed that there was a requirement for a group in the base form (or the usual kinetic equivalent), pK_a 8.3 for the L-cysteine reaction and pK_a 9.1 for the 2-mercaptoethanol reaction. Thus these reactions of low-molecular-weight thiols probably occur by attack of the thiolate anion on the disulphide, the un-ionized forms of these thiols are unreactive and this disulphide cannot effectively

Fig. 1. pH-dependences of k_{+2} and k_{-2} for the reversible reaction of papain with 5,5'-dithiobis-(2-nitrobenzoate) dianion at 25.0°C and 10.1

The points (●, k_{+2} ; ▲, k_{-2}) were obtained from computer analysis of experimental findings in terms of eqn. (1); the lines (—, k_{+2} ; ----, k_{-2}) are theoretical for:

$$k_{+2} = \frac{k_{+2} - k_{+2}(\text{obs.})}{1 + [\text{H}^+]/K_a} + k_{+2}(\text{obs.})$$

where $k_{+2} = 284 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_{+2}(\text{obs.}) = 40 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $\text{p}K_a = 8.0$ and for:

$$k_{-2} = \frac{k_{-2}}{1 + K_a/[\text{H}^+]}$$

where $k_{-2} = 11200 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $\text{p}K_a = 4.4$.

increase its electrophilicity towards thiolate ions by protonation on S. Under the conditions used ($[\text{disulphide}] = 10\text{--}300 \mu\text{M}$; $[\text{thiol}] = 5\text{--}20 \mu\text{M}$), no change in the position of equilibrium with change in pH was observed.

In the pH range 7.5–8.6 the papain reaction obeyed second-order kinetics, and with $[\text{papain}] = 5\text{--}30 \mu\text{M}$ and $[\text{disulphide}] = 10\text{--}600 \mu\text{M}$ K_{eq} did not vary with pH. In the pH range 5.0–7.3, however, K_{eq} was observed to decrease with decreasing pH. Robyt *et al.* (1971) reported that the reaction of papain with 5,5'-dithiobis-(2-nitrobenzoate) dianion is complicated by reaction of 5-mercapto-2-nitrobenzoate dianion with the cystinyl disulphide bonds

of papain. However, Brocklehurst *et al.* (1972) have reported definitive evidence showing that this complication does not occur.

The pH-dependence of K_{eq} is that predicted for a thiol–disulphide interchange in which the thiols involved in the forward and the reverse reactions have non-identical $\text{p}K_a$ values (see Smith *et al.*, 1964). The pH– $\text{p}K_{\text{eq}}$ data interpreted in terms of Dixon's (1953) rules are in accord with an equilibrium in which, in the pH range 5–7, the products bear a net charge of –1 relative to the reactants and there is a $\text{p}K_a$ value unique to the reactant state of 8.0 (that of the papain thiol group) and a $\text{p}K_a$ value unique to the product state of 4.4 (that of the thiol group of 5-mercapto-2-nitrobenzoate monoanion). By contrast with the pH– $\text{p}K_{\text{eq}}$ data, the pH-dependences of k_{+2} and k_{-2} , the apparent (see Brocklehurst & Little, 1972) second-order rate constants that constitute K_{eq} , are not those that would be predicted for a simple thiol–disulphide interchange. These would be expected to be sigmoidal pH– k_{+2} and pH– k_{-2} profiles with each thiol required in the base form for reactivity, with $\text{p}K_a$ 8–9 for the papain thiol group and $\text{p}K_a$ 4.4 for the thiol group of 5-mercapto-2-nitrobenzoate monoanion.

In the pH range 5.5–8.5 (see Fig. 1) the reaction of the papain thiol group with 5,5'-dithiobis-(2-nitrobenzoate) dianion is described by eqn. (2), in which k_{+2} (the apparent second-order rate constant for reaction of the papain thiolate ion) = $284 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_{+2}(\text{obs.})$ (the rate constant for reaction of the unionized thiol groups of papain) = $40 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $\text{p}K_a$ (the apparent dissociation constant of the papain thiol group) = 8.0:

$$k_{+2} = \frac{k_{+2} - k_{+2}(\text{obs.})}{1 + [\text{H}^+]/K_a} + k_{+2}(\text{obs.}) \quad (2)$$

Below pH 5.5 the pH-dependence of k_{+2} is not described by eqn. (2); in the pH range 5.0–5.5 k_{+2} appears to increase with decreasing pH.

In the pH range 5.0–7.3 the apparent second-order rate constant for the reverse reaction (k_{-2}) increases with decreasing pH. The data can be fitted to a sigmoid curve, $\text{p}K_a$ 4.4, $k_{-2} = 11.2 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$, with 5-mercapto-2-nitrobenzoic acid formally required for reactivity as the monoanion. In the pH range 5.5–8.5 the reversible reaction of papain with 5,5'-dithiobis-(2-nitrobenzoate) dianion may be described by eqn. (3):

The pH-independent reaction (pH 5.5–6.5) of the papain thiol group with 5,5'-dithiobis-(2-nitrobenzoate) dianion may be interpreted in a manner analogous to that for the principal reaction of the XH state of the reaction of papain with 2,2'-dipyridyl disulphide (see Brocklehurst & Little, 1972), i.e. reaction of the un-ionized thiol group of cysteine-25 assisted by general base catalysis by histidine-159.

By contrast with dipyridyl disulphides, 5,5'-dithiobis-(2-nitrobenzoate) dianion cannot readily increase its electrophilicity by protonation. If the increase in k_{+2} , which is apparent below pH 5.5 (see Fig. 1), is real, this could result from a decrease in the dissociation constant of the papain–reagent complex, possibly resulting from hydrogen-bonding involving the reagent carboxyl group(s). Accurate kinetic analysis of this system is not possible below pH 5 with conventional spectrophotometric techniques.

Molecular interpretations that are in accord with the equilibrium and kinetic data (see Fig. 1 and eqn. 3) are given in eqns. (4) and (5):

The reverse reaction depends kinetically on the thiolate ion concentration and the H⁺ concentration. In eqn. (5) this reaction is represented as the attack of the 5-mercapto-2-nitrobenzoate dianion on the mixed-disulphide linkage assisted by general acid catalysis by the imidazolium ion of histidine-159. This is the microscopic reverse of the reaction of the un-ionized thiol group of cysteine-25 assisted by general base catalysis by the imidazole group of histidine-159.

The value of k_{-2} ($11.2 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$) given in Fig. 1 and eqns. (3) and (5) was obtained by using as a model to fit the data in Fig. 1 a group of pK_a 4.4 required in the acid form. According to eqn. (5), however, the pH- k_{-2} profile should be part of a bell-shaped profile, and thus k_{-2} will be the value associated with this (and the appropriate ionic forms of the reactants) and not with the sigmoid profile of Fig. 1. The value of k_{-2} for eqn. (5) therefore will depend on the pK_a of the imidazole group of histidine-159 in the mixed disulphide. The evidence presented above suggests that this pK_a is below 5.0. It is of note that the group required in the base form for the deacylation of *trans*-cinnamoyl-papain has pK_a 4.69 (Bender & Brubacher, 1964), and this group could be the imidazole group of histidine-159.

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