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# Reactivities of the Various Protonic States in the Reactions of Papain and of L-Cysteine with 2,2′- and with 4,4′-Dipyridyl Disulphide: Evidence for Nucleophilic Reactivity in the Un-ionized Thiol Group of the Cysteine-25 Residue of Papain Occasioned by its Interaction with the Histidine-159–Asparagine-175 Hydrogen-Bonded System

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One of the unresolved questions central to the mechanism of the catalytic action of papain (EC 3.4.4.10) concerns the state of the nucleophilic thiol group of cysteine-25 when it becomes acylated during catalysis [compare Hussain & Lowe (1968) and Drenth *et al.* (1970)]. Part of the ambiguity arises because of the probable intermediacy of a tetrahedral adduct in acylation and the uncertainty as to whether the rate-limiting step at a given pH is formation of the adduct or its breakdown. If it could be established that the un-ionized cysteine-25–histidine-159–asparagine-175 hydrogen-bonded system does possess nucleophilic character, then it is likely that the thiolate ion of cysteine-25 would be at least equally capable of attacking the electrophilic centre of a substrate to form a tetrahedral adduct. Thus the pH–rate profile for formation of the tetrahedral adduct might be expected to be of double sigmoid form, the rate increasing with increasing pH. The fall in acylation rate at pH values above 6 (see, e.g., Whitaker & Bender, 1965) could then be interpreted as a change in rate-limiting step from formation of the tetrahedral adduct at pH values below 6 to elimination of the first product at pH values above 6.

We have already reported (Brocklehurst & Little, 1970) an unusually rapid reaction of the papain thiol group with 2,2′-dipyridyl disulphide in acidic media. We now report a kinetic and mechanistic analysis of the reactions of papain and of L-cysteine with 2,2′- and with 4,4′-dipyridyl disulphide. This provides compelling evidence that the thiol group of cysteine-25 in papain possesses nucleophilic reactivity in its un-ionized form and that this probably results from its interaction with the histidine-159–asparagine-175 system.

## *Materials and methods*

These have been described previously (Brocklehurst & Little, 1970).

## *Results and discussion*

In the pH range 2–10 both low-molecular-weight thiols and the thiol group of cysteine-25 in papain react stoicheiometrically with 2,2′- and with 4,4′-dipyridyl disulphide to produce mixed disulphides and chromophoric thiopyridones (see Brocklehurst & Little, 1970). For these reactions plots of second-order rate constants ($k_{+2}$), calculated from total concentrations of the reactants, against pH (or $\log k_{+2}$ versus pH) reveal a number of significantly reactive protonic states in the reactants (for examples see Fig. 1).

The most straightforward profile is that for the reaction of L-cysteine with 4,4′-dipyridyl disulphide (Fig. 1*a*). Two reactive states (designated X and XH to represent their relative stoicheiometries with respect to protons) are evident. X is interpreted as reaction of the thiolate ion of L-cysteine with unprotonated 4,4′-dipyridyl disulphide (4-Py–S–S–4-Py) ($k_{+2}=1.92\times10^4\,M^{-1}\cdot s^{-1}$). In this reaction the interpretation of XH is relatively unambiguous. Thus the lack of a reactive XH state in the reaction of L-cysteine with 5,5′dithiobis-(2-nitrobenzoic acid) demonstrates both the lack of nucleophilic character in a simple un-ionized thiol group and the inability of the disulphide bond to increase its electrophilicity by protonation on S (Little & Brocklehurst, 1972). In the reaction with 4,4′-dipyridyl disulphide, therefore, the XH state represents reaction of the L-cysteine thiolate ion with the reagent singly protonated on N (4-Py–S–S–4-PyH$^+$).

The wide separation of the acid dissociation constants of the protonated pyridyl disulphides (Py–S–S–PyH$^+$) [$K_{a_{II}}$(Py–S–S–Py)] and the thiol (RSH) [$K_a$(SH)] (see Fig. 1 legend) means that the reagents exist predominantly as non-ionic forms in the pH region in which reactivity is attributable mainly to the XH state (pH 5–6). If, therefore, in a given system the only reactive form of the XH state is in fact Py–S–S–PyH$^+$ and thiolate ion (RS$^-$), the second-order rate constant for the reaction of these ionic forms, $k_{+2}(\pm)$, is related to that calculated for the XH state from the total concentrations (which approximate [Py–S–S–Py] and [RSH]; see above), namely $k_{+2}$(obs.), by:

$$k_{+2}(\pm) = k_{+2}(\text{obs.})\cdot\frac{[\text{Py–S–S–Py}][\text{RSH}]}{[\text{Py–S–S–PyH}^+][\text{RS}^-]} = k_{+2}(\text{obs.})\cdot\frac{K_{a_{II}}(\text{Py–S–S–PyH}^+)}{K_a(\text{SH})} \quad (1)$$

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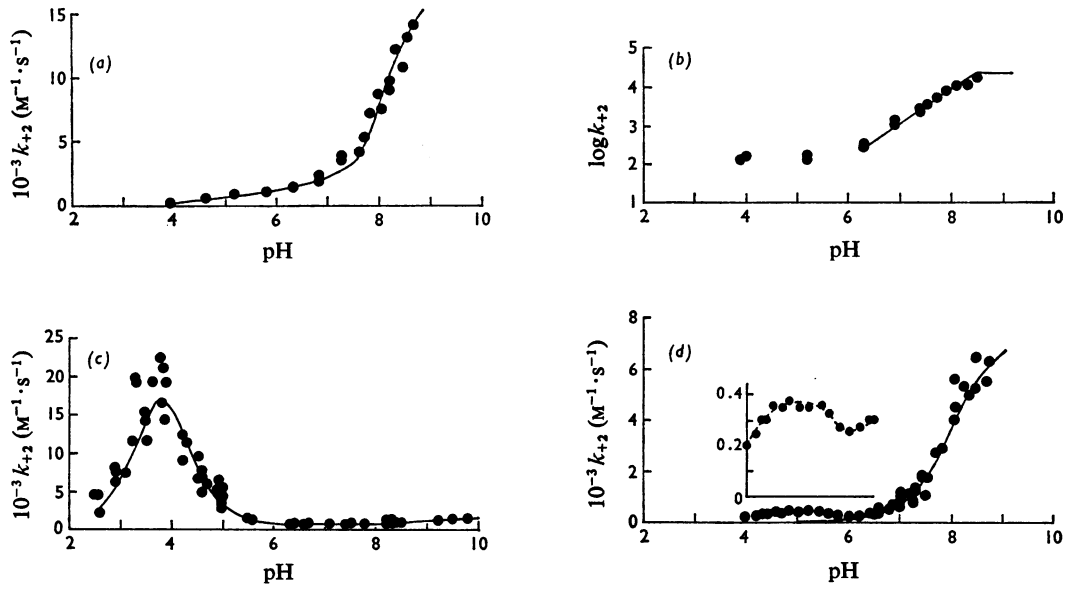


Fig. 1. *pH–rate profiles for the reactions of* L-*cysteine and of papain with* 2,2′- *and with* 4,4′-*dipyridyl disulphide at* 25.0°*C and I* 0.1

The buffers used were: sodium formate, sodium acetate, phosphate ($KH_2PO_4$+NaOH) and tris–HCl. The points are experimental and the lines theoretical for:

(*a*) L-cysteine–4,4′-dipyridyl disulphide:

$$k_{+2} = k_{+2}(\text{obs.})/(1+[H^+]/K_{a_2}+K_{a_3}/[H^+])+k_{+2}/(1+[H^+]/K_{a_3});$$
$$k_{+2}(\text{obs.}) = 1.2\times10^3\,M^{-1}\cdot s^{-1};\ k_{+2}=1.92\times10^4\,M^{-1}\cdot s^{-1};\ pK_{a_2}=5.1;\ pK_{a_3}=8.25$$

(*b*) L-cysteine–2,2′-dipyridyl disulphide:

$$k_{+2} = k_{+2}(\text{obs.})+k_{+2}/(1+[H^+]/K_{a_3});$$
$$k_{+2}(\text{obs.}) = 1.30\times10^2\,M^{-1}\cdot s^{-1};\ k_{+2}=3.0\times10^4\,M^{-1}\cdot s^{-1};\ pK_{a_3}=8.35$$

(*c*) papain–2,2′-dipyridyl disulphide:

$$k_{+2} = k'_2/(1+[H^+]/K_{a_1}+K_{a_2}/[H^+])+k_{+2}(\text{obs.})/(1+[H^+]/K_{a_2}+K_{a_3}/[H^+])+k_{+2}/(1+[H^+]/K_{a_3});$$
$$k'_{+2}=5\times10^4\,M^{-1}\cdot s^{-1};\ k_{+2}(\text{obs.})=7\times10^2\,M^{-1}\cdot s^{-1};$$
$$k_{+2} = 1.7\times10^3\,M^{-1}\cdot s^{-1};\ pK_{a_1}=pK_{a_2}=3.8;\ pK_{a_3}=8.8$$

(*d*) papain–4,4′-dipyridyl disulphide:
rate equation is of the same form as for (*c*); the continuous line is for $k_{+2}=k_{+2}/(1+[H^+]/K_{a_3})$; $k_{+2}=7.4\times10^3\,M^{-1}\cdot s^{-1}$; $pK_{a_3}=8.0$; apparent $pK_a$ values determined spectrophotometrically: 2,2′-dipyridyl disulphide, $pK_{a_{II}}=2.45$; 4,4′-dipyridyl disulphide, $pK_{a_I}=4.0$ and $pK_{a_{II}}=5.1$ [Long (1961) gives $pK_{a_{II}}$ for L-cysteine=8.36].

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In the L-cysteine–4,4′-dipyridyl disulphide reaction, $k_{+2}$(obs.) (=$1.2\times10^3\,M^{-1}\cdot s^{-1}$) obtained experimentally converts by use of eqn. (1) into $k_{+2}(\pm)=1.7\times10^6\,M^{-1}\cdot s^{-1}$. In the reaction of L-cysteine with 2,2′-dipyridyl disulphide (Fig. 1*b*) $k_{+2}$ for the X state (reaction of the $RS^-$ with 2-Py–S–S–S–2-Py) is $3.0\times10^4\,M^{-1}\cdot s^{-1}$. For the XH state $k_{+2}$(obs.)= $130\,M^{-1}\cdot s^{-1}$. If, as in the L-cysteine–4,4′-dipyridyl disulphide reaction, the only reactive form of the XH state is Py–S–S–$PyH^+$ and $RS^-$, $k_{+2}(\pm)=1.2\times10^8\,M^{-1}\cdot s^{-1}$. This value, which is approx. $10^2$ times that for the L-cysteine–4,4′-dipyridyl disulphide reaction, may reflect greater electrophilicity of the reacting S atom in 2-Py–S–S–2-$PyH^+$ compared with that in 4-Py–S–S–4-$PyH^+$ or significant reactivity in the 2-Py–S–S–2-Py–RSH form of the XH state of

this reaction. The latter could be accounted for by a six-membered cyclic transition state. If there is significant reactivity in two forms of the XH state, $k_{+2}(\pm)$ is related to $k_{+2}$(obs.) by:

$$k_{+2}(\text{obs.}) = \frac{k_{+2}(0)\cdot[\text{Py–S–S–Py}][\text{RSH}] + k_{+2}(\pm)\cdot[\text{Py–S–S–PyH}^+][\text{RS}^-]}{[\text{Py–S–S–Py}_T][\text{RSH}_T]} \quad (2)$$

in which $k_{+2}(0)$ is the second-order rate constant for the reaction of the non-ionic forms Py–S–S–Py and RSH and the subscript T denotes total concentrations. Since in the XH state, to a close approximation, $[\text{Py–S–S–Py}_T]=[\text{Py–S–S–Py}]$ and $[\text{RSH}_T]=[\text{RSH}]$, eqn. (2) becomes:

$$k_{+2}(\text{obs.}) = k_{+2}(0) + k_{+2}(\pm)\cdot\frac{[\text{Py–S–S–PyH}^+][\text{RS}^-]}{[\text{Py–S–S–Py}][\text{RSH}]} \quad (3)$$

Thus if both forms of a given XH state have significant reactivity, calculation of $k_{+2}(\pm)$ by use of eqn. (1) will yield a high estimate of this rate constant.

The most compelling evidence for nucleophilic reactivity in the un-ionized thiol group of papain arises from (i) comparison of the relative reactivities of the X and XH states of the L-cysteine–2,2′-dipyridyl disulphide reaction with those of the corresponding states of the papain–2,2′-dipyridyl disulphide reaction together with (ii) observation of high reactivity in the $XH_2$ state of the papain–2,2′-dipyridyl disulphide reaction (Fig. 1*c*). It is important to note that apparent second-order rate constants for the enzyme reactions are probably ratios of the first-order rate constants for the thiol–disulphide interchanges within enzyme–reagent complexes and the dissociation constants of the complexes. In the X state the reactivity of papain (the thiolate ion of cysteine-25) towards 2-Py–S–S–2-Py ($k_{+2}=1.7\times10^3\,\text{M}^{-1}\cdot\text{s}^{-1}$) is 18-fold lower than that of the L-cysteine thiolate ion. This may be due in part to an unfavourable binding constant. Comparison of $k_{+2}$ for the X state of the L-cysteine–2,2′-dipyridyl disulphide reaction with $k_{+2}(\pm)$ for the XH state of this reaction gives the maximum enhancement factor in the reactivity of 2-Py–S–S–2-Py towards $RS^-$ consequent on protonation on N as $4\times10^3$. If a six-membered cyclic transition state operates in the XH state of this reaction, this rate enhancement factor could be considerably smaller, possibly as small as 70, as in the L-cysteine–4,4′-dipyridyl disulphide reaction. If it is assumed that the above 'maximum' rate enhancement of $4\times10^3\times k_{+2}$ applies in the papain–2,2′-dipyridyl disulphide reaction, $k_{+2}(\pm)$ should be, at most, $4\times10^3\times1.7\times10^3\,\text{M}^{-1}\cdot\text{s}^{-1}=6.8\times10^6\,\text{M}^{-1}\cdot\text{s}^{-1}$, if the only reactive form of the XH state of the papain reaction is $RS^-$ and 2-Py–S–S–2-$PyH^+$. By using eqn. (1) this value of $k_{+2}(\pm)$ converts into $k_{+2}(\text{obs.})=19\,\text{M}^{-1}\cdot\text{s}^{-1}$. The value of $k_{+2}$(obs.) obtained from the experimental findings (Fig. 1*c*), however, is $700\,\text{M}^{-1}\cdot\text{s}^{-1}$. By using eqn. (1) this value of $k_{+2}$(obs.) converts into $k_{+2}(\pm)=2.5\times10^8\,\text{M}^{-1}\cdot\text{s}^{-1}$.

It is unlikely that this large discrepancy between the observed reactivity of the XH state of the papain–2,2′-dipyridyl disulphide reaction and the maximum reactivity predicted from the L-cysteine–2,2′-dipyridyl disulphide reaction is due to better binding of 2-Py–S–S–2-$PyH^+$. The effect of the protonation of 2-Py–S–S–2-Py might be to decrease the dissociation constant of the enzyme–reagent complex if 2-Py–S–S–2-$PyH^+$ were hydrogen-bonded to the enzyme, but this would also decrease the electrophilicity of the reagent and the effects would tend to cancel. Also, if the enhanced reactivity found in the XH state of the papain–2,2′-dipyridyl disulphide reaction is the result of better binding in this state and the reactive form of this state is $RS^-$ and 2-Py–S–S–2-$PyH^+$, it is difficult to account for the high reactivity of the $XH_2$ state (see Fig. 1*c*) of this reaction.

Thus the view is compelled that the high reactivity of the XH state of the papain–2,2′-dipyridyl disulphide reaction results from abnormally high reactivity of the un-ionized papain thiol group with 2-Py–S–S–2-Py. This is most readily explained in terms of general base catalysis by the imidazole group of the histidine-159–asparagine-175 hydrogen-bonded pair. We reject the other obvious general base, the N atom of one of the pyridine rings of 2,2′-dipyridyl disulphide, because of the higher reactivity of the $XH_2$ state compared with the XH state in the papain–4,4′-dipyridyl disulphide reaction (see below) and the reactivity of the XH state in the papain–5,5′-dithiobis-(2-nitrobenzoic acid) reaction (see Little & Brocklehurst, 1972), and also on the basis of results found with bromelain, another thiol protease supposedly similar to papain (K. Brocklehurst, E. M. Crook & M. Kierstan, unpublished work). For the reactions of 2,2′-dipyridyl disulphide with the thiol groups of two types of bromelain that differ markedly probably in the $pK_a$ of the active-centre histidine residue, the pH–rate profiles differ in the manner predicted by this difference in $pK_a$.

The rate enhancement factor (approx. 70) on going from the XH state to the $XH_2$ state of the papain–2,2′-dipyridyl disulphide reaction [i.e. from $k_{2+}(0)\simeq 680\,\text{M}^{-1}\cdot\text{s}^{-1}$ to $k'_{+2}$ for $XH_2=5\times10^4\,\text{M}^{-1}\cdot\text{s}^{-1}$; see Fig. 1(*c*)] is the same as that observed on going from

the X state to the XH state in the L-cysteine–4,4′-dipyridyl disulphide reaction. The $XH_2$ state is interpreted as reaction of the thiol group of the cysteine-25–histidine-159–asparagine-175 system of papain with 2-Py–S–S–2-$PyH^+$. The loss of reactivity in the $XH_3$ state is considered to arise from protonation of histidine-159, which prevents it from generating nucleophilic character in the thiol group of cysteine-25. One consequence of this interpretation is that the effective protonation of histidine-159 in the enzyme–reagent adsorptive complex is described by an apparent kinetic $pK_a$ value of 3.8 (Fig. 1*c*). This value presumably describes the dissociation of histidine-159 in the adsorptive complex perturbed by the pre-equilibrium of the adsorption step. The smaller rate enhancement observed on going from the XH to the $XH_2$ state in the papain–2,2′-dipyridyl disulphide reaction compared with that observed on going from the X to the XH state in the L-cysteine–2,2′-dipyridyl disulphide reaction may be accounted for either in terms of a decrease of the electrophilicity of 2-Py–S–S–2-$PyH^+$ by binding to the enzyme or by the incursion of the cyclic mechanism in the L-cysteine reaction.

In a previous paper (Brocklehurst & Little, 1970) we tentatively suggested that the lack of high reactivity in the papain–4,4′-dipyridyl disulphide reaction at low pH implied that the thiol–imidazole interaction in papain might be generated by the binding of 2-Py–S–S–2-$PyH^+$ to the enzyme. It is important to report that a more complete analysis of the papain–4,4′-dipyridyl disulphide reaction (Fig. 1*d*) has revealed reactivity in the $XH_2$ state of this reaction, which strongly suggests that the cysteine-25–histidine-159 interaction occurs in the papain–4-Py–S–S–4-$PyH^+$ adsorptive complex as well as in the papain–2-Py–S–S–2-$PyH^+$ complex. This, together with the demonstration of abnormally high reactivity of the XH state in the papain–2,2′-dipyridyl disulphide reaction, means that these results provide no evidence of a requirement for a conformational change consequent on binding of 2-Py–S–S–2-$PyH^+$ to the enzyme to provide the postulated cysteine-25–histidine-159 interaction.

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