Evidence for a close similarity in the catalytic sites of papain and ficin in near-neutral media despite differences in acidic and alkaline media

Kinetics of the reactions of papain and ficin with chloroacetate

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1. The pH-dependences of the second-order rate constants (k) for the alkylation by chloroacetate of the active-centre thiol groups of papain (EC 3.4.22.2) and ficin (EC 3.4.22.3) were determined over a wide range of pH at 25°C at I 0.1. 2. The main feature of both pH- k profiles is a striking rate maximum at pH 6 (characterizing parameters in both cases pK_i approx. 3.5, pK_{II} approx. 8.4 and pH-independent rate constant k_{XH} 2.5–3.0 M⁻¹·s⁻¹). 3. The profile for the ficin reaction contains a plateau at high pH, with k_x 0.10 M⁻¹·s⁻¹; if an analogous plateau exists in the papain reaction, k_x is much lower, <0.02 M⁻¹·s⁻¹. 4. Both enzymes appear to contain closely similar thiolate-imidazolium interactive systems at pH 6, but differences in their behaviour in more-acidic media and in alkaline media suggest differences in interaction with the postulated carboxylate component of the putative catalytic triad.

As part of an investigation aimed at the identification of essential features of cysteine proteinase mechanism we have previously studied the reactions of the active-centre thiol groups of the supposedly analogous enzymes papain (EC 3.4.22.2) and ficin (EC 3.4.22.3) with a number of reactivity probes designed to detect kinetic consequences of dispositions and interactions of the components of the catalytic diads, imidazole-thiol, or catalytic triads, imidazole-thiol-carboxy (Brocklehurst & Malthouse, 1980, and references cited therein). The existence of the catalytic diad, imidazole-thiol, as a minimal common feature of both enzymes is well established. There exists both structural evidence for the close proximity of these groups and kinetic evidence for the presence of an interactive system in each enzyme, in which the sulphur atom possesses nucleophilic character in weakly acidic media. The presence and function of the active-centre carboxy groups is less certain. The structural evidence suggests that residue 158 in papain and the analogous residue in ficin are aspartic acid residues, and kinetic evidence supports the assignment of residue 158 in papain as aspartic acid. Thus in its reactions with the thiol-specific reactivity probes 2,2'-dipyridyl disulphide and 4-chloro-7-nitrobenzofurazan, the reactivity of papain is modulated by two groups with pK_a values close to 4 in addition to the pK_a value of 8–9. Modulation by one pK_a of 4 and

one pK_a of 8–9 is interpretable in terms of the thiol-imidazole diad. The additional pK_a close to 4 requires an additional ionizing group, the only probable candidate in papain being aspartic acid-158. With papain, inhibition of the reaction of its interactive diad with the 2,2'-dipyridyl disulphide cation (Brocklehurst & Malthouse, 1980) and acceleration of the reaction of its interactive diad with 4-chloro-7-nitrobenzofurazan (Shipton *et al.*, 1976), both consequent on a protonation associated with a pK_a value of 4, strongly suggest the possibility that thiol reactivity in papain can be influenced markedly by the state of ionization of the side chain of aspartic acid-158. The apparent inhibition of the reaction with 2,2'-dipyridyl disulphide by the acidic form of aspartic acid-158, in which the two ionizations with pK_a values close to 4 are positively co-operative, is particularly noteworthy because of the support that it appears to give to the two-state (UP/DOWN) mechanism of the papain catalytic act proposed by Angelides & Fink (1979).

The fact that analogous kinetic evidence for a second group with a pK_a close to 4 is not obtained from studies on the reactions of ficin with the same reactivity probes (Malthouse & Brocklehurst, 1976; Shipton *et al.*, 1976) must raise doubts either about a common catalytic mechanism or about the postulated UP/DOWN mechanism postulated for papain.

One of the possibilities discussed by Brocklehurst & Malthouse (1980) is that an active-centre carboxy group does exist in ficin but that its pK_a may be lower than 3, in which case it would not be detected by the particular reactivity probes discussed above.

In the present paper, kinetic study of ficin and papain with the small anionic alkylating agent, chloroacetate, whose interactions with enzyme should be restricted to the catalytic site [cf. the studies reported by Brocklehurst & Malthouse (1980) and Brocklehurst *et al.* (1981)], demonstrates close similarities between the interactive systems of papain and ficin at pH about 6, where the modulating effect of the imidazolium ion is dominant, but differences at higher pH consistent with either a lack of a carboxylate anion in ficin and the presence of such a group in papain or dispositions of thiol-imidazole diads and carboxylate anions that are different in the two active centres at some pH values.

Materials and methods

Materials

Spray-dried latex of *Carica papaya* was supplied by Powell and Scholefield (Liverpool, U.K.) and the dried latex of *Ficus glabrata* was obtained from Hughes and Hughes (Romford, Essex, U.K.). Chloroacetic acid was the AnalaR product of BDH Chemicals), and α -N-benzoyl-DL-arginine *p*-nitroanilide hydrochloride was obtained from Sigma Chemical Co. The buffers used in kinetic studies were as previously described (Stuchbury *et al.*, 1975).

Isolation of the enzymes

Papain contaminated only by its oxidation products was isolated as described previously (Baines & Brocklehurst, 1979). Covalent chromatography was performed as a batchwise process (Stuchbury *et al.*, 1975).

Ficin was isolated by essentially the method described by Malthouse & Brocklehurst (1976).

Kinetics of the reactions of papain and ficin with chloroacetate

These reactions were allowed to proceed at 25°C at 1.0 l in 3 ml reaction mixtures, buffered at various pH values between 2.7 and 11.0, containing 10–20 μ M enzyme and 3.5 mM-chloroacetate. After various time intervals 0.2 ml samples were removed and assayed for catalytic activity towards 0.91 mM- α -N-benzoylarginine *p*-nitroanilide at 25°C in 0.1 M-Tris/HCl buffer, pH 7.3, by recording the linear increase in A_{412} ($\Delta\epsilon_{412} = 13\,500\text{ M}^{-1}\cdot\text{cm}^{-1}$) on the 0.02 or 0.05 range of a Cary 118C spectrophotometer (chart speed 20–40 s/cm). The stock solution of the substrate was prepared by dissolving

43.5 mg of solid substrate in 1 ml of dimethyl sulphoxide and making up the volume to 100 ml with the Tris/HCl buffer. The decrease in the initial rate of catalysis consequent on alkylation of the essential thiol group obeyed first-order kinetics. Activity loss was monitored essentially to completion (zero activity) in the pH range 3–9, and for at least 50% of the alkylation reaction at pH values <3 and >9, where reaction is slower. Where necessary the rates of activity loss were corrected for pH-induced denaturation. Activity loss in the absence of chloroacetate was negligible at most pH values; at pH 11.0 the rate of denaturation of ficin (7 μ M) is about 20% of the rate of activity loss in the presence of 3.5 mM-chloroacetate. At pH 11.0 the rates of denaturation of papain and ficin are closely similar (approx. $7 \times 10^{-5}\text{ s}^{-1}$), but the rate of denaturation of papain is comparable with the rate of alkylation by 3.5 mM-chloroacetate because the latter is much lower than the rate of alkylation of ficin. Division of the first-order rate constant for activity loss by the concentration of chloroacetate provided the second-order rate constant for the alkylation reaction.

The characterizing parameters of the pH-*k* profile were determined by an optimization procedure (Shipton & Brocklehurst, 1978).

Results and discussion

The existence of nucleophilic character in two ionization states of the papain active centre is well established (see Brocklehurst *et al.*, 1981). When an electrophilic probe reagent is not specifically activated at low pH, reactions of the papain thiol group with such reagents are characterized by pH-rate profiles that either are of double sigmoid form, the rate increasing with increasing pH, or contain a bell-shaped component, in weakly acidic media, sometimes with a clearly detectable plateau at high pH (see, e.g., Chaiken & Smith, 1969*a,b*; Polgar, 1973; Halasz & Polgar, 1976; Shipton & Brocklehurst, 1977). Reaction in the high-pH plateau (the X state) is interpreted as attack by the uncomplicated thiolate anion, and reaction in the other reactive state in weakly acidic media (the XH state) as attack by the thiolate anion of the interactive thiol-imidazole/thiolate-imidazolium system. A number of authors (e.g. Wallenfels & Eisele, 1968; Chaiken & Smith, 1969*b*; Jolley & Yankeelov, 1972) have reported the existence of abnormally high XH-state reactivities leading to striking bell-shaped components in pH-rate profiles in reactions of the papain thiol group with anionic alkylating agents such as chloroacetate, and it has been generally considered that these result from interaction of the carboxylate anion of the reagent with the imidazolium ion of the interactive system of papain (see Polgar & Halasz, 1978). The small anionic reagent chloroacetate

Fig. 1. Logarithmic plot showing the dependence on pH of the second-order rate constants for the reactions of chloroacetate with papain and ficin at 25°C and 1.0 l.

The points are experimental: ●, ficin reaction; ■, papain reaction. The straight lines are of slope +1, 0 and -1 respectively. The parameters $pK_I = 3.5$, $pK_{II} = 8.4$ and $k_{XH} = 2.6 \text{ M}^{-1} \cdot \text{s}^{-1}$ were obtained by optimization of the data for the ficin reaction as described by Shipton & Brocklehurst (1978); the value of $k_X = 0.1$ for the ficin reaction was obtained by inspection; for the papain reaction the value of k at pH 11.0 is $0.02 \text{ M}^{-1} \cdot \text{s}^{-1}$, and k_X for this reaction is thus less than this value.

therefore seemed a suitable reactivity probe with which to compare both the catalytically competent interactive systems of papain and ficin, which predominate at pH values near to 6, and the active centres at high pH, where the histidine side chain is no longer cationic, without possible complications from interaction beyond the catalytic site in the binding regions of the active centre.

The pH-dependences of the second-order rate constants (k) for the reactions of chloroacetate with the active-centre thiol groups of papain and ficin at 25°C at 1.0 l are shown in Fig. 1.

The parameters that characterize the reactivity of the XH state of the ficin reaction ($k_{XH} = 2.6 \text{ M}^{-1} \cdot \text{s}^{-1}$) and its formation from the unreactive XH_2 state that contains no thiolate anion ($pK_I = 3.5$) are in good agreement with the corresponding parameters reported by Polgar & Halasz (1978) ($pK_I = 3.61$, $k_{XH} = 2.4 \text{ M}^{-1} \cdot \text{s}^{-1}$) for the papain

reaction, which they studied in the pH range 3–5.5. The limited values collected for the papain reaction at pH values 4–7.5 in the present study (Fig. 1) are in reasonable agreement with the data of Polgar & Halasz (1978). The value of pK_{II} of Fig. 1 (8.4) for both papain and ficin reactions is in the region expected for pK_{II} of the active centres of these enzymes (see, e.g., Hollaway *et al.*, 1964; Brocklehurst *et al.*, 1981). The X-state reactivities of both papain (Chaiken & Smith, 1969*b*) and ficin (Hollaway *et al.*, 1964) towards the neutral alkylating agent chloroacetamide are about 50 times higher than the reactivities of simple low-molecular-weight thiolate anions (for typical values see Polgar & Halasz, 1978). For the reaction with the chloroacetate anion, however, the X-state reactivity of papain ($<0.02 \text{ M}^{-1} \cdot \text{s}^{-1}$) is about the same as or less than the reactivity of a low-molecular-weight thiolate anion (approx. 0.01 – $0.02 \text{ M}^{-1} \cdot \text{s}^{-1}$), whereas that of ficin ($0.10 \text{ M}^{-1} \cdot \text{s}^{-1}$) is about 5–10 times higher than the reactivity of a typical low-molecular-weight thiolate anion. Clearly, the advantage afforded to the reactions of papain with neutral alkylating agents is offset by an adverse interaction in the reaction of chloroacetate with papain at high pH, and this effect is not exhibited to the same degree in the corresponding ficin reaction. The lower relative reactivity of papain towards chloroacetate suggests an additional inhibitory factor, and this could be electrostatic repulsion of chloroacetate by the carboxylate anion of aspartic acid-158 of papain if this repulsion does not have a close analogue in the ficin reaction. This would support the conclusion inferred from reactivity-probe studies at low pH (see Brocklehurst & Malthouse, 1980) that either ficin lacks a carboxylate anion in its active centre or the influence of such a group in ficin on reaction at the thiolate ion is different from that in papain. The close similarity in the XH-state reactivities of papain and ficin towards chloroacetate suggests the presence in each enzyme of a similar interactive S^-/ImH^+ system. The lack of evidence for modulation of the reactivity of this diad in ficin by a carboxy group demands further study of those properties of papain that seem to suggest a two-state mechanism.

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