the purification of the bl protein described here should facilitate the investigation of its involvement in the resistance mechanism as well as elucidation of the nature of the bonds joining the bl monomers together in the bl and b3 proteins.

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Intramolecular Inhibition by Enzyme of Site-Specific Modification
Reactions can Mask pKₐ Values Characteristic of the Reaction Pathway:
Do the Side Chains of Aspartic Acid-158 and Lysine-156 of Papain Form an Ion-Pair?

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We have studied the reactions of papain (EC 3.4.22.2) and ficin (EC 3.4.22.3) with the thiol-specific two-protonic-state reactivity probe 2,2'-dipyridyl disulphide (2-Py-S-S-2-PyH⁺, 2-Py-S-S-2-Py⁺H⁺) (Brocklehurst, 1974; Malthouse & Brocklehurst, 1976; M. Shipton & K. Brocklehurst, unpublished work). Because the bell-shaped component of the pH-rate profile of the papain reaction in acidic media does not reflect the pKₐ value of 2-Py-S-S-2-PyH⁺ (2.45) but rather two pKₐ values close to 4, we described the pH-dependence of the apparent second-order rate constant (k) of the reaction (eqn. 1) in terms of Scheme 1(a).

\[ k = \frac{\bar{k}_{\text{NH}_2}}{1 + \frac{[H^+]}{K_{\text{II}}} + \frac{[H^+]^2}{K_{\text{III}}} + \frac{[H^+]^3}{K_{\text{IV}}}} + \frac{\bar{k}_X}{1 + \frac{[H^+]}{K_{\text{II}}} + \frac{[H^+]^2}{K_{\text{III}}} + \frac{[H^+]^3}{K_{\text{IV}}}} \]  

At 25°C, pH 1.0, the values of the parameters of eqn. (1) are: \( \bar{k}_{\text{NH}_2} = 4.2 \times 10^4 \text{M}^{-1} \cdot \text{s}^{-1} \), \( \bar{k}_X = 1.7 \times 10^3 \text{M}^{-1} \cdot \text{s}^{-1} \), \( pK_{\text{II}} = 3.85 \), \( pK_{\text{III}} = 3.9 \) and \( pK_{\text{IV}} = 8.8 \). In terms of Scheme 1(a), the increase in \( k \) in acidic media was attributed to assistance to the reaction of neutral 2-Py-S-S-2-Py provided by the un-ionized carboxyl group of aspartic acid-158, as envisaged also for the reaction of papain with 4-chloro-7-nitrobenzofurazan (Allen & Lowe, 1973, Shipton et al., 1976).

The pH-dependence of \( k \) for the reaction of ficin with 2-Py-S-S-2-Py, however, does appear to reflect the pKₐ value of 2-Py-S-S-2-PyH⁺, and the bell-shaped component of the pH–k profile was described by eqn. (2) and Scheme 1(b).

\[ k = \frac{\bar{k}_{\text{NH}_2}}{1 + \frac{[H^+]}{K_{\text{II}}} + \frac{[H^+]^2}{K_{\text{III}}} + \frac{[H^+]^3}{K_{\text{IV}}}} + \frac{K_e}{1 + K_e + K_r} \]  

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Scheme 1. Kinetic models for the reactions of an enzyme, \( E-EH_3 \), with a two-protonic-reactivity probe, \( R+RH \)

\( K_e, K_{el}-K_{elIII} \) and \( K_r \) are molecular acid dissociation constants and \( k_X-k_{XH_2} \) are pH-independent rate constants. In using Scheme I(c) to describe reactions of papain and ficin with 2-Py–S–S–2-Py, the reactive components of the states characterized by \( k_{XH_2} \) and \( k_{XH} \) are considered to contain an undissociated carboxyl group and those of the states characterized by \( k_{XH_2}^{*} \) and \( k_{XH}^{*} \) a carboxylate anion. Free protons and formal charges of the various ionization states are omitted for clarity.
At 25°C, pH 0.1 the values of the parameters of eqn. (2) are: $pK_r = 2.42$, $pK_e = 3.82$ and $\bar{k}_{XH} = 2.9 \times 10^6$ M$^{-1}$s$^{-1}$. The value of $\bar{k}_{XH}$ is thus 100 times the value of $\bar{k}_{XH}$ for the papain reaction, despite the similarity in the values of the reactivities of the other protonic states of the two reactions (for the ficin reaction $\bar{k}_{XH} = 400$ M$^{-1}$s$^{-1}$, $\bar{k}_X = 2.2 \times 10^3$ M$^{-1}$s$^{-1}$ and $pK_{eII} = 8.61$).

In view of the close similarity of papain and ficin in many respects (Glazer & Smith, 1971), it seems reasonable to ask why the 2-Py-S-S-2-PyH$^+$ cation reacts rapidly with the ficin thiol group [$k_{XH}$ (Scheme 1b) = $2.9 \times 10^6$ M$^{-1}$s$^{-1}$], but appears not to do so with the papain thiol group. We have evaluated the reactions, therefore, by using the more general kinetic model of Scheme 1(c), where account is taken of reaction of both 2-Py-S-S-2-Py (R) and 2-Py-S-S-2-PyH$^+$ (RH) with enzyme forms in which a neighbouring carboxyl group is (a) ionized and (b) un-ionized.

In terms of Scheme 1(c) the pH-dependence of $k$ (when $K_r \gg K_e$; $pK_r = 2.45$, $pK_{eII} = 3.8$–3.9) is given by eqn. (3).

$$k = \frac{\bar{k}_{XH} \cdot K_e}{K_r} \left(1 + \left[\frac{[H^+]}{[H^+] + K_e + K_e \cdot K_{eII}}\right] \right) + \frac{\bar{k}_{XH} \cdot K_{eII} \cdot K_r}{K_e} \left(1 + \left[\frac{[H^+]}{[H^+] + K_{eII} + K_e \cdot K_{eII}}\right] \right)$$

Profiles of $k$ versus pH for the reaction of 2-Py-S-S-2-Py with both papain and ficin have been plotted by using $pK_r = 2.45$, $pK_{eII} = 3.85$, $\bar{k}_{XH} = 850$ M$^{-1}$s$^{-1}$ for the papain reaction and $\bar{k}_{XH} = 400$ M$^{-1}$s$^{-1}$ for the ficin reaction, and various values of the other parameters of eqn. (3). For the ficin reaction, a good fit to the experimental data is obtained by using $k_{XH} = 400$ M$^{-1}$s$^{-1}$ (or $k_{XH} = 0$) and $k_{XH} = k_{XH}^* = 2.2 \times 10^6$ M$^{-1}$s$^{-1}$. With these values, the first term of eqn. (3) is dominant at pH values less than 3.8, and conventional analysis of the plotted bell provides $pK_r \approx 2.4$, $pK_{eII} \approx 3.8$ and $\bar{k} \approx 1 \times 10^5$ M$^{-1}$s$^{-1}$. For the papain reaction, a good fit to the experimental data (i.e. for the bell, $pK_r = pK_{eII} = 3.85$, and $\bar{k} \approx 4 \times 10^4$ M$^{-1}$s$^{-1}$) is obtained by using $k_{XH}^* \approx 1 \times 10^6$ M$^{-1}$s$^{-1}$ (i.e. only about a factor of two lower than the value for the ficin reaction), provided that the second term of the eqn. (3) is allowed to dominate the profile (e.g. $k_{XH} \approx 1 \times 10^3$ M$^{-1}$s$^{-1}$, $k_{XH}^* = 1 \times 10^3$ M$^{-1}$s$^{-1}$). Thus the data can accommodate the postulate that the reactivities of papain and ficin towards the 2-Py-S-S-2-PyH$^+$ are, after all rather similar as long as it is assumed that protonation of the carboxylate ion of aspartate-158 of papain greatly inhibits the reaction, whereas no such inhibition occurs in the ficin reaction. One possible mechanism for the specific inhibition of the papain reaction is suggested by a known difference in primary structure. Residue 156 of papain is lysine, whereas the analogous residue in ficin is serine (Husain & Lowe, 1968, 1970). The cationic side chain of lysine-156 could lower the reactivity of the active-centre thiol towards the cationic-probe molecule and the inhibition might be alleviated by interaction of this side chain with the anionic form of the side chain of aspartic acid-158.

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