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Chemical evidence for the pH-dependent control of ion-pair geometry in cathepsin B

Benzofuroxan as a reactivity probe sensitive to differences in the mutual disposition of the thiolate and imidazolium components of cysteine proteinase catalytic sites

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1. Benzofuroxan reacts with the catalytic-site thiol group of cathepsin B (EC 3.4.22.1) to produce stoichiometric amount of the chromophoric reduction product, *o*-benzoquinone dioxime. In a study of the pH-dependence of the kinetics of this reaction, most data were collected for the bovine spleen enzyme, but the more limited data collected for the rat liver enzyme were closely similar both in the magnitude of the values of the second-order rate constants (k) and in the shape of the pH–k profile. 2. In acidic and weakly alkaline media, the reaction is faster than the reactions of benzofuroxan with some other cysteine proteinases. For example, in the pH region around 5–6, the reaction of cathepsin B is about 10 times faster than that of papain, 15 times faster than that of stem bromelain and 6 times faster than that of ficin. 3. The pH-dependence of k for the reaction of cathepsin B with benzofuroxan was determined in the pH range 2.7–8.3. In marked contrast with the analogous reactions of papain, ficin and stem bromelain [reported by Shipton & Brocklehurst (1977) Biochem. J. **167**, 799–810], the pH–k profile for the cathepsin B reaction contains a sigmoidal component with pK_a 5.2 in which k increases with decrease in pH. This modulation of the reactivity of the catalytic-site $-S^-/-ImH^+$ ion-pair state of cathepsin B (produced by protonic dissociation from $-SH/-ImH^+$ with pK_a approx. 3) towards a small, rigid, electrophilic reagent, in a reaction that appears to involve both components of the ion-pair for efficient reaction, suggests that the state of ionization of a group associated with a molecular pK_a of approx. 5 may control ion-pair geometry. This might account for the remarkable finding [reported by Willenbrock & Brocklehurst (1984) Biochem. J. **222**, 805–814] that, although the ion-pair appears to be generated in cathepsin B as the pH is increased across pK_a 3.4, catalytic competence is not generated until the pH is increased across pK_a 5–6.

INTRODUCTION

The cysteine proteinases constitute the group of endopeptidases whose members rely for catalytic activity on the presence of a thiol group of a cysteine residue in the enzyme molecule. The group includes papain (EC 3.4.22.2) and actinidin (EC 3.4.22.14), for which three-dimensional structures deduced from X-ray-diffraction data at high resolution (0.165 nm and 0.17 nm respectively) and complete amino acid sequences are available, calotropin DI (from the Indian madar plant, *Calotropis gigantea*), for which X-ray-diffraction data at 0.32 nm resolution are available but as yet no amino acid sequence, and cathepsin B (EC 3.4.22.1), cathepsin H and stem bromelain (EC 3.4.22.4), for which complete or almost complete amino acid sequences are available (see Kamphuis *et al.*, 1985). The X-ray-diffraction data show that for papain, actinidin and calotropin DI each enzyme consists of a single polypeptide chain folded to form two domains with a deep cleft between them, containing on one wall the catalytic-site cysteine residue, and on the other the catalytic-site histidine residue. The alignment and detailed comparison of the amino acid sequences of papain, actinidin, stem bromelain, cathepsin B and cathepsin H, made by Kamphuis *et al.* (1985), suggest that these five cysteine proteinases would be predicted to have a common folding pattern and rather similar three-dimensional structures. The accumulated data from structural and mechanistic studies on these enzymes suggest a catalytic mechanism with common basic features, but with variations for some of the enzymes contributed by effects from the electric field in the catalytic-site region, the consequences of protonic dissociation remote from the catalytic site, and the coupling of enzyme–substrate binding interactions with catalytic-site chemistry (Brocklehurst, 1986*a*,*b*). A central common feature of cysteine proteinase mechanism is the formation of a nucleophilic interaction system, involving the side chains of the catalytic-site cysteine and histidine residues, by protonic dissociation with pK_a 3–4. Such interactive systems are readily detected and characterized by using disulphides containing the 2-mercaptopyridine leaving group as two-protonic-state reactivity probes (Brocklehurst, 1982), and a plausible component of these systems is the $-S^-/-ImH^+$ ion-pair. Acylation of the thiolate anion component of the ion-pair by the carbonyl portion of a substrate, assisted by general acid catalysis provided by the imidazolium ion component, is generally

Abbreviations used: Z-, benzyloxycarbonyl-; -NNap, 2-naphthylamide.

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considered to provide a basis for the mechanism of the catalytic act.

Previous work in this laboratory (Willenbrock & Brocklehurst, 1984, 1985*a*,*b*) suggests that cathepsin B may possess characteristics that make it particularly suitable for studies designed to reveal additional details of the mechanism of the acylation process. Evidence from kinetics of leupeptin binding (Baici & Gyger-Marazzi, 1982) and from nucleophile competition studies (Bajkowski & Frankfater, 1983*a*,*b*) had provided evidence for the expected acyl-enzyme mechanism, possibly involving tetrahedral intermediates. The particular value of cathepsin B for mechanistic study is that formation of $-S^-/-ImH^+$ ion-pair is quite distinct from another pH-dependent process that is required to endow cathepsin B with catalytic competence towards the naphthylamide substrate, Z-Arg-Arg-NNap. Two-protonic-state reactivity-probe kinetics, with 2,2′-dipyridyl disulphide, show that nucleophilic character is generated in the sulphur atom of cathepsin B by protonic dissociation with pK_a 3.4, presumably to form the $-S^-/-ImH^+$ ion-pair (Willenbrock & Brocklehurst, 1984), as is the case (pK_a 3–4) for many other cysteine proteinases. Substrate-catalysis kinetics, however, show that ion-pair formation is not a sufficient condition for catalytic competence in cathepsin B, because catalytic activity is not generated as the pH is raised across pK_a 3.4, but rather as it is raised across pK_a 5–6 (5.1 for $k_{cat.}$ and 5.6 for $k_{cat.}/K_m$ for the bovine spleen enzyme and 5.8 for $k_{cat.}/K_m$ for the rat liver enzyme; Willenbrock & Brocklehurst, 1984, 1985*a*,*b*). One possible explanation of the need for additional protonic dissociation following ion-pair formation is that the particular geometry needed for reaction of both components of the ion-pair with the scissile bond of the substrate may be controlled by the state of ionization of the additional group of pK_a 5–6. By contrast, the less demanding role for $-ImH^+$ in merely maintaining $-S^-$ at low pH appears to be all that is required for rapid reaction with the 2,2′-dipyridyl disulphide monocation at low pH.

The separation of these pH-dependent events in cathepsin B contrasts markedly with the well-known situation in papain, where increase in $k_{cat.}/K_m$ as the pH is increased up to pH 6 appears to be essentially synchronous with generation of the $-S^-/-ImH^+$ ion-pair, pK_a 3–4. The nature of the further modulation of these events in papain by another, positively co-operative, protonic dissociation with pK_a 3–4 (Brocklehurst & Little, 1972; Sluyterman & Wijdenes, 1973; Shipton & Brocklehurst, 1978; Lewis *et al.*, 1978; Brocklehurst & Malthouse, 1980) has never been established, although it has usually been attributed to a field effect contributed by the carboxy group of Asp-158.

In an attempt to define in chemical terms the nature of the activation process detected, by steady-state kinetic study of the catalytic act, as an obligatory protonic dissociation with pK_a 5–6, we studied the reaction of cathepsin B with benzofuroxan, and the results of that study are presented and discussed in the present paper. Following the introduction of this reagent as a convenient chromophoric oxidizing agent for thiol groups in enzymes and other proteins (Shipton *et al.*, 1977), it was shown to exhibit considerable selectivity for $-S^-/-ImH^+$ ion-pairs as against uncomplicated thiolate anions, not associated with a cationic acid partner (Shipton & Brocklehurst, 1977). This selectivity might be expected to require a rather precise geometric arrangement of the $-S^-$ and $-ImH^+$ components to permit rate-determining attack of $-S^-$ on benzofuroxan assisted by association of $-ImH^+$ with the *N*-oxide oxygen atom, and evidence that this may be so has been reported (Salih & Brocklehurst, 1983).

We here report that the reaction of the ion-pair of cathepsin B with benzofuroxan is modulated markedly by the state of ionization of a group associated with a pK_a of 5.2. This modulation, which is not found for the corresponding reaction of papain, provides evidence that the pH-dependent activation of cathepsin B may indeed involve a change in the geometry of the catalytic-site ion-pair.

MATERIALS AND METHODS

Materials

Cathepsin B was isolated both from bovine spleen and from rat liver as described by Willenbrock & Brocklehurst (1984). The synthesis and the characterization of benzofuroxan, and the buffers used in the kinetic study, were described by Shipton *et al.* (1977).

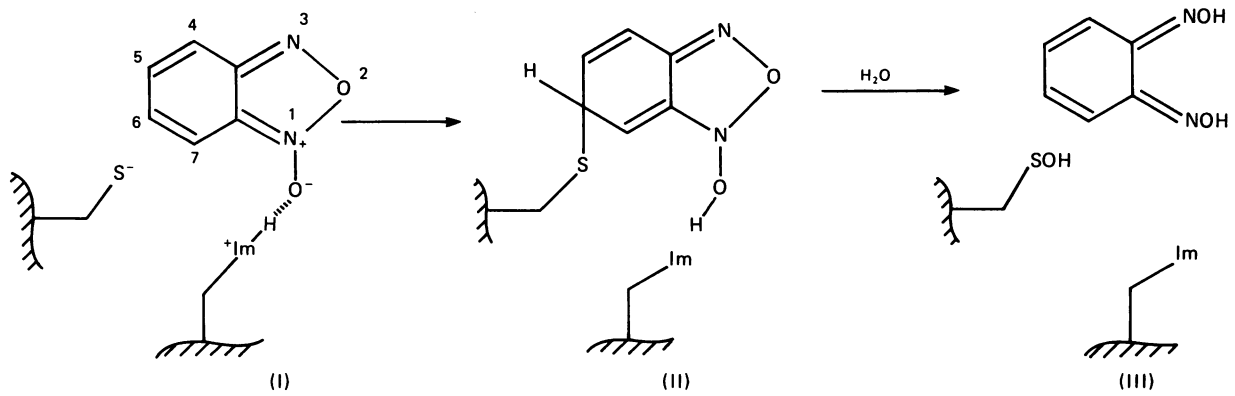
Kinetics

Complete progress curves were recorded for the reaction of cathepsin B with benzofuroxan at 25 °C at *I* 0.1 in aqueous buffers containing 6.7% (v/v) ethanol by monitoring the increase in absorbance at 416 nm, the isosbestic point in the pH-dependent spectrum of the product, *o*-benzoquinone dioxime, with a Cary 118C spectophotometer. Pseudo-first-order conditions were maintiained by using benzofuroxan in several-hundred-fold excess over cathepsin B (see the Results and discussion section). The spectral changes were quantified by using $\epsilon_{416} = 5110\ M^{-1}\cdot cm^{-1}$ (Shipton *et al.*, 1977). At pH values below 3.0 the amplitude of the absorbance change for the reaction decreased progressively with decrease in pH. This phenomenon has been observed in reactions of a number of cysteine proteinases with various reactivity probes, and is reasonably ascribed to denaturation leading to loss or substantial decrease in nucleophilic reactivity by perturbation of the geometry of the catalytic site. Such a situation may be described by two first-order processes, each producing a different product (see Frost & Pearson, 1961), i.e. pseudo-first-order reaction of the enzyme with benzofuroxan producing absorbance at 416 nm and a first-order denaturation process resulting in no increase in A_{416}. Reactions with diminished amplitudes of ΔA_{416} still obeyed good first-order kinetics, but the observed (pseudo-)first-order rate constant, $k_{obs.}$, is now the sum of the pseudo-first-order rate constant for *o*-benzoquinone dioxime production, k_r, and the first-order rate constant for the denaturation process. The value of k_r may be calculated from $k_r = k_{obs.}\cdot\Delta A'/\Delta A$, where $\Delta A'$ is the diminished amplitude of the absorbance change for the reaction at a given pH below 3.0, and ΔA is the corresponding absorbance change observed at higher pH values, where no denaturation occurs.

Other aspects of data analysis were performed as described by Salih & Brocklehurst (1983).

RESULTS AND DISCUSSION

The finding that a naphthylamide substrate fails to acylate the nucleophilic $-S^-/-ImH^+ \leftrightharpoons -SH/-Im$ cata-



Scheme 1. Reaction of an ion-pair form of a cysteine proteinase with benzofuroxan

The reaction is shown as involving nucleophilic attack by $-S^-$ at C-6 of benzofuroxan together with association and proton transfer between $-ImH^+$ and the *N*-oxide oxygen atom (I); C-6 is only one of the possible electrophilic centres in benzofuroxan (Shipton & Brocklehurst, 1977), but the gross geometry of cysteine proteinase catalytic sites inferred from structural studies on several of these enzymes (see the Introduction) suggests that simultaneous attack at this position and interaction of $-ImH^+$ with $-N^+-O^-$ should be possible. Following the formation of the adduct (II), attack by water would provide the observed products (III), i.e. inactive enzyme (the sulphenic acid shown probably undergoes further oxidation to sulphinic acid $-SO_2H$) and *o*-benzoquinone dioxime (see Shipton *et al.*, 1977).

lytic-site system of cathepsin B (produced from SH/ImH^+ across pK_a 3.4) unless additional protonic dissociation with pK_a 5–6 has occurred (Willenbrock & Brocklehurst, 1984, 1985*a*,*b*) suggests as one possibility that the latter process may control ion-pair geometry in this enzyme. We considered that evidence in support of this suggestion might be obtained from kinetic study of the reaction of cathepsin B with benzofuroxan, an electrophilic reagent of rigid structure, in which both $-S^-$ and $-ImH^+$ of the enzyme should be required simultaneously for efficient reaction (see Scheme 1). Evidence that benzofuroxan exerts selectivity for the protonic states of papain, ficin and stem bromelain that would be expected to contain $-S^-/-ImH^+$ ion-pair, as against those that contain uncomplicated $-S^-/-Im$ thiolate anion, is provided by the pH-dependence of the second-order rate constant (k) for the reaction (Fig. 1*a*). Thus the protonic state in the bell-shaped component with pK_I 3.6 and pK_{II} 8–9 is of higher reactivity than that in the plateau region approached at high pH across the pK_a of 8–9 during conversion of $-SH/-Im \rightleftharpoons -S^-/-ImH^+$ into $-S^-/-Im$. This shape of the pH–k profile, which derives from the combination of a bell-shaped component, with k optimal at pH approx. 6, and a sigmoidal component, with k increasing with increasing pH, is characteristic also of reactions of several cysteine proteinases with certain haloalkanoates (see, e.g., Wallenfels & Eisele, 1968; Chaiken & Smith, 1969*b*; Brocklehurst *et al.*, 1982). It contrasts with that of pH–k profiles for the reactions of cysteine proteinases with the corresponding haloalkanamides, which are of double sigmoidal form, k increasing with increasing pH (see, e.g., Chaiken & Smith, 1969*a*; Polgár, 1973; Halász & Polgár, 1976). The possibility for electrostatic interaction between catalytic site and the anionic alkylating agents is considered to produce the greater reactivity of the ion-pair state of the enzyme relative to that of the $-S^-/-Im$ state.

The reactions of cathepsin B with benzofuroxan were studied under pseudo-first-order conditions, with enzyme concentration approx. 1 μM and benzofuroxan concentration several-hundred-fold higher. The reactions obeyed good first-order kinetics with respect to time and, at pH 5, the first-order rate constant increased linearly with increase in benzofuroxan concentration up to 1 mM. Thus under these conditions the reaction is also first-order with respect to concentration in benzofuroxen, and study of the pH-dependence of k ($16\ M^{-1}\cdot s^{-1}$ at pH 5) provides macroscopic pK_a values characteristic of the free enzyme molecule (Brocklehurst & Dixon, 1977; Brocklehurst, 1979).

The pH-dependence of k for the reaction of benzofuroxan with cathepsin B (Fig. 1*b*) contrasts markedly with those for the corresponding reactions of papain and stem bromelain (Fig. 1*a*) in that a kinetically important pK_a of approx. 5 is revealed for the cathepsin B reaction. Most of the data were collected for reaction of the bovine spleen enzyme, but the more limited data obtained with the rat liver enzyme, for which an amino acid sequence is available (Takio *et al.*, 1983), suggests similar reactivity and pH-dependence characteristics.

The benzofuroxan reactivity probe shows that for papain, ficin, stem bromelain (Fig. 1*a*) and cathepsin B (Fig. 1*b*) nucleophilic character is generated by protonic dissociation with pK_a 3–4, which is interpreted as the formation of a protonic state containing the $-S^-/-ImH^+$ ion-pair (see Scheme 1). For the first three enzymes the reactivity of $-S^-/-ImH^+$ is expressed optimally at pH values around 5–7 and falls to a lower value as the pH is raised across pK_a 8–9. This decrease in reactivity is interpreted as loss of the assistance to the reaction of $-S^-$ and $-ImH^+$ becomes $-Im$. For the cathepsin B reaction, the value of pK_I (3.1) is in good agreement with the pK_a value for $-S^-/-ImH^+$ ion-pair formation (3.3) deduced from studies with 2,2′-dipyridyl disulphide (Willenbrock & Brocklehurst, 1984). The particularly striking feature of Fig. 1(*b*) is that the reactivity of the $-S^-/-ImH^+$ ion-pair of cathepsin B is clearly modulated by the effect of protonic dissociation of pK_a 5.2 before the reactivity decreases again as the pH is increased across pK_a approx. 8.5, presumably as $-S^-/-Im$ is produced.

The pK_a value (5.2) characteristic of the modulation of $-S^-/-ImH^+$ reactivity towards benzofuroxan is

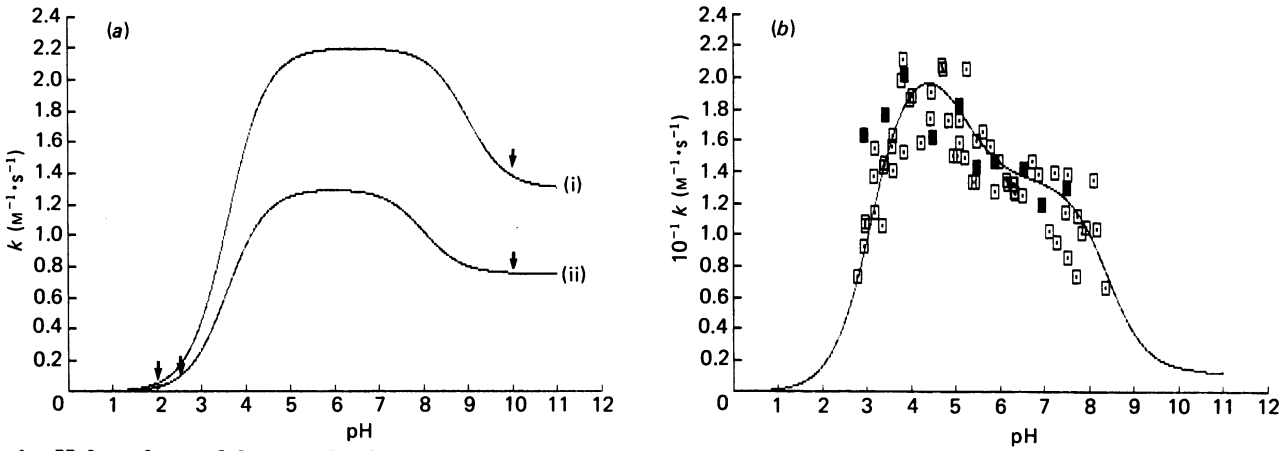


Fig. 1. pH-dependence of the second-order rate constant for reactions of benzofuroxan with (*a*) (i) papain, (*a*) (ii) stem bromelain and (*b*) cathepsin B from bovine spleen (□) and from rat liver (■) at 25 °C at *I* 0.1 in aqueous buffers containing 6.7% (v/v) ethanol

(*a*) Theoretical curves, which gave good fits to the experimental data reported by Shipton & Brocklehurst (1977); the arrows indicate the pH ranges over which the data were collected. The curves were computed by using a BBC microcomputer and:

$$k = \tilde{k}_1/[1+(10^{-pH})(10^{pK_I})+(10^{-pK_{II}})(10^{pH})]+\tilde{k}_2/[1+(10^{-pH})(10^{pK_{II}})]$$

where, for the reaction of papain, $k_1 = 2.2\ M^{-1}\cdot s^{-1}$, $k_2 = 1.3\ M^{-1}\cdot s^{-1}$, $pK_I = 3.6$ and $pK_{II} = 9.0$, and for the reaction of stem bromelain $k_1 = 1.3\ M^{-1}\cdot s^{-1}$, $k_2 = 0.75\ M^{-1}\cdot s^{-1}$, $pK_I = 3.6$ and $pK_{II} = 8.0$. The pH–*k* profile for the reaction of ficin with benzofuroxan (not shown) is of similar form, with $k_1 = 3.5\ M^{-1}\cdot s^{-1}$, $k_2 = 2.1\ M^{-1}\cdot s^{-1}$, $pK_I = 3.2$ and $pK_{II} = 8.2$. (*b*) The points are experimental and the line is theoretical for:

$$k = \tilde{k}_1/[1+(10^{-pH})(10^{pK_I})+(10^{-pK_{II}})(10^{pH})]+\tilde{k}_2/[1+(10^{-pH})(10^{pK_{II}})+(10^{-pK_{III}})(10^{pH})]+\tilde{k}_3/[1+(10^{-pH})(10^{pK_{III}})]$$

where $k_1 = 21.5\ M^{-1}\cdot s^{-1}$, $k_2 = 13.5\ M^{-1}\cdot s^{-1}$, $k_3 = 1.3\ M^{-1}\cdot s^{-1}$, $pK_I = 3.1$, $pK_{II} = 5.2$ and $pK_{III} = 8.4$. The instability of cathepsin B in alkaline media prevented collection of data at pH values higher than 8.3, and the value of k_3 was set equal to the value of k_2 for the corresponding reaction of papain [*a*(i)].

reasonably close to the value (5.6) that characterizes generation of catalytic activity, reflected in $k_{cat.}/K_m$, of the bovine spleen enzyme towards Z-Arg-Arg-NNap. The variation of ion-pair reactivity of cathepsin B towards the small, rigid, electrophilic reactivity probe, benzofuroxan, across a pK_a of 5–6 supports the idea that the absolute dependence of catalytic activity on protonic dissociation across a pK_a of similar value may derive from the need to control ion-pair geometry in the catalytic act. It does not seem to be a difficulty that the ion-pair reactivity towards benzofuroxan decreases as the pH is raised across the pK_a of similar value. There is no reason to suppose that transition-state geometry for the benzoforuxan reaction is similar in important respects to that for the general acid-catalysed acylation reaction of the catalytic act. The important observation is that the reactivity of the ion-pair towards a rigid electrophilic reagent with reasonably demanding requirements for the optimal disposition of $-S^-$ and $-ImH^+$ changes across a pK_a value similar to that characteristic of protonic dissociation obligatory for catalytic competence. The identity of the group associated with the pK_a of 5–6 remains to be established. It must be somewhat remote from the immediate catalytic site, because the usual candidate for an ionizing group additional to –SH and –Im in the cysteine proteinases, the aspartic acid residue adjacent on the *N*-terminal side to the catalytic-site histidine residue, e.g. in papain and in ficin, is glycine in cathepsin B (see Takio *et al.*, 1983), and there is no obvious substitute for such a group close to the catalytic site that can be deduced from the amino acid sequence of cathepsin B, the postulated common folding pattern of the cysteine proteinases and the three-dimensional structures of papain and actinidin.

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REFERENCES

Baici, A. & Gyger-Marazzi, M. (1982) Eur. J. Biochem. **129**, 33–41

Bajkowski, A. S. & Frankfater, A. (1983*a*) J. Biol. Chem. **258**, 1645–1649

Bajkowski, A. S. & Frankfater, A. (1983*b*) J. Biol. Chem. **258**, 1650–1655

Brocklehurst, K. (1979) Biochem. J. **181**, 775–778

Brocklehurst, K. (1982) Methods Enzymol. **87C**, 427–469

Brocklehurst, K. (1986*a*) in Cysteine Proteinases and their Inhibitors (Turk, V., ed.), Walter de Gruyter, Berlin, in the press

Brocklehurst, K. (1986*b*) in Enzyme Mechanism (Page, M. I. & Williams, A., eds.), Royal Society of Chemistry, London, in the press

Brocklehurst, K. & Dixon, H. B. F. (1977) Biochem. J. **167**, 859–862

Brocklehurst, K. & Little, G. (1972) Biochem. J. **128**, 471–474

Brocklehurst, K. & Malthouse, J. P. G. (1980) Biochem. J. **191**, 707–718

Brocklehurst, K., Mushiri, S. M., Patel, G. & Willenbrock, F. (1982) Biochem. J. **201**, 101–104

Chaiken, I. M. & Smith, E. L. (1969*a*) J. Biol. Chem. **244**, 5087–5094
Chaiken, I. M. & Smith, E. L. (1969*b*) J. Biol. Chem. **244**, 5095–5099
Frost, A. A. & Pearson, R. G. (1961) Kinetics and Mechanism, pp. 160–162, John Wiley and Sons, New York
Halász, P. & Polgár, L. (1976) Eur. J. Biochem. **71**, 571–575
Kamphuis, I. G., Drenth, J. & Baker, E. N. (1985) J. Mol. Biol. **182**, 317–329
Lewis, S. D., Johnson, F. A., Ohno, A. K. & Shafer, J. A. (1978) J. Biol. Chem. **253**, 5080–5086
Polgár, L. (1973) Eur. J. Biochem. **33**, 104–109
Salih, E. & Brocklehurst, K. (1983) Biochem. J. **213**, 713–718
Shipton, M. & Brocklehurst, K. (1977) Biochem. J. **167**, 799–810
Shipton, M. & Brocklehurst, K. (1978) Biochem. J. **171**, 385–401
Shipton, M., Stuchbury, T., Brocklehurst, K., Herbert, J. A. L. & Suschitzky, H. (1977) Biochem. J. **161**, 627–637
Sluyterman, L. A. Æ. & Wijdenes, J. (1973) Biochim. Biophys. Acta **302**, 95–101
Takio, K., Towatari, T., Katunuma, N., Teller, D. C. & Titani, K. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 3666–3670
Wallenfels, K. & Eisele, B. (1968) Eur. J. Biochem. **3**, 267–275
Willenbrock, F. & Brocklehurst, K. (1984) Biochem. J. **222**, 805–814
Willenbrock, F. & Brocklehurst, K. (1985*a*) Biochem. J. **227**, 511–519
Willenbrock, F. & Brocklehurst, K. (1985*b*) Biochem. J. **227**, 521–528

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