Evidence that the lack of high catalytic activity of thiolsubtilisin towards specific substrates may be due to an inappropriately located proton-distribution system

Demonstration of highly nucleophilic character of the thiol group of thiolsubtilisin in the catalytically relevant ionization state of the active centre by use of a two-protonic-state reactivity probe

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The active centre of the semi-synthetic enzyme thiolsubtilisin was investigated by studying the kinetics of the reaction of the thiol group of cysteine-221 with the thiol-specific two-protonic-state reactivity probe 2,2'-dipyridyl disulphide. The three-states criterion [Brocklehurst (1974) *Tetrahedron* **30**, 2397–2407] was used to provide definitive evidence of the existence of a thiol–imidazole interactive system in acidic media in which the sulphur atom possesses highly nucleophilic character. The lack of catalytic competence of thiolsubtilisin despite its possession of the requisite nucleophilic capability is discussed. The exceedingly high rate of reaction of thiolsubtilisin with 2,2'-dipyridyl disulphide at pH 4–5 is shown to constitute a rapid and convenient active-site titration in which intact thiol–imidazole interaction is detected even in the presence of other thiols.

The study of analogous enzymes has much to contribute to the elucidation of enzyme mechanism (see, e.g., Garavito *et al.*, 1977; Brocklehurst *et al.*, 1980), and may be the only practicable way at present to study the effect of structural variation in active centres on catalytic competence. The possibility of converting a serine residue into a cysteine residue in peptides with retention of optical activity (Photaki & Bardakos, 1965; Zioudrou *et al.*, 1966) led to the preparation of the so-called 'semi-synthetic enzyme' thiolsubtilisin (see Tsai & Bender, 1979; Philipp *et al.*, 1979, and references cited therein), and thus provided a valuable link for comparative mechanistic study of serine proteinases and cysteine proteinases. Similar, though more limited, studies to those reported previously on thiolsubtilisin have been conducted also on thioltrypsin prepared from *Streptomyces griseus* trypsin (Yokosawa *et al.*, 1977). The inability of thiolsubtilisin to catalyse the

hydrolysis of specific non-activated esters and amides, which results mainly from an unobservably low acylation rate (Neet *et al.*, 1968; Tsai & Bender, 1979), is a striking observation. This finding is particularly noteworthy because evidence from (a) an X-ray-crystallographic study (see Lowe, 1976), (b) the reactivities of subtilisin and thiolsubtilisin towards highly specific peptide chloromethyl ketone derivatives and (c) the spectra and deacylation rates of pairs of *S*- and *O*-arylacryloyl-enzymes (Tsai & Bender, 1979) strongly suggests that the conformations both of the subtilisin molecule in general and of the active-centre region in particular remain essentially unchanged when the active-centre serine residue is converted into a cysteine residue. If the lack of catalytic competence in thiolsubtilisin cannot be attributed to site distortion, two obvious explanations remain: (i) the active-centre sulphur atom of thiolsubtilisin may not be rendered nucleophilic by active-centre interaction in acidic and neutral media, and (ii) the proton-distributing site in the active centre may not be operative.

In the present paper compelling evidence is reported that the active-centre thiol group of thiolsubtilisin possesses highly nucleophilic charac-

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ter, not only in the uncomplicated thiolate ion that predominates at high pH, but also in an interactive active-centre system that predominates in neutral and weakly acidic media, where high catalytic competence might have been expected. The inability of this highly nucleophilic sulphur atom of thiolsubtilisin to become acylated by specific ester and amide substrates is discussed.

Materials and methods

Subtilisin BPN' (type VII) from *Bacillus amyloliquefaciens* (a crystallized and freeze-dried product) and phenylmethanesulphonyl fluoride were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). The potassium salt of ethanethioic acid (thiolacetic acid) was obtained from Merck (Darmstadt, W. Germany). 2,2'-Dipyridyl disulphide was obtained from Aldrich (Gillingham, Dorset, U.K.) and purified and characterized as described previously (Brocklehurst & Little, 1973).

Thiolsubtilisin was prepared from subtilisin BPN' by the procedure described by Polgar & Bender (1967, 1969), in which the serine hydroxy group is allowed to react with phenylmethanesulphonyl fluoride, the phenylmethanesulphonyl group is displaced by reaction with thiolacetate and the resulting acetylthiolsubtilisin is allowed to deacylate spontaneously. The product obtained from 500 mg of subtilisin BPN' contained in 10 ml of solution was desalted in two 5 ml batches each by sequential passage through two Sephadex G-25 columns (25 cm $\times$ 2.5 cm), the first equilibrated and eluted with 0.1 M-KCl containing 1 mM-EDTA and the second with 10 mM-sodium phosphate buffer, pH 6.5. Protein concentration was determined spectrophotometrically by using $\epsilon_{278} = 32300 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Neet *et al.*, 1968). Thiol concentration was determined by using 2,2'-dipyridyl disulphide at pH 4, 6 and 8 as described by Brocklehurst & Little (1973), by using $\epsilon_{343} = 8080 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Stuchbury *et al.*, 1975), and thiolsubtilisin preparations always contained 0.9–1.0 mol of thiol with high reactivity at pH 4.0 per mol of protein. The same thiol content was obtained when the titration was performed at pH 8.0.

Kinetics of the reactions of thiolsubtilisin with 2,2'-dipyridyl disulphide at 25°C at I 0.1 were studied by stopped-flow spectral analysis at 343 nm, as described for the analogous reactions of ficin (EC 3.4.22.3) by Malthouse & Brocklehurst (1976) with [enzyme] 9–12 μM and [2,2'-dipyridyl disulphide] 0.1–0.8 mM in the mixing chamber. The progress curves were all first-order with respect to time in thiolsubtilisin, and the reaction is also first-order with respect to concentration in 2,2'-dipyridyl disulphide at least up to 0.8 mM.

Results and discussion

Characterization of the Cys-221–His-64 system of thiolsubtilisin

Although studies of the reaction of thiolsubtilisin with alkylating agents (see, e.g., Polgar *et al.*, 1973; Halasz & Polgar, 1977; Tsai & Bender, 1979) suggest the possibility of interaction of the thiol group with another active-centre residue, Tsai & Bender (1979) have pointed out that it cannot be concluded on the present evidence whether there is interaction of Cys-221 and His-64. The lack of a hydrogen bond between the serine hydroxy group and the histidine imidazole group of the so-called 'charge-relay systems' of the much-studied serine proteinases, including subtilisin (Matthews *et al.*, 1977; Kraut, 1977), adds to the uncertainty. Also, the Cys-221–His-64 system of thiolsubtilisin is probably somewhat similar to the Cys-25–His-159 system of the natural cysteine proteinase papain (EC 3.4.22.2) as it exists at high pH, i.e. the UP conformation (thought not identical with it; see Garavito *et al.*, 1977), and Angelides & Fink (1979a) have suggested that there may be little or no interaction of the thiol and imidazole groups of papain in this UP conformation (see below).

Studies on the natural cysteine proteinases such as papain and ficin (EC 3.4.22.3) [see Brocklehurst & Malthouse (1980), and references cited therein] and on a minimal catalytic-centre model, 2-benzimidazolylmethanethiol (see Brocklehurst *et al.*, 1979a), have established a sensitive method for the detection and characterization of multiple nucleophilic states in thiol-containing active centres (Brocklehurst, 1974, 1979a; Brocklehurst *et al.*, 1979a). The method consists of measuring the apparent (see Brocklehurst, 1979b) second-order rate constants for the reaction of the enzyme thiol group with a thiol-specific two-protonic-state electrophile (see Brocklehurst *et al.*, 1979a) such as 2,2'-dipyridyl disulphide, usually in three pH regions around pH 4, 6 and 9 respectively (see Scheme 1). Three reactive ionization states in pH regions around pH 4, 6 and >9 with $k_{\text{pH}4} > k_{\text{pH}6} < k_{\text{pH}9}$ are characteristic of the existence of two nucleophilic states of the enzyme. The nucleophilic state at $\text{pH} > 9$ contains the simple thiolate ion, with all other ionizing active-centre residues proton-deficient. The nucleophilic state that predominates at pH values around 6, whose reaction is magnified at pH values around 4 by protonation of the probe reagent to provide the highly electrophilic 2,2'-dipyridyl disulphide monocation, is the one in which the sulphur atom of the active centre is rendered nucleophilic by interaction with at least one other active-centre residue. This is the catalytically competent state in the natural cysteine proteinases, and an important component of this state may be the thiolate–

Scheme 1. Basis of the detection and characterization of the catalytically relevant nucleophilic state ($\text{ImH}^+ \text{S} = \text{Im} \cdots \text{HS}$) of a cysteine proteinase by use of 2,2'-dipyridyl sulphide as a two-protonic-state reactivity probe and the three-states criterion

Im represents the imidazole group of a histidine residue. The rate constants, $\tilde{k}_x$ – $\tilde{k}_{xH_2}$, are the four pH-independent rate constants that characterize the reaction of a typical cysteine proteinase with 2,2'-dipyridyl disulphide. Usually $\tilde{k}_x > \tilde{k}_{xH} < \tilde{k}_{xH_2}$, and $\tilde{k}_{xH_1}$ is negligible, i.e. there are three reactive protonic states and one essentially unreactive state. Such a set of rate constants provides for a striking bell-shaped curve in acidic media with a pH optimum around pH 3–4 [because $\text{p}K_r = 2.45$ (Brocklehurst & Little, 1973) and $\text{p}K_{e1}$ is often approx. 4–5] and a sigmoidal component with $\text{p}K_{e1}$ 8–10. When the thiol group is not part of an interactive system, the second nucleophilic state does not exist, and there is no bell-shaped component characterized by $\tilde{k}_{xH_2}$. Rather, the observed rate constant decreases with decrease in pH along a double-sigmoidal curve where $\tilde{k}_x > \tilde{k}_{xH}$ and $\tilde{k}_{xH_2} \approx 0$. Whereas for the thiol group of an interactive system $\tilde{k}_{xH}$ contains contributions from the reactions of both cationic and neutral forms of the probe reagent, for a 'bare' or non-interactive thiol group $\tilde{k}_{xH}$ characterizes only the reaction of the cationic reagent with low residual concentrations of simple thiolate anion. When the $\text{p}K_a$ of the thiol group is approx. 6.5, as it is in some aromatic thiols, the decrease in thiolate anion concentration in acidic media is compensated for more or less exactly by the increase in the electrophilicity of the probe reagent, and $\tilde{k}_x \approx \tilde{k}_{xH}$ (Brocklehurst *et al.*, 1980).

imidazolium ion pair (see Scheme 1). The striking increase in rate in acidic media is not observed in reactions of 2,2'-dipyridyl disulphide either with simple low-molecular-weight thiols or with the inessential, non-interactive, thiol groups of chymopapains A and B (B. S. Baines & K. Brocklehurst, unpublished work). Observation of a marked increase in rate at low pH in reactions of 2,2'-dipyridyl disulphide with enzyme active centres is characteristic of the existence in the enzyme of a nucleophilic state in approximately neutral media, provided by interaction with another acid/base system in the enzyme, additional to the uncomplicated thiolate ion that predominates at high pH.

For the reaction of the thiol group of thiolsubtilisin with 2,2'-dipyridyl disulphide, the striking rate maximum at pH values around 4 (see Table 1) provides definitive evidence that the side chain of cysteine-221 does indeed interact with another acid/base group, most probably the imidazole group of histidine-64, to provide a highly nucleophilic state that predominates in weakly acidic media, where catalytic activity might have been expected. The resemblance of the pH-dependence of k for the reaction of 2,2'-dipyridyl disulphide with thiol-

subtilisin to the analogous pH-dependences for the reactions of this reagent with the natural cysteine proteinases papain and ficin is evident from Table 1.

Now that the provision of a highly nucleophilic state of the sulphur atom of thiolsubtilisin in acidic media by active-centre interaction has been convincingly established, it is important to consider why this high nucleophilicity cannot be expressed in the successful acylation of this nucleophilic centre by specific non-activated ester and amide substrates. As Tsai & Bender (1979) have pointed out, the low basicity of the sulphur nucleophile in the natural cysteine proteinases is probably compensated for by protonation of the leaving group either before or during formation of the transition state or tetrahedral intermediate. In catalysis by the serine proteinases such as subtilisin itself the probable sequence of events involves bond formation between the serine oxygen atom and the carbonyl carbon atom of the substrate followed by protonation of the leaving group (Fastrez & Fersht, 1973; Bizzozero & Zweifel, 1975). Although some current thinking involves attack of the non-hydrogen-bonded hydroxy group of the enzyme on the bound, strained, substrate (see Kraut, 1977), versions of the base-catalysis mechanism are still being put forward (see Kossiakoff & Spencer, 1980). Because attack of the hard (Edwards & Pearson, 1962; Pearson & Songstad, 1967) nucleophile (serine oxygen) on the electrophilic centre of the substrate does not require prior or synchronous protonation of the leaving group, it is no detriment that in the serine proteinases the active-centre imidazolium ion appears to be inappropriately located (see, e.g., Bizzozero & Zweifel, 1975; James, 1980) for synchronous protonation as determined by stereoelectronic theory (Deslongchamps *et al.*, 1972, 1973, 1975). When the hard highly basic oxygen nucleophile in subtilisin is replaced by the soft weakly basic sulphur nucleophile in thiolsubtilisin, prior or synchronous protonation of a non-activated ester or amide substrate is required to permit acylation of the thiol group, and the inability of the imidazolium ion to participate in the reaction in this way probably explains the failure of thiolsubtilisin to act as a catalyst. In the case of the natural cysteine proteinase papain it has been suggested that the crystallographically determined active-centre conformation may not persist in neutral and weakly acidic media (Angelides & Fink, 1978) and that the active-centre imidazolium ion may move through rather a large distance to take up a position in which it can supply the necessary prior or synchronous protonation of substrate (Brocklehurst & Malthouse, 1978, 1980; Angelides & Fink, 1978, 1979a,b; Brocklehurst *et al.*, 1979a,b). There is no reason to suppose that this type of conformational change is required by subtilisin. If that is the case, it would not be available therefore in thiolsubtilisin,

Table 1. *Second-order rate constants (k) for the reactions of 2,2'-dipyridyl disulphide with the active-centre thiol groups of thiolsubtilisin, ficin and papain at 25°C and at 10.1*

Enzyme	pH	$10^{-4}k$ ($M^{-1} \cdot s^{-1}$)	Reference
Thiol-subtilisin	2.57	3.5	Present work
	2.93	4.9	
	3.33	8.6	
	4.00	8.8	
	5.00	8.6	
	5.90	4.4	
	6.97	3.4	
	7.78	3.5	
	9.52	10.2	
Ficin	2.10	2.8	Malthouse & Brocklehurst (1976)
	2.61	4.8	
	2.83	7.1	
	2.90	7.8	
	3.82	6.2	
	4.50	2.5	
	7.00	0.4	
	7.81	0.4	
	9.50	2.0	
Papain	2.50	0.2	Shipton & Brocklehurst (1978)
	3.11	0.4	
	3.50	1.0	
	4.00	1.3	
	4.51	0.9	
	5.82	0.2	
	6.85	0.1	

where it would be required to complete the acylation step of the catalytic act.

The disulphide reactivity probe 2,2'-dipyridyl disulphide effectively separates the two components of the active-centre chemistry that pertain to the catalytic act. The electrophilic character of the reagent, being a different type from that present in a specific substrate (soft rather than hard), coupled with the ability of the reagent to increase its electrophilicity markedly consequent on protonation on the ring nitrogen atoms, permits the revelation of the highly nucleophilic character of the active-centre sulphur atom in acidic media without requiring the proton-distributing site of the active centre (the imidazolium ion of His-64) to be operative as a proton-donor.

Active-site titration of thiolsubtilisin

Despite the lack of catalytic activity of thiolsubtilisin towards non-activated ester and amide substrates, it is still possible to study the deacylation process and some aspects of the acylation process by using activated ester substrates (see Philipp *et al.*, 1979, and references cited therein). In connection with such studies it is valuable to be able to determine the concentration of sites that contain the interactive thiol-imidazole/thiolate-imidazolium system discussed above, which is the only valid concept at present of the thiolsubtilisin 'active centre' despite its lack of full catalytic competence. The concentration of such sites in thiolsubtilisin preparations is readily determined by spectroscopic titration at 343 nm with 2,2'-dipyridyl disulphide at pH 4–5. This method was described by Brocklehurst & Little (1973) for titration of the active sites of natural cysteine proteinases, and is able to discriminate between fully active enzyme and both enzyme that contains an active-centre thiol group, but is not catalytically active because of conformational distortion that has destroyed the thiol-imidazole interactive system, and contaminant thiols of low relative molecular mass.

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