Chymopapain A

Purification and investigation by covalent chromatography and characterization by two-protonic-state reactivity-probe kinetics, steady-state kinetics and resonance Raman spectroscopy of some dithioacyl derivatives

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1. Chymopapain A was isolated from the dried latex of papaya (*Carica papaya*) by ion-exchange chromatography followed by covalent chromatography by thiol–disulphide interchange. 2. The latter procedure was used (a) to produce fully active enzyme containing one essential thiol group per molecule of protein, (b) to establish that the chymopapain A molecule contains, in addition, one non-essential thiol group per molecule and (c) to recalculate the literature value of ϵ_{280} for the enzyme as $36000 \text{ M}^{-1} \cdot \text{cm}^{-1}$. 3. The Michaelis parameters for the hydrolysis of L-benzoylarginine *p*-nitroanilide and of benzyloxycarbonyl-lysine nitrophenyl ester at 25 °C, and I 0.1 at several pH values catalysed by chymopapain A, papaya proteinase Ω , papain (EC 3.4.22.2) and actinidin (EC 3.4.22.14) were determined. Towards these substrates chymopapain A has $k_{\text{cat.}}/K_m$ values similar to those of actinidin and of papaya proteinase Ω and significantly lower than those of papain or ficin. 4. The environment of the catalytic site of chymopapain A is markedly different from those of other cysteine proteinases studied to date, as evidenced by the pH-dependence of the second-order rate constant (k) for the reaction of the catalytic-site thiol group with 2,2'-dipyridyl disulphide. The striking bell-shaped component that is a characteristic feature of the reactions of S^-/ImH^+ (thiolate/imidazolium) ion-pair components of many cysteine-proteinase catalytic sites with the 2,2'-dipyridyl disulphide univalent cation is not present in the pH– k profile for the chymopapain A reaction. The result is consistent with the presence of an additional positive charge in, or near, the catalytic site that repels the cationic form of the probe reagent. 6. Resonance Raman spectra were collected at pH values 2.5, 6.0 and 8.0 for each of the following dithioacyl derivatives of chymopapain A: *N*-benzoylglycine-, *N*-(β -phenylpropionyl)glycine- and *N*-methoxycarbonylphenylalanylglycine-. The main conclusion of the spectral study is that in each case the acyl group binds as a single population known as conformer B in which the glycinic N atom is in close contact with the thiol S atom of the catalytic-site cysteine residue, as is the case also for papain and other cysteine proteinases studied. Thus the abnormal catalytic-site environment of chymopapain A detected by the reactivity-probe studies, which may have consequences for the acylation step of the catalytic act, does not perturb the conformation of the bound acyl group at the acyl-enzyme-intermediate stage of catalysis.

INTRODUCTION

The cysteine proteinases appear to constitute a group of enzymes in which there is both extensive sequence homology and structural variation in mechanistically relevant regions of the molecule (see Willenbrock & Brocklehurst, 1984; 1985*a, b*). Such structural differences must account for the differences in active-centre characteristics noted between various members of the group (see the references given above and Brocklehurst *et al.*, 1983, 1984*a*; Salih & Brocklehurst, 1983). This type of natural structural variation in the enzyme molecule should contribute to the understanding of the molecular basis of enzyme catalysis and may suggest additional structural changes to be made by site-directed mutagenesis to test specific features of mechanistic hypotheses.

The present paper is concerned with the little-studied cysteine proteinase chymopapain A isolated from the dried latex of papaya (*Carica papaya*). In ion-exchange profiles of aqueous extracts of dried papaya latex, this enzyme is eluted after papain and before the complex band containing multiple forms of chymopapain B and is easily recognized and isolated. The early work on this enzyme was summarized by Baines & Brocklehurst (1982*a*) (see also Brocklehurst & Salih, 1983; Brocklehurst *et al.*, 1984*b*), but in view of recent discussion of the various chymopapain fractions (Buttle & Barrett, 1984; Brocklehurst *et al.*, 1985*a, b*; Barrett & Buttle, 1985), additional comment may be helpful. The designation of chymopapains as A or B was made originally by Kunimitsu & Yasunoba (1967) on the basis of basicity, chymopapain A being eluted before chymopapain B in an

Abbreviations used: f.p.l.c., fast protein liquid chromatography; SP-, sulphopropyl-; rR, resonance Raman; L-Bz-Arg-*p*Nan, L-benzoylarginine *p*-nitroanilide; Z-Lys-ONp, benzyloxycarbonyl-lysine phenyl ester; SH_e, essential thiol group; SH_n, non-essential thiol group.

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ion-exchange procedure. The A and B classification is worth preserving, even now that improvements in chromatographic techniques have revealed a multiplicity of chymopapain forms. This is because the catalytic-site characteristics of chymopapain A (the early-to-be-eluted form in dried latex) are distinctly different from those of the various forms of chymopapain B (the later-to-be-eluted forms in dried latex), and both A and B forms are present in many types of commercial dried papaya latex (Brocklehurst *et al.*, 1980, 1984b; Baines & Brocklehurst, 1982a). The reaction of the catalytic-site thiol group of a chymopapain of the B type with a two-protonic-state reactivity probe (see Brocklehurst, 1982) such as 2,2'-dipyridyl disulphide, is characterized by a plot of second-order rate constant (k) against pH that has a shape found also for the analogous reactions of many other cysteine proteinases. The main features are a striking rate optimum at pH 3-4, a shallow minimum at pH approx. 6 and a plateau of only slightly higher reactivity in alkaline media approached across a pK_a of approx. 9 (see Brocklehurst *et al.*, 1985b). It is clearly established in the present paper that the pH- k profile for the reaction of 2,2'-dipyridyl disulphide with the catalytic-site thiol group of chymopapain A, however, has a markedly different shape: the bell-shaped component of high reactivity at low pH is missing in the pH- k profile for the chymopapain A reaction. These considerable differences in pH- k profile shape and in the magnitude of values of k at some pH values provide a simple and convenient method of identifying catalytic sites as being characteristic of chymopapain A or of chymopapain B. Barrett & Buttle (1985), however, contend that our method of identifying forms of chymopapain by the use of two-protonic-state reactivity probes may not be easy to reproduce in other laboratories and cite Khan & Polgár (1983) in support of this contention. In fact, Khan & Polgár (1983, p. 335, column 1, paragraph 2, lines 1-5) wrote: "In our opinion the last three of the above criteria, i.e. the analysis of the dipyridyl disulfide reactions, may offer the simplest and most reliable methods to distinguish the different forms of chymopapain.", a view that coincides with that provided by our own experience. There is indeed complete agreement between the rather limited results of Khan & Polgár (1983) and the more extensive data obtained in this laboratory (see the Results and discussion section) on the pH-dependent reactivity characteristics of the catalytic-site thiol group of the early-to-be-eluted chymopapain towards 2,2'-dipyridyl disulphide in acidic media. These characteristics provide two of the three criteria that Khan & Polgár regard as valuable, i.e. relative reactivities of chymopapains A and B towards 2,2'-dipyridyl disulphide in acidic media and the shapes of the pH- k profiles in acidic media. A related approach to the study of these enzymes involves analysis of activity loss/thiol modification data by the method of Tsou Chen-Lu (1962) and provides the third of the criteria mentioned by Khan & Polgár. When this approach is applied to the chymopapains in alkaline media, a complex situation exists because both the catalytic-site thiol groups and the non-essential thiol groups of these enzymes react readily with 2,2'-dipyridyl disulphide, and in some cases the pH- k profiles cross. It is this type of analysis at a particular pH value in the region where pH- k profiles cross that can provide different forms of Tsou Chen-Lu (1962) plot on either side of the pH- k cross-over point (see Baines & Brocklehurst,

1982a; Khan & Polgár, 1983; Brocklehurst *et al.*, 1984b; and cf. Polgár, 1984) and may appear to suggest different conclusions unless the complete pH- k profiles have been determined and each assigned to the appropriate reaction, i.e. of the essential and non-essential thiol groups respectively. By far the clearest and simplest method of distinguishing between chymopapains A and B involves determination of pH- k profiles for reactions with 2,2'-dipyridyl disulphide in acid media by stopped-flow spectral analysis.

The present paper deals with the early-to-be-eluted form of chymopapain from dried latex, which is designated as 'A-type', both in the original sense used by Kunimitsu & Yasunoba (1967) and now also on the basis of its atypical reactivity characteristics towards 2,2'-dipyridyl disulphide. This enzyme is easily identified in ion-exchange profiles by both criteria and would be difficult to confuse with later-to-be-eluted forms in the complex elution band containing chymopapain B. It is not the purpose of the present paper to deal extensively with chymopapain forms that are eluted after this first chymopapain or with chymopapain forms present in fresh latex (see Buttle & Barrett, 1984; Brocklehurst *et al.*, 1985b). It is noteworthy, however, that multiple forms of chymopapain eluted over the whole of the chymopapain region exist in fresh non-fruit papaya latex and that all of them possess the unusual catalytic-site reactivity characteristics typical of chymopapain A (Brocklehurst *et al.*, 1985a, b). Closely similar elution profiles are obtained by f.p.l.c. on a Mono S column and by ion-exchange chromatography on a column of SP-Sephadex C-50 (Brocklehurst *et al.*, 1985b; E. Salih & K. Brocklehurst, unpublished work). Methods for the isolation of all of the major components of papaya latex have now been developed (E. Salih & K. Brocklehurst, unpublished work) by using ion-exchange chromatography with appropriate salt gradients, and these methods are at least as effective as f.p.l.c. both in resolution and in speed of operation. Treatment of this mixture of proteins with a low- M_r thiol such as cysteine results in the production of some chymopapain forms with B-type catalytic-site reactivity characteristics (E. Salih, K. Brocklehurst, R. McKee & H. Smith, unpublished work). The structural relationships between A- and B-types of chymopapain remain to be established but the suggestion by Buttle & Barrett (1984) that forms other than chymopapain B are artefacts produced during the commercial preparation of papaya latex can now be discounted after our analysis of fresh non-fruit latex. The conclusion suggested by Buttle & Barrett (1984) that all forms of chymopapain are products of a single gene, on the basis of their experiments with polyclonal antibodies, must be regarded as premature. Their results show only that the various chymopapains possess regions of close structural similarity. This finding certainly provides no evidence for different gene products among the chymopapains, but neither does it establish them as products of a single gene. Whether or not these enzymes are different gene products, A- and B-types of chymopapain possess markedly different catalytic-site reactivities, which must derive from structural differences, and thus the two types of isolatable enzyme require distinctive labels. Our experiments on cysteine treatment of chymopapain mixtures isolated from fresh papaya latex show that forms with the B type of catalytic-site reactivity can be generated by some sort of post-translational modification,

but whether this involves conversion of A-forms into B-forms is not yet established.

The present paper reports the purification of fully active chymopapain A by covalent chromatography and characterization of the enzyme by a number of techniques including two-protonic-state reactivity-probe kinetics (Brocklehurst, 1982) and resonance Raman (rR) spectroscopy (see e.g. Carey & Storer, 1983). The kinetic study suggests that the catalytic site of this enzyme may be exposed to an electrostatic field that is quite different from that experienced by other cysteine proteinases studied to date, and this may have consequences for the catalytic mechanism. The rR study shows that, in some dithioacyl derivatives of chymopapain A, the dithioacyl group is bound in a single conformation known as conforner B (Carey & Storer, 1983; Storer *et al.*, 1983) in which the glycinic N atom of the acyl group is in close contact with the thiol sulphur atom of the catalytic-site cysteine residue. In this respect chymopapain A is similar to other cysteine proteinases (see Carey *et al.*, 1983; Brocklehurst *et al.*, 1984c and references cited therein). These findings strengthen the view that although the differences between various cysteine proteinases can have consequences for the acylation process of the catalytic act (see Willenbrock & Brocklehurst, 1984; 1985a, b), the chemistry of the deacylation process is probably highly conserved (Brocklehurst *et al.*, 1984c).

MATERIALS AND METHODS

Materials

The four cysteine proteinases used in the present work were isolated as described previously: for papain by Baines & Brocklehurst (1979), for actinidin by Brocklehurst *et al.* (1981), for ficin by Malthouse & Brocklehurst (1976), for papaya proteinase Ω (papaya peptidase A) by Baines & Brocklehurst (1982b), and for partially active chymopapain A by Baines & Brocklehurst (1982a). For the rR experiments, papain was purified from Sigma twice-crystallized material by using Hg-agarose chromatography as described by Sluyterman & Wijdenes (1970). The resulting Hg-papain was activated with 2-mercaptoethanol (2 μ l/ml) for 30 min and eluted from a column (15 cm $\times$ 1 cm) of Sephadex G-15 with distilled water. Chymopapain A was received in the Ottawa laboratory from London as an $(\text{NH}_4)_2\text{SO}_4$ precipitate. It was diluted with distilled water and activated and isolated in active, activator-free form as described for papain.

The thionoester substrates were prepared as described by Ozaki *et al.* (1982) and by Carey *et al.* (1984).

Sephacrose 2B-glutathione-2-pyridyl disulphide gel for use in covalent chromatography was prepared as described by Brocklehurst *et al.* (1974). Other chromatographic materials were obtained from Pharmacia (G.B.) Ltd., and most other chemicals were from Sigma or BDH. 2,2'-Dipyridyl disulphide (Aldrich) was purified as described previously (Baines & Brocklehurst, 1982b).

Methods

Spectroscopic and kinetic measurements. rR spectra were recorded by mixing sequentially 270 μ l of enzyme ($A_{280} \approx 8$), 30 μ l of 50 mM-substrate in acetonitrile and 100 μ l of buffer as described previously (see, e.g., Brocklehurst *et al.*, 1984c). The instrumentation was described in detail by Carey & Sans Cartier (1983). Other

spectroscopic and kinetic measurements were made by using either a Cary 118 spectrophotometer or Dionex stopped-flow equipment at 25 °C (I 0.1).

Stock solutions of Z-Lys-ONp (Sigma) were made in acetonitrile/water (19:1, v/v), which were stored at 4 °C for several weeks without deterioration. Concentrations were determined by measuring A_{320} after complete cysteine-proteinase-catalysed hydrolysis and by using:

$$\epsilon_{320} = \tilde{\epsilon}_{320} + \frac{\tilde{\epsilon}'_{320} - \tilde{\epsilon}_{320}}{(1 + \frac{K}{[\text{H}^+]})}$$

where $\tilde{\epsilon}_{320} = 1400 \text{ M}^{-1} \cdot \text{cm}^{-1}$, $\tilde{\epsilon}'_{320} = 8100 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and $\text{p}K = 7.2$, which was used also to quantify kinetic runs. Stock solutions of L-Bz-Arg-pNan were made by dissolving the solid substrate in 1.5 ml of dimethyl sulphoxide and then adding this gradually with stirring to 98.5 ml of water. The concentration was determined by measuring A_{410} after complete cysteine-proteinase-catalysed hydrolysis and by using $\epsilon_{410} = 8800 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Erlanger *et al.*, 1961).

Individual Michaelis parameters for the various cysteine-proteinase-catalysed hydrolyses were obtained from initial-rate measurements with a range of substrate concentrations that spanned provisional estimates of K_m ; adherence to the Michaelis-Menten equation was established by the linearity of plots of s_0/v versus s_0 and values of K_m and V_{max} were then determined by computer fit to the hyperbolic form of the Michaelis-Menten equation. Values of k_{cat} were calculated by dividing values of V_{max} by enzyme catalytic-site concentrations determined by spectrophotometric titration with 2,2'-dipyridyl disulphide at pH 4 (see Brocklehurst & Little, 1973). In some cases k_{cat}/K_m was determined directly from plots of v versus s_0 , using $s_0 \ll K_m$, when v appears to increase linearly with increase in s_0 .

Other materials and methods involved in the kinetic studies with 2,2'-dipyridyl disulphide, including buffers, spectral data and details of data processing have been described previously [see Baines & Brocklehurst (1982b) and references cited therein]. All kinetic experiments were carried out at 25.0 °C and I 0.1.

Preparation and characterization of chymopapain A by covalent chromatography. These experiments were carried out by using the Sepharose 2B-glutathione-2-pyridyl disulphide gel described previously (Brocklehurst *et al.*, 1973, 1974) and the gel used in the present work contained about 200 μM of pyridyl disulphide sites as determined by reaction at pH 8.0 with low- M_r thiol (2-mercaptoethanol) and spectral analysis of the gel-free supernatant at 343 nm. The covalent attachment of chymopapain A was carried out in three different ways.

(i) Covalent attachment at pH 4.0 with both thiol groups of the enzyme unblocked. Activated, activator-free chymopapain A [10 ml in 0.1 M-KCl of, e.g., 120 μM -protein containing 1.4 mol of thiol/mol of protein (calculated by using $\epsilon_{280} = 46400 \text{ M}^{-1} \cdot \text{cm}^{-1}$; see the Results and discussion section), of which close to 50% was essential thiol] was mixed with 30 ml of the pyridyl disulphide gel that had been pre-equilibrated in 0.1 M-sodium acetate buffer, pH 4.0, containing 1 mM-EDTA. The mixture was stirred slowly with a magnetic stirrer for 1 h and was then packed into a column (11 cm $\times$ 2.5 cm) and washed with

the sodium acetate buffer until the eluate had negligible A_{280} . The column was then re-equilibrated with 0.1 M-Tris/HCl buffer, pH 8.0, containing 1 mM-EDTA, and the covalently bonded protein was eluted with 10 mM-cysteine in the Tris/HCl buffer. Ten 5 ml fractions were collected and those with high A_{280} (10 ml) were pooled. The pooled fraction was freed from excess cysteine and pyridine-2-thione by gel filtration through a column (17 cm $\times$ 2.5 cm) of Sephadex G-25 and evaluated for thiol content, thiol-reactivity characteristics and catalytic-activity characteristics.

(ii) Covalent attachment at pH 4.0 specifically through the essential thiol group (SHe). To provide for specific attachment of chymopapain A through its essential thiol group, the non-essential thiol group (SHn) was blocked by reaction with 2,2'-dipyridyl disulphide at high pH where SHn reacts more rapidly than SHe. A sample of the activated, activator-free enzyme described in (i) was adjusted to pH 10.2 by addition of carbonate buffer and an aqueous solution of 2,2'-dipyridyl disulphide (75 μ M) was added with good stirring in 0.1 ml portions until spectrophometric titration of a sample with excess 2,2'-dipyridyl disulphide showed that all of the non-essential thiol had been blocked. At this stage the reaction mixture contains, as protein species, chymopapain A with both SHe and SHn blocked as mixed disulphides and chymopapain A with only SHn blocked and free SHe, but a negligible amount of enzyme with only SHe blocked. The mixture of protein forms was subjected to covalent chromatography and gel filtration and analysis as described in (i).

(iii) Covalent attachment at pH 4.0 specifically through the non-essential thiol group. Reaction at pH 4.0 of a sample of chymopapain A with 2,2'-dipyridyl disulphide in amount equal to the amount of SHe present results in the specific and stoichiometric derivatization of SHe as a mixed disulphide and leaves SHn in its original state. The protein was subjected to covalent chromatography, gel filtration and analysis as described in (i).

RESULTS AND DISCUSSION

Preparation and characterization of chymopapain A by covalent chromatography

(1) Preparation of fully active chymopapain A containing two thiol groups per molecule. Preparations of chymopapain A obtained from dried papaya latex by ion-exchange chromatography (Baines & Brocklehurst, 1982a) contain two populations of thiol group that are readily distinguished by their reactivity characteristics towards the two-protonic-state thiol titrant and reactivity probe, 2,2'-dipyridyl disulphide. One type is in a molecular environment that provides for relatively high reactivity at pH 4 (k approx. $2 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$), and this group is essential for catalytic activity. The other type, which is not essential for activity, reacts 200–250 times more slowly with 2,2'-dipyridyl disulphide at pH values around 4. Both types of thiol group react very rapidly (k approx. 1×10^4 – $2 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$) at pH 8.

The enzyme prepared by ion-exchange chromatography from various types of dried papaya latex usually contains 1.3–1.6 mol of thiol per mol of protein as judged by the extent of release of pyridine-2-thione from 2,2'-dipyridyl disulphide at pH 8, assuming A_{280} for the enzyme to be $46400 \text{ M}^{-1} \cdot \text{cm}^{-1}$. This is the value that is obtained from

the values of $A_{1\text{cm}}^{1\%}$ and M_r reported by Robinson (1975) (see Baines & Brocklehurst, 1982a). In most preparations of chymopapain A, we have found that the two populations of thiol groups are present in essentially equal amounts.

It seems appropriate to apply covalent chromatography by thiol-disulphide interchange (Brocklehurst *et al.*, 1973, 1985c) to prepare fully active enzyme, to check the literature value of ϵ_{280} , to confirm the assumption that both types of thiol group reside in the same protein molecule and to investigate the distribution of oxidation states in the preparation of partially active chymopapain A. All of the experiments described relate to enzyme that contained close to equal amounts of both populations of thiol group before covalent chromatography.

Covalent chromatography was carried out in three ways as described in the Materials and methods section. In all three methods activated (i.e. reduced) activator-free chymopapain A prepared by ion-exchange chromatography was used as starting material. Thus any thiol groups that had existed in reversibly oxidized forms as mixed disulphides were in the thiol state before the start of the experiment.

In method (i) the fully reduced enzyme was applied to the Sepharose-glutathione-2-pyridyl disulphide gel. This method should permit attachment of the enzyme to the gel by either the SHn groups or the SHe groups or possibly both. In method (ii) the SHn groups were reversibly blocked as mixed disulphides by reaction with 2,2'-dipyridyl disulphide before reaction with the gel. In this method, therefore, attachment was possible only through the SHe groups. Thus any inactive protein molecules derived from chymopapain A, in which the catalytic-site thiol group had been irreversibly oxidized to sulphinic acid, would not be able to bond to the gel through their SHn groups. In method (iii), the SHe groups were reversibly blocked in an analogous way before attachment to the gel of any protein molecules containing the SHn type of group. The selectivity of thiol-group blocking depends on the different pH-dependent reactivity characteristics of the two types of thiol group towards 2,2'-dipyridyl disulphide.

The three methods of attachment and the consequences for the selectivity of attachment among the postulated oxidation states of partially active chymopapain A are illustrated in Scheme 1. A typical set of results showing thiol contents before and after covalent chromatography by method (i) is given in Table 1. Closely similar results were obtained also by using methods (ii) and (iii), i.e. methods involving specific attachment of the protein by SHe and by SHn groups respectively. It is important to note that in all cases the ratio of amount of SHe to the amount of SHn (R) is essentially unchanged by any of the three methods of covalent chromatography, although the thiol contents and hence the specific activities based on A_{280} are subsequently increased. This lack of variation in R when methods (ii) and (iii) are used demonstrates that molecular species in which only one of the two types of thiol group exists in an irreversibly oxidized state (e.g. sulphinic acid) exist in the enzyme prepared by ion-exchange chromatography to only a negligible extent. Every active enzyme molecule in the preparations used in the present work must contain both the catalytic-site thiol group (SHe) and the SHn group. The inactive protein separated from the active enzyme by covalent chromatography must be either chymopapain A in which both thiol

Scheme 1. Isolation and characterization of fully active chymopapain A by covalent chromatography

The gel used in the covalent chromatography process was Sepharose 2B–glutathione–2-pyridyl disulphide gel, i.e.:

'e' and 'n' denote respectively essential and non-essential thiol groups and their derivatives; methods 1–3, which permit covalent attachment by both thiol groups, by only SHe and by only SHn respectively are described in the Materials and methods section. Of the predicted protein products, only (I), i.e. chymopapain A containing both SHe and SHn is isolated; the unsymmetrical oxidation states (II) and (III) appear to exist to a negligible extent in the enzyme prepared by ion-exchange chromatography.

groups have been irreversibly oxidized or some other non-thiol-containing protein.

The fact that both the SHe and the SHn groups are present in chymopapain A prepared by covalent chromatography by methods (ii) and (iii) demonstrates that both thiol groups are present in the same enzyme molecule. Before these results were obtained by using covalent chromatography, the possibility existed that the two populations of thiol group detected by differential reactivity characteristics towards 2,2'-dipyridyl disulphide existed separately in different types of protein molecule that were not separated by the ion-exchange procedure.

(2) The value of ϵ_{280} for chymopapain A. Chymopapain A prepared by covalent chromatography [particularly by methods (ii) and (iii)] should contain 2.0 mol of thiol/mol of protein. When the thiol content is calculated by using $\epsilon_{280} = 46400 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (see above), values substantially greater than 2.0 are obtained. Analysis of the stopped-flow spectrophotometric record for the reaction of chymopapain A with the thiol-specific reagent 2,2'-dipyridyl disulphide at pH 8.2 (see Fig. 1) shows the progress curve to be composed of two phases of equal amplitude. The combination of thiol titre of enzyme prepared by covalent chromatography and the nature of the progress curves

Table 1. Results from a typical experiment showing the effect on its thiol content of subjecting chymopapain A to covalent chromatography

Enzyme preparation	[Protein] (μM) from A_{280}		Thiol content (mol/mol of protein)					
			SHe		SHn			
	*Using $\epsilon_{280} = 4.64 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$	*Using $\epsilon_{280} = 3.6 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$	[SHe] (μM)	[SHn] (μM)	*Using $\epsilon_{280} = 4.6 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$	*Using $\epsilon_{280} = 3.6 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$	*Using $\epsilon_{280} = 4.6 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$	*Using $\epsilon_{280} = 3.6 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$
Before covalent chromatography	121	156	77	80	0.6	0.5	0.7	0.5
After covalent chromatography	16	21	20	22	1.3	1.0	1.4	1.0

* $\epsilon_{280} = 4.64 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ is the value, calculated from the values of $A_{1\%}^{1\text{cm}}$ and M_r reported by Robinson (1975); $\epsilon_{280} = 3.6 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ is the value calculated to provide a total thiol content for chymopapain A after covalent chromatography of 2.0 mol of thiol/mol of protein. The specific activity of the preparation towards L-Bz-Arg-pNan at pH 6.0, 25 °C, 10.1, expressed as mol of *p*-nitroaniline produced/min per mg of protein increased after covalent chromatography in proportion to the increase in essential thiol content. [SHe] was determined from the rapid phase of the increase in A_{343} consequent on reaction with 2,2'-dipyridyl disulphide at pH 4.0. The sum of [SHn] and [SHe] was calculated from the increase in A_{343} when an analogous titration was carried out at pH 8.0.

compels us to take the view that the literature value of ϵ_{280} is too high. A value of $\epsilon_{280} = 36000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ is required to provide a thiol content of 2.0 mol of thiol/mol of protein.

Experience suggests that some preparations of chymopapain A prepared from commercial dried latex by ion-exchange chromatography will probably be found to contain approx. 1 mol of thiol/mol of protein calculated by using $\epsilon_{280} = 36000 \text{ M}^{-1} \cdot \text{cm}^{-1}$. It is important to stress, however, that such preparations will almost certainly be only approx. 50% active, because both types of thiol, i.e. essential and non-essential, will probably be present in about equal amount.

Characterization of the catalytic site of chymopapain A by using 2,2'-dipyridyl disulphide as a two-protonic-state reactivity probe

Disulphides containing the 2-pyridyl group, including the symmetrical, commercially available compound, 2,2'-dipyridyl disulphide, have proved to be valuable reactivity probes for the characterization of cysteine proteinase catalytic sites (see Brocklehurst, 1982). Although the reactions of chymopapain A with such disulphides are more complex than those of many other cysteine proteinases because this enzyme contains an additional thiol group per molecule, this is not a problem for studies in acidic media because the essential thiol group reacts much more rapidly with 2,2'-dipyridyl disulphide than does the non-essential thiol group. Thus kinetic analysis at pH values < 6 is not complicated by the reaction of the non-essential thiol group. The stopped-flow spectrophotometric record is monophasic, and when the reaction is carried out under pseudo-first-order conditions, the conventional logarithmic plot is linear (see, e.g., Fig. 2) and provides the pseudo-first-order rate constant for the reaction of the catalytic-site thiol group. As has been found for all other cysteine proteinases studied to date, the reaction is

first-order with respect to concentration in the reactant present in excess, 2,2'-dipyridyl disulphide, and is thus second-order over all the range of concentration permitted by solubility of 2,2'-dipyridyl disulphide, i.e. up to approx. 1 mM. The pH-dependence in acidic media of the second-order rate constant, k , for the reaction of the essential thiol group of chymopapain A with 2,2'-dipyridyl disulphide is shown in Fig. 3. Analogous pH- k profiles for two other cysteine proteinases isolated from dried papaya latex, papain and papaya proteinase Ω (originally called 'papaya peptidase A'; see Brocklehurst *et al.*, 1985a) are shown in Fig. 4. The unusual form of the pH-dependence of k for the chymopapain A reaction which was suggested by a preliminary study (Brocklehurst *et al.*, 1980) has now been firmly established in the present work by the extensive kinetic data shown in Fig. 3. The lack of a bell-shaped component in the pH- k profile for the chymopapain A reaction is noteworthy. A pronounced bell-shaped component with maximal values of k in the pH range 3–4 (see Fig. 4) is a striking feature of the pH- k profiles for the reactions of 2,2'-dipyridyl disulphides with all other cysteine proteinases studied to date, i.e. papain, actinidin, bromelain, ficin [see Brocklehurst (1982) and references therein], papaya proteinase Ω (Baines & Brocklehurst, 1982b) and cathepsins B and H (Willenbrock & Brocklehurst, 1984, 1985a), with the semi-synthetic enzyme thiolsubtilisin (Brocklehurst & Malt-house, 1981) and with the minimal cysteine-proteinase catalytic-site model, benzimidazol-2-ylmethane thiol (Brocklehurst *et al.*, 1979). The high reactivity in these bell-shaped components is considered to involve reaction of the thiolate anion of the catalytic site thiolate/imidazolium ion-pair (ImH^+/S^-) (or analogue in the case of the model compound) with the 2,2'-dipyridyl disulphide monocation. The value of k ($2.5 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$) for the chymopapain A reaction at pH values around 5–6 (which is considerably higher than values for reactions of simple low- M_r thiols and thus the

Fig. 1. (A) Stopped-flow record and (b) corresponding first-order logarithmic plots for the reactions of both thiol groups of chymopapain A with 2,2'-dipyridyl disulphide at 25 °C, I 0.1 and pH 8.2

The biphasic progress curve that arises from the existence of two parallel pseudo-first-order reactions producing the common product pyridine-2-thione was resolved into two linear logarithmic plots by using the procedure of Frost & Pearson (1961). $[2,2'\text{-dipyridyl disulphide}]_0 = 220 \mu\text{M}$; $[\text{SHe}] = 3.6 \mu\text{M}$; $[\text{SHn}] = 3.7 \mu\text{M}$.

values for the reaction of the non-essential thiol group) suggests that a catalytic-site ImH^+/S^- ion-pair might well exist in this enzyme and be capable of reacting with the neutral 2,2'-dipyridyl disulphide molecule. The fact that k then decreases with decrease in pH in the manner shown in Fig. 3 could be due to the poor binding of the 2,2'-dipyridyl disulphide monocation to the catalytic-site region of this enzyme. The increase in reactivity predicted from the increase in electrophilicity of the reagent consequent on protonation could be offset by the decreased binding ability of the cationic form relative to that of the neutral form of the reagent. This might occur if the catalytic-site region of chymopapain A contains an additional positive charge not present in the other cysteine proteinases mentioned above. The presence of such a positive charge may well have consequences for the catalytic mechanism of this enzyme, particularly in the control of the relative dispositions of the thiolate and imidazolium components of the ion-pair. The possibility of control of ion-pair geometry in the cysteine proteinases by both local field effects and by long-range effects has been discussed by Willenbrock & Brocklehurst (1985b)

and it seems possible that further study of the unusual catalytic-site field of chymopapain A might contribute to the understanding of these phenomena.

Comparison of the Michaelis parameters for chymopapain A-catalysed hydrolysis with those of some other cysteine proteinases

Very little has been reported on the catalytic characteristics of chymopapain A (see Cayle *et al.*, 1964; Ebata & Takahashi, 1966). To compare the catalytic characteristics of chymopapain A towards substrates commonly used to assay cysteine proteinases, the Michaelis parameters for the hydrolysis of L-Bz-Arg-pNan and of Z-Lys-ONp catalysed by chymopapain A, papaya proteinase Ω , papain, actinidin and ficin were determined and are presented in Table 2. When $k_{\text{cat.}}/K_m$ is used to measure catalytic effectiveness, chymopapain A appears to be roughly comparable with papaya proteinase Ω and with actinidin, but substantially worse as a catalyst for

Fig. 2. Stopped-flow record and corresponding first-order logarithmic plot for the reaction of the essential thiol group of chymopapain A with 2,2'-dipyridyl disulphide at 25 °C, I 0.1 and pH 3.5

The very much slower reaction of the non-essential thiol group, which can be monitored by using either a slower sweep time or a conventional spectrophotometer, is not shown. $[2,2'\text{-dipyridyldisulphide}]_0 = 220 \mu\text{M}$; $[\text{SHe}] = 3.6 \mu\text{M}$; $[\text{SHn}] = 3.7 \mu\text{M}$.

Fig. 3. pH-dependence in acidic media of the second-order rate constant (k) for the reaction of the catalytic-site thiol group of chymopapain A with 2,2'-dipyridyl disulphide at 25 °C, I 0.1

The points are experimental and the line is theoretical for:

$$k = \frac{\bar{k}}{1 + \frac{[H^+]}{K}}$$

in which $\bar{k} = 2600 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $pK = 2.35$. At higher pH values the value of k for the reaction of the catalytic-site thiol group increases markedly with increases in pH (e.g., at pH 8.2, $\bar{k} = 19900 \text{ M}^{-1} \cdot \text{s}^{-1}$, see Fig. 1) and the reaction of SHn becomes fast enough to cause the progress curves to become biphasic (Fig. 1).

the hydrolysis of both L-Bz-Arg-pNan and Z-Lys-ONp than papain and, in the case of the latter substrate, than ficin.

Investigation of some dithioacyl-chymopapain A derivatives by using rR spectroscopy

The unusual catalytic-site environment of chymopapain A detected by the reactivity-probe study discussed above points to the need for structural studies on this enzyme. As one approach to obtain structural data in solution, rR spectra were obtained for dithioacyl-chymopapain A derivatives. Lowe & Williams (1965) showed that thionoester substrates $[R-C(=S)-OR']$ react with the thiol group of papain to produce dithioacyl-enzymes $[R-C(=S)-S-Enzyme]$ as intermediates in the catalytic process. The dithioester intermediate differs by the substitution of $C=S$ for $C=O$, from the thioester $[R-C(=O)-S-Enzyme]$ postulated to occur on the catalytic pathway of the cysteine-proteinase-catalysed hydrolysis of both ester and amide substrates. It seems likely, however, that information gained by study of dithioacyl-enzymes will contribute to our understanding of the interaction of the cysteine proteinases with more natural substrates (see Brocklehurst *et al.*, 1984c). The approach used to study dithioacyl-enzymes by the rR method has been discussed by Storer *et al.* (1979, 1983) and by Carey & Storer (1983) (see also Brocklehurst *et al.*, 1984c).

Fig. 4. pH-dependence in acid media of the second-order rate constant (k) for the reaction of 2,2'-dipyridyl disulphide with the catalytic-site (and only) thiol group of (a) papaya proteinase Ω (papaya peptidase A) and (b) papain at 25 °C, I 0.1

The points are new experimental data for the reaction of papaya proteinase Ω and lines are theoretical: (a) for the reaction of papaya proteinase Ω , and (b) for the reaction of papain, for:

$$k = \frac{\bar{k}_1}{1 + \frac{[H^+]}{K_I} + \frac{K_{II}}{[H^+]}} + \frac{\bar{k}_2}{1 + \frac{[H^+]^2}{K_I K_{II}} + \frac{[H^+]}{K_{II}} + \frac{K_{III}}{[H^+]}} + \frac{\bar{k}_3}{1 + \frac{[H^+]}{K_{III}} + \frac{K_{IV}}{[H^+]}} + \frac{\bar{k}_4}{1 + \frac{[H^+]}{K_{IV}}}$$

in which for (a): $\bar{k}_1 = 2.65 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$, $\bar{k}_2 = 0$, $\bar{k}_3 = 500 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\bar{k}_4 = 2000 \text{ M}^{-1} \cdot \text{s}^{-1}$, $pK_I = 2.45$, $pK_{II} = 3.55$, pK_{III} (not determined because $\bar{k}_2 = 0$) and pK_{IV} (not shown) = 8.1, and for (b) $\bar{k}_1 = 3.7 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\bar{k}_2 = 2.5 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\bar{k}_3 = 750 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\bar{k}_4 = 1.7 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$, $pK_I = 3.85$, $pK_{II} = 3.9$, $pK_{III} = 5.3$ and pK_{IV} (not shown) = 8.8 (see Willenbrock & Brocklehurst, 1985a). The new data for the reaction of papaya proteinase Ω are broadly similar to those reported previously (Baines & Brocklehurst, 1982b), but provide somewhat different values for the characterizing parameters of the pH-dependence of k . In particular the previous data provided a value for pK_I of 3.88, which might have been significantly different from pK_{II} of the 2,2'-dipyridyl disulphide dication (2.45). The data now reported convincingly adhere to a pH-dependence that suggests that the bell-shaped component in acidic media involves reaction of the S^-/ImH^+ ion-pair of papaya proteinase Ω with the 2,2'-dipyridyl disulphide monocation without intervention of an additional ionizing group in the enzyme, an intervention that has been convincingly demonstrated only for papain among the cysteine proteinases studied to date (see Brocklehurst & Malthouse, 1980).

Table 2. Kinetic parameters characteristic of hydrolyses catalysed by some cysteine proteinases at 25 °C and *I* 0.1

k_{cat} and K_m were determined as described in the Materials and methods section; when these parameters were not determined separately, k_{cat}/K_m was determined from values of s_0 , e_T and v measured with $s_0 \ll K_m$, where v was shown to increase linearly with increase in s_0 .

Enzyme	Substrate	pH	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m ($\text{M}^{-1} \cdot \text{s}^{-1}$)
Chymopapain A	L-Bz-Arg-pNan	9.2	0.05	1.3×10^{-2}	4.1
		7.4	0.03	5.0×10^{-3}	6.6
		6.1	ND*	ND	6.0
		5.0	ND	ND	5.6
		4.0	0.03	8.6×10^{-3}	3.1
Actinidin	L-Bz-Arg-pNan	7.1	0.014	1.8×10^{-3}	7.8
		5.8	ND	ND	7.2
Papaya proteinase Ω	L-Bz-Arg-pNan	9.2	0.15	1.6×10^{-2}	9.4
		7.4	ND	ND	15.6
		4.0	0.14	1.8×10^{-2}	7.8
Papain	L-Bz-Arg-pNan	6.9	ND	ND	145
		6.2	ND	ND	145
		5.0	ND	ND	110
Actinidin	Z-Lys-ONp	6.9	50	9×10^{-5}	5.6×10^5
		6.0	52	7.5×10^{-5}	6.9×10^5
		4.9	44	8.3×10^{-5}	5.3×10^5
Chymopapain A	Z-Lys-ONp	6.9	15.5	1.0×10^{-5}	1.55×10^6
		6.0	15.5	7.5×10^{-6}	2.1×10^6
		4.9	14	7.5×10^{-6}	1.9×10^6
Papaya proteinase Ω	Z-Lys-ONp	5.9	49	2.8×10^{-5}	1.8×10^6
		5.3	49	3.5×10^{-5}	1.4×10^6
Ficin	Z-Lys-ONp	6.9	90	1.0×10^{-5}	9.0×10^6
		6.0	75	7.0×10^{-6}	1.1×10^7
		4.9	62	7.0×10^{-6}	8.9×10^6
Papain	Z-Lys-ONp	6.9	90	3.2×10^{-6}	2.8×10^7
		6.0	83	2.0×10^{-6}	4.2×10^7
		4.9	49	2.0×10^{-6}	2.5×10^7

* ND, not determined.

In the present work rR data were obtained for three dithioacyl chymopapain A derivatives, namely *N*-benzoylglycine-, *N*-(β -phenylpropionyl)glycine- and *N*-methoxycarbonylphenylalanylglycine-dithioacyl-chymopapain A. The rR spectra of *N*-benzoylglycine-dithioacyl-chymopapain A at pH values 2.5, 6.0 and 8.0 are compared with those of the corresponding papain derivative at pH 6.0 in Fig. 5. No major differences are seen between the four spectra shown in Fig. 5, i.e. the spectrum of the chymopapain A derivative is essentially pH-independent and is very similar to that of the papain derivative at pH 6.0. Thus the spectral analysis for the latter and the resulting conclusions regarding conformation discussed by Storer *et al.* (1983) apply also to the chymopapain A derivative. In each case the acyl group binds as a single population of a conformation known as a conformer B in which the glycinic N atom is in close contact with the thiol sulphur atom of the catalytic-site cysteine residue. This conclusion is in agreement with the results reported previously (Carey *et al.*, 1983) for rR studies on analogous derivatives of a 'chymopapain' preparation obtained from Sigma Chemical Co. The commercial product currently supplied by Sigma as 'chymopapain' is now known (E. Salih & K. Brocklehurst, unpublished work) to contain not only chymopapain A but also B-type chymopapains and a substantial

quantity of papaya proteinase Ω (originally called 'papaya peptidase A' by Schack, 1967; see Brocklehurst *et al.*, 1985a). Previously only partial spectra and data obtained near to neutral pH were reported for this mixture of enzymes. The new data presented in Fig. 5 show for the first time the characteristic features at 671 cm^{-1} and 953 cm^{-1} (see Storer *et al.*, 1983) and cover the pH range 2.5–8.0 over which the rR spectra do not change substantially. Thus the conformation of the dithioacyl group would seem to be unaffected by any ionizations in the enzyme that occur in this pH region.

Close inspection of the chymopapain A spectra seen in Fig. 5 reveals that the feature near 1170 cm^{-1} is less well resolved and has a greater relative intensity compared with its counterpart in the papain spectrum. This difference may reflect a slightly changed conformation in the *N*-benzoyl portion of the substrate (see Storer *et al.*, 1983) with, possibly, the bound *N*-benzoyl group experiencing a wider range of environments in the case of chymopapain A than in the case of papain.

In the present work the rR data for the dithio species involving chymopapain A and *N*-(β -phenylpropionyl)glycine and *N*-methoxycarbonylphenylalanylglycine at pH 6.0 were also compared with those for the corresponding papain intermediates presented previously (Storer *et al.*, 1983; Carey *et al.*, 1984). Spectral quality

Fig. 5. rR spectra of *N*-benzoylglycinedithioacyl-chymopapain A and of the analogous derivative of papain

rR conditions: 90 mW, 324 nm excitation, 12 cm⁻¹ spectral slit; 20 s acquisition time.

was as good as, or better than, that shown in Fig. 5, but for both of these additional substrates no significant spectral differences, in terms of band positions or band shape, could be detected. Thus these spectra are not shown. Again, the conclusions stated for the *N*-benzoylglycine-substrate obtain; there is a single population of conformer B. Taken together, the results for all three substrates suggest that the marked differences in catalytic-site properties between chymopapain A and many other cysteine proteinases, detected by the two-protonic-state reactivity probe, are not reflected by changes in conformation of the bound acyl group when the acyl-enzyme intermediate has been formed.

The *N*-methoxyloxycarbonylphenylalanylglycine [CH₃OC(=O)-Phe-Gly] acyl group has a high specificity for papain, since it satisfies the requirement of that enzyme for a hydrophobic side chain in the substrate that can bind in the S₂-subsite (Berger & Schechter, 1970). The rR data allow us to estimate the degree of acylation of the enzymes by the substrate, by comparing the intensities of the dithioacyl-enzyme rR bands to those of acetonitrile used as a solvent for the substrate. The comparison shows that the degree of acylation for CH₃OC(=O)-Phe-Gly with papain and chymopapain A are very similar.

The rR data obtained for the chymopapain A derivatives add further support to the growing evidence [see Brocklehurst *et al.* (1984c) and references cited therein] that the chemistry of the deacylation process is highly conserved among the cysteine proteinases. The differences detected among the various cysteine proteinases by reactivity-probe techniques include the unusual

electrostatic field deduced to be present in the catalytic site of chymopapain A and the absolute requirement in cathepsin B for an additional deprotonation to endow the S⁻/ImH⁺ ion pair with catalytic competence (Willenbrock & Brocklehurst, 1984, 1985b). The influence of such variations in chemical characteristics and hence in structure would seem to be confined to some part of the catalytic act that precedes deacylation. It seems entirely reasonable that acylation might be particularly susceptible to modulation by natural structural change in these enzymes, because it is at this stage of catalysis that kinetic specificity is manifested in papain-catalysed hydrolysis (Lowe & Yuthavong, 1971a, b).

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REFERENCES

- Baines, B. S. & Brocklehurst, K. (1979) *Biochem. J.* **177**, 541–548
- Baines, B. S. & Brocklehurst, K. (1982a) *J. Protein Chem.* **1**, 119–139
- Baines, B. S. & Brocklehurst, K. (1982b) *Biochem. J.* **205**, 205–211

- Barrett, A. J. & Buttle, D. J. (1985) *Biochem. J.* **228**, 527
- Berger, A. & Schechter, I. (1970) *Philos. Trans. R. Soc. London Ser. B* **257**, 249–264
- Brocklehurst, K. (1982) *Methods Enzymol.* **87C**, 427–469
- Brocklehurst, K. & Little, G. (1973) *Biochem. J.* **133**, 67–80
- Brocklehurst, K. & Malthouse, J. P. G. (1980) *Biochem. J.* **191**, 707–718
- Brocklehurst, K. & Malthouse, J. P. G. (1981) *Biochem. J.* **193**, 819–823
- Brocklehurst, K. & Salih, E. (1983) *Biochem. J.* **213**, 559–560
- Brocklehurst, K., Carlsson, J., Kierstan, M. P. J. & Crook, E. M. (1973) *Biochem. J.* **133**, 573–584
- Brocklehurst, K., Carlsson, J., Kierstan, M. P. J. & Crook, E. M. (1974) *Methods Enzymol.* **34B**, 531–544
- Brocklehurst, K., Stuchbury, T. & Malthouse, J. P. G. (1979) *Biochem. J.* **183**, 233–238
- Brocklehurst, K., Baines, B. S. & Mushiri, M. S. (1980) *Biochem. J.* **189**, 189–192
- Brocklehurst, K., Baines, B. S. & Malthouse, J. P. G. (1981) *Biochem. J.* **197**, 739–746
- Brocklehurst, K., Mushiri, S. M., Patel, G., Willenbrock, F. (1983) *Biochem. J.* **209**, 873–879
- Brocklehurst, K., Salih, E. & Lodwig, T. S. (1984a) *Biochem. J.* **220**, 609–612
- Brocklehurst, K., Baines, B. S., Salih, E. & Hatzouli, C. (1984b) *Biochem. J.* **221**, 553–554
- Brocklehurst, K., Carey, P. R., Lee, H. H., Salih, E. & Storer, A. C. (1984c) *Biochem. J.* **223**, 649–657
- Brocklehurst, K., Salih, E., McKee, R. & Smith, H. (1985a) *Biochem. J.* **228**, 525–527
- Brocklehurst, K., McKee, R., Salih, E. & Smith, H. (1985b) *J. Protein Chem.* **4**, 103–127
- Brocklehurst, K., Carlsson, J. & Kierstan, M. P. J. (1985c) *Top. Enzyme Ferment. Biotechnol.* **10**, 146–188
- Buttle, D. J. & Barrett, A. J. (1984) *Biochem. J.* **223**, 81–88
- Carey, P. R. & Sans Cartier, L. R. (1983) *J. Raman Spectrosc.* **14**, 271–275
- Carey, P. R. & Storer, A. C. (1983) *Acc. Chem. Res.* **16**, 455–460
- Carey, P. R., Ozaki, Y. & Storer, A. C. (1983) *Biochem. Biophys. Res. Commun.* **117**, 725–731
- Carey, P. R., Angus, R. H., Lee, H. H. & Storer, A. C. (1984) *J. Biol. Chem.* **259**, 14357–14360
- Cayle, T. C., Saletan, L. T. & Lopez Ranos, B. (1964) *Wallerstein Lab. Commun.* **27**, 87–96
- Ebata, M. & Takahashi, Y. (1966) *Biochim. Biophys. Acta* **118**, 201–203
- Erlanger, B. F., Kokowsky, M. & Cohen, W. (1961) *Arch. Biochem. Biophys.* **95**, 271–278
- Frost, A. A. & Pearson, R. G. (1961) *Kinetics and Mechanism*, 2nd edn., pp. 160–165, John Wiley and Sons, London
- Khan, I. U. & Polgár, L. (1983) *Biochim. Biophys. Acta* **760**, 350–356
- Kunimitsu, D. K. & Yasunoba, K. T. (1967) *Biochim. Biophys. Acta* **139**, 405–417
- Lowe, G. & Williams, A. (1965) *Biochem. J.* **96**, 189–193
- Lowe, G. & Yuthavong, Y. (1971a) *Biochem. J.* **124**, 107–115
- Lowe, G. & Yuthavong, Y. (1971b) *Biochem. J.* **124**, 117–122
- Malthouse, J. P. G. & Brocklehurst, K. (1976) *Biochem. J.* **159**, 221–234
- Ozaki, Y., Pliura, D. H., Carey, P. R. & Storer, A. C. (1982) *Biochemistry* **21**, 3102–3108
- Polgár, L. (1984) *Biochem. J.* **221**, 555–556
- Robinson, G. W. (1975) *Biochemistry* **14**, 3695–3700
- Salih, E. & Brocklehurst, K. (1983) *Biochem. J.* **213**, 713–718
- Schack, P. (1967) *C.R. Trav. Lab. Carlsberg* **36**, 67–83
- Sluyterman, L. A. Æ. & Widjenes, J. (1970) *Biochim. Biophys. Acta* **200**, 593–595
- Storer, A. C., Murphy, W. F. & Carey, P. R. (1979) *J. Biol. Chem.* **254**, 3163–3165
- Storer, A. C., Lee, H. & Carey, P. R. (1983) *Biochemistry* **22**, 4789–4796
- Tsou Chen-Lu (1962) *Sci. Sin.* **11**, 1535–1558
- Willenbrock, F. & Brocklehurst, K. (1984) *Biochem. J.* **222**, 805–814
- Willenbrock, F. & Brocklehurst, K. (1985a) *Biochem. J.* **227**, 511–519
- Willenbrock, F. & Brocklehurst, K. (1985b) *Biochem. J.* **227**, 521–528

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