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COMPARISON OF THE SUBSTRATE CONFORMATIONS IN THE ACTIVE SITES OF PAPAIN, CHYMOPAPAIN, FICIN AND BROMELAIN BY RESONANCE RAMAN SPECTROSCOPY

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SUMMARY: The resonance Raman spectra of several enzyme-substrate intermediates of papain, chymopapain, ficin and bromelain are reported. The intermediates are dithioacyl enzymes formed during the catalyzed hydrolysis of N-acylglycine thionoester substrates. Interpretation of the resonance Raman spectra allows us to compare, for the first time, the substrate geometries in a series of functioning intermediates from different enzymes. The substrates assume essentially identical conformations for papain, chymopapain and ficin and a similar, but not identical, conformation in the active site of bromelain. Each dithioacyl enzyme population appears to be made up of a single homogeneous conformational state. This state has been characterised in earlier studies of dithioacyl papains. It is designated as conformer B and is characterized by an attractive contact between the substrate's glycinic N atom and the active site cysteine S atom. It is now apparent that conformer B is of general significance in the mechanism of cysteine proteases.

The purpose of this communication is to demonstrate that it is possible to compare the conformations of a substrate bound to a series of cysteine proteases. Since these enzyme-substrate complexes are catalytically viable intermediates, knowledge of the substrate conformations provides valuable information on the comparative mechanisms of the cysteine proteases. These enzymes depend for their catalytic activity on the thiol group of a cysteine residue (1,2). During the reaction with a peptide or ester substrate a thiol ester intermediate is formed between the substrate and the cysteine -SH. By using thionoester substrates it is possible to generate in a reaction mixture dithioester intermediates which have an intense absorption band near 315nm (3). In turn, this absorbance may be used to obtain resonance Raman (RR) spectra of the dithioester transient (4). By this means, we are able to observe the vibrational spectrum of the group undergoing transformation in the active site and to use this spectrum to form a detailed picture of the conformation in and near the dithioester bonds (5).

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There have been several recent reports from this laboratory on using the RR-dithioester approach to delineate conformational events in papain's active site (6-9). In this paper we make a comparison between the papain findings and new data for ficin, chymopapain and bromelain. There have been a number of comparisons of the properties of these enzymes using kinetic (10,11) and reactivity probes (12). Now we are able to present direct evidence, for which there is no precedent, on the conformations of four glycine based substrates bound to papain, chymopapain, ficin and bromelain.

EXPERIMENTAL: Papain (2X crystallised, EC 3.4.22.1), chymopapain (EC 3.4.22.6), ficin (2X crystallised, EC 3.4.22.3) and bromelain (EC 3.4.22.4) were purchased from the Sigma Chemical Co. The papain was purified by affinity chromatography as described previously (6). The other enzymes were purified using the mercurial-agarose column method described by Sluyterman and Widnes (13). The purified enzymes were stored in their inactive mercurial forms and concentrated and activated prior to use as described by Ozaki et al. (6). The thionoester substrates N-benzoylglycine, N-phenylacetylglycine, N-(β-phenylpropionyl)glycine and N-carbobenzoxyglycine methyl thionoester were synthesised as described in reference (6).

Spectroscopic studies were carried out on reaction mixtures consisting of ~150 μM enzyme and 5-10 mM substrate in a buffer solution containing 20% by volume of acetonitrile, 5 mM EDTA and 50 mM sodium phosphate at pH 6.8. Under these conditions an approximately constant concentration of the dithioacyl-enzyme (O.D. at 315 nm 0.2-1.5) is produced for the few minutes required to record the RR spectrum. Each partial spectrum shown in Figures 1 and 2 was obtained using a fresh reaction mixture and took about 5 min to scan. The 1140 cm^{-1} or 600 cm^{-1} band was scanned through about 4 min after starting the reaction for papain, chymopapain and ficin and about 8 min after mixing for bromelain.

Raman measurements were made by using a Spex 0.5 m double spectrometer with direct current detection. The excitation source was a 324 nm Kr^+ line (25-40 mW) from a Coherent Radiation 2000K laser. Peak frequencies were calibrated by using emission lines from a Ne lamp and are believed to be accurate to ±2 cm^{-1} for well resolved features. A rotating cell assembly was employed to prevent sample photodegradation in the beam. UV absorption spectra were obtained using a Cary 118 or 219 spectrophotometer.

RESULTS AND DISCUSSION: Each of the enzyme-substrate intermediates studied here has an absorption band in the 309-316 nm region due to the dithioester chromophore. Excitation into this transition with 324 nm Kr^+ irradiation allowed us to record the RR spectra shown in Figures 1 and 2. For each substrate, the four dithioacyl enzyme RR spectra are similar. Thus, band assignments may be made by reference to those for dithioacyl-papains which have been analysed extensively (5-9). The most intense peak in the RR spectra occurs near 1135 cm^{-1}; it has been designated Band II in earlier publications

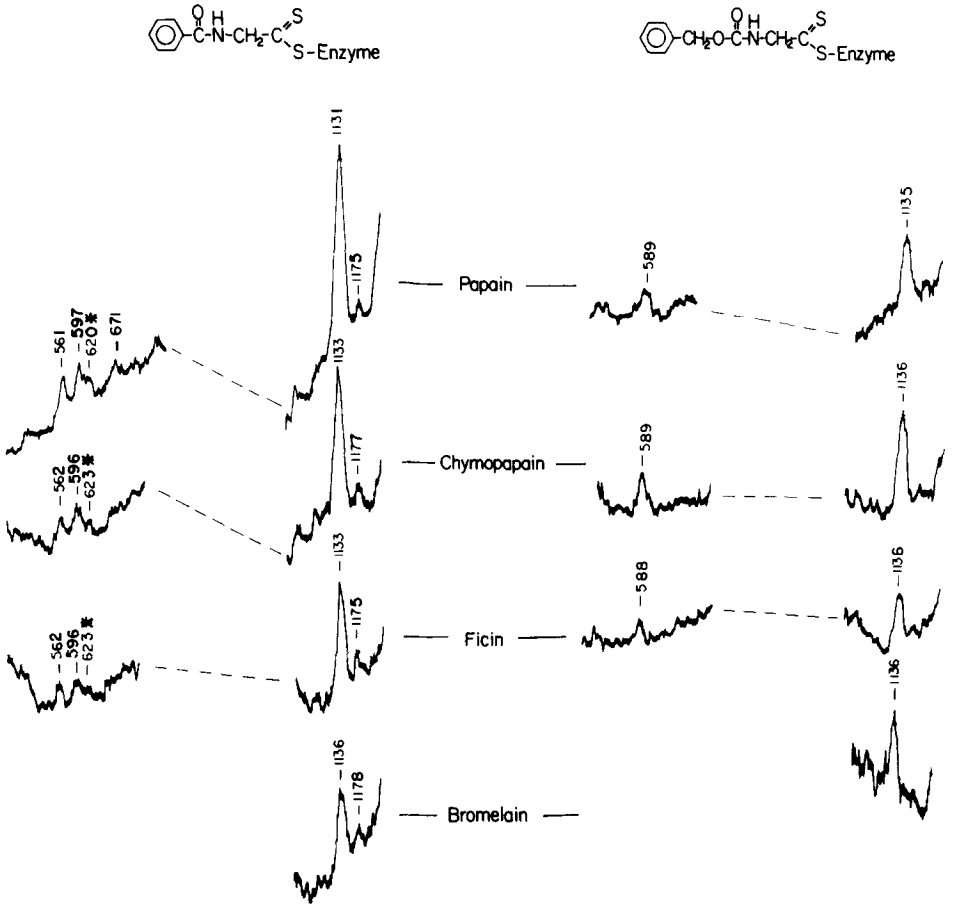

Figure 1. Resonance Raman spectra of dithioacyl enzyme intermediates formed between papain, chymopapain, ficin and bromelain and the substrates N-benzoylglycine and N-carbobenzoxyglycine methyl thionoesters. Peak near 620 cm^{-1}, denoted by an asterisk, is due to excess substrate.

(6,7,14) and contains significant contributions from C=S and C-C(=S) bond stretching motions. The normal mode giving rise to the medium intensity band seen near 1095 cm^{-1} in Figure 2, called Band III, also involves the motion of the thiocarbonyl carbon atom but, at the same time, is vibrationally delocalised into the NH-C-C(=S) portion (8,9). The weak feature seen in the N-benzoylglycine and N-(β-phenylpropionyl)glycine dithioacyl enzyme spectra near 1175 cm^{-1} is due to a mode highly delocalised through the glycine dithioester moiety. The band seen in all spectra near 600 cm^{-1} is also highly delocalised but the feature seen in some of the better quality RR spectra near 680 cm^{-1} is a reasonable group frequency corresponding to the S-C stretching motion in the C(=S)S-C (cysteine) linkage.

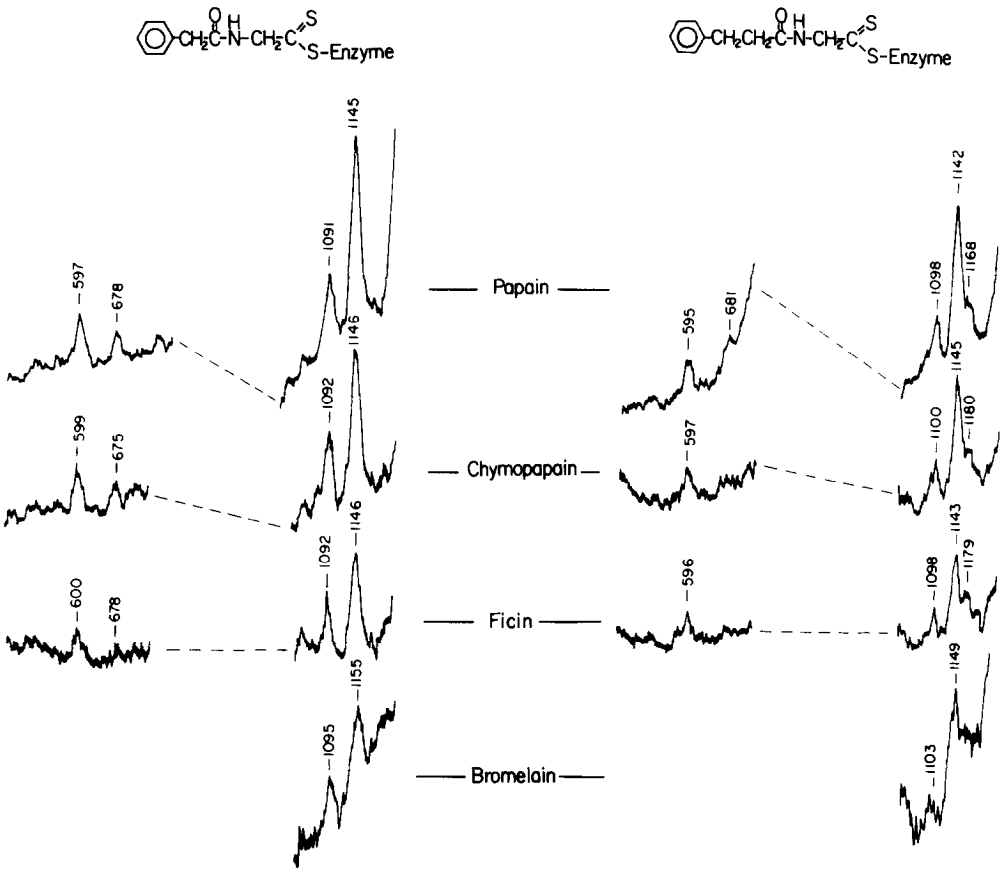

Figure 2. Resonance Raman spectra of dithioacyl enzyme intermediates formed between papain, chymopapain, ficin and bromelain and the substrates N-phenylacetylglycine and N-(β-phenylpropionyl)glycine methyl thionoesters.

Although the vibrational description of the RR peaks is complex it is important to emphasise that the different conformational states of N-acylglycine dithioesters have characteristic and separate RR signatures. The relationship between spectral signature and conformational states has been established by joint X-ray crystallographic and Raman studies (7,15) and by experiments involving isotopic replacements (8,9). By these means the RR spectra of N-acylglycine dithioacyl papains are fairly well understood. The papain spectra (e.g. in Figures 1 and 2) are due to the substrate assuming a single conformational state in the active site, known as conformer B. In this conformation the torsional angles about the $NH-CH_2-C(=S)$ linkages assume values such that the nitrogen atom is in close contact with the cysteine (thiol) S atom. Evidence has been presented for dithioacyl papains (9) that

this is a relaxed conformational state involving little or no geometric distortion. In other words, papain selects one of the available relaxed conformation states available to N-acylglycine dithioesters. There is evidence, however, that the torsional angle about the $C(=S)S-CH_2C$ bond is not relaxed. It appears to be significantly distorted from the preferred conformation of 180°. The RR spectra can be understood in terms of variations about the torsional angles discussed. There is no evidence for spectral perturbations due to e.g. charge, or dipole, or H-bonding effects.

The papain results and conclusions can now be compared to the results for chymopapain, ficin and bromelain. For chymopapain and ficin, the RR spectrum of each dithioacyl enzyme is very similar to the spectrum of the corresponding dithioacyl papain. The number of peaks, their frequency, and with one exception their relative intensities, are the same within the limits of experimental reproducibility. Thus, the immediate conclusion is that ficin and chymopapain, just as in the case of papain, select out one of the preferred conformational states of N-acylglycine dithioesters, namely conformer B. Furthermore, since the peak positions in the RR spectra are sensitive to changes in torsional angles in the N-acylglycine dithioester group, we can state that the conformations of any one substrate in the active site of papain, chymopapain and ficin are very similar. For example, since the frequency of the intense band ocurring near 1135 cm^{-1} is very sensitive to changes in the $NHCH_2-C(=S)$ torsional angle, it is likely that a variation of more than roughly 15° in the value of this angle in going from the acyl-papain to the chymopapain, or ficin analog would result in a measurable frequency difference for the band near 1135 cm^{-1}.

The only detectable difference in the papain, chymopapain, ficin series concerns the relative intensity of the feature near 1095 cm^{-1} seen in Figure 2. The effect is especially pronounced for the $PhCH_2C(=O)NHCH_2C(=S)S-$ acyl group, for which the average of several measurements of the ratio of the intensities of the 1145 to the 1091 cm^{-1} bands gave 2.40±0.20 (papain), 2.15±0.20 (chymopapain) and 1.75±0.25 (ficin). However, since this effect is

seen in peak intensities only, the changes in the electronic ground state chemistry must be minimal. The intensity changes may be due to an effect which is most pronounced in the excited state or to a very minor conformational change in or about the $-C(=O)NHCH_2C(=S)$ bonds. In any event, it does not alter the central conclusion that the substrates are binding in a single population of essentially identical conformer Bs in the three enzymes.

The data for bromelain are of inferior quality compared to those from the other enzyme intermediates and it was not possible to obtain reliable measurements in the 600 cm^{-1} region. However, the spectra in Figures 1 and 2 show clearly that the dithioacyl bromelains are in a class of their own in that, although they show conformer B signatures, the peak frequencies in each case (except the $PhCH_2OC(=O)NHCH_2C(=S)S-$) are at higher values compared to the other dithioacyl enzymes. Since the RR peak positions in the bromelain case are not those for a relaxed conformer B (9) we can say that for these dithioacyl enzymes the substrate is binding in a perturbed conformer B state. It should also be added that, for the bromelain case, the poor spectral quality mitigates against the detection of a a small population of non-B conformers - it would be difficult to detect a non-B population which is less than 20% of the B population.

Taken together the present findings for sixteen dithioacyl enzymes involving papain, chymopapain, ficin and bromelain provide strong evidence for the universality of B-type conformers for glycine-based acyl groups in cysteine proteases. The detailed chemistry of B-type conformations is presently under study. We know that the N•••S(thiol) contact, characteristic of conformer B, is a HOMO•••LUMO attraction involving the N lone pair electrons and a σ^* orbital of the S-C bond (which is a cysteine linkage in the active site). The geometry of the interaction is maintained within fine limits for different N-acylglycine dithioesters (15). Moreover, recent kinetic and RR studies have shown the N•••S contact is broken in the rate determining step for deacylation of N-acylglycine dithiopapains (16). However, even with these recent findings we do not understand completely the

catalytic significance of conformer B. The present data emphasises that our understanding of the reaction pathway of these enzymes will only be complete when the chemistry of B-type conformations is fully elucidated.

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