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Identity of Acyl Group Conformations in the Active Sites of Papain and Cathepsin B by Resonance Raman Spectroscopy*

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Resonance Raman spectroscopic data provide conclusive evidence for the existence of an acyl-enzyme intermediate during the reaction of a thionoester substrate, *N*-methyloxycarbonylphenylalanylglycine methyl thionoester ($CH_3OC(=O)$-Phe-$NHCH_2C(=S)OCH_3$), with cathepsin B from porcine spleen. The resonance Raman spectrum of $CH_3OC(=O)$-Phe-$NHCH_2C(=S)$S-cathepsin B, where the thiol S is from the active-site cysteine residue, is compared to that of the corresponding papain acyl-enzyme. Within the limits of experimental error (±2 cm^{-1} for peak positions), there are no detectable spectral differences. Since the resonance Raman spectrum is sensitive to the torsional angles in the glycinic bonds and the cysteine linkages, the conformations are identical in those parts of the acyl-enzymes where chemical transformation occurs. A conformational analysis of the model compound $CH_3OC(=O)$-Phe-$NHCH_2C(=S)SC_2H_5$ demonstrates that the dithioacyl group in both dithioacyl-enzymes is present as a single population of a form known as conformer B. Conformer B is characterized by a small torsional angle about the glycinic $NHCH_2$-CS(thiol) bond such that the nitrogen and S (thiol) atoms are in close contact. This conformer is widespread among the dithioacyl intermediates of plant cysteine proteinases, and it is apparent that the same chemistry is retained in a mammalian cysteine proteinase. Steady-state kinetic parameters are also reported for $CH_3OC(=O)$-Phe-$NHCH_2C(=S)OCH_3$ reacting with papain and cathepsin B. The similarity of the k_{cat} values, 0.53 and 1.15 s^{-1}, for papain and cathepsin B, respectively, provides further evidence for a conserved deacylation process.

Cathepsin B is a thiol-dependent lysosomal proteinase which has been isolated from a variety of animal tissues including human liver (1), bovine spleen (2), porcine liver (3) and kidney (4), rat liver (5), and rabbit liver (6). Increasing attention has been given to the possible biological importance of cathepsin B with its implication in, for example, intracellular protein turnover (7, 8), the processing of hormones (9), inflammation (10), tumor invasion and metastasis (11), and collagenolysis (6).

Cathepsin B is one of a number of lysosomal cysteine proteinases, and it is usually assumed that this class of mammalian enzymes bears a resemblance structurally and mechanistically to the plant cysteine proteinases of which papain is the best known. Initially, this assumption for cathepsin B was based on the identification of a catalytically essential active-site cysteine (12) and similar properties to papain with regard to inhibitors (13, 14), substrate specificity (13), and optimal pH range for enzymatic activity (15). The analogy was further strengthened by striking amino acid sequence homologies, which include the essential active-site residues, between papain and cathepsin B from rat liver (5).

During the past several years, resonance Raman spectroscopy has been developed as a means of obtaining precise conformational information for the acyl group within a catalytically viable acyl-enzyme intermediate (16, 17). The technique has been particularly effective for cysteine proteinases since the cysteine sulfur atom is used to generate part of the chromophore required for the RR[1] effect (17). By this means, the conformations of glycine-based acyl groups in a number of plant cysteine proteinases, papain, papaya peptidase II, and actinidin (18), chymopapain, bromelain, and ficin (19) have been compared. Each acyl group tested was found to take up an identical, or very similar, conformational state in the active sites of every plant cysteine proteinase studied. This conformational state, known as conformer B, has unusual spectroscopic and chemical properties (20–22). For different enzymes, these properties provide a critical test of *functional* homology at the acyl-enzyme stage of the reaction. In the present work, RR spectroscopy is used to compare the acyl-enzymes derived from papain and porcine spleen cathepsin B. The spectral signatures are found to be identical, and thus the characteristic acyl-enzyme chemistry found in plant cysteine proteinases has been conserved for cathepsin B.

EXPERIMENTAL PROCEDURES

Materials

Cathepsin B was purified from pig spleen following the procedure of Barrett (1). Minor differences in our preparation included the use of Triton X-100 instead of Arquad 2C-50 in the extraction procedure and a single-step acetone precipitation using 1.3 volumes of −5 °C acetone to 1 volume of cooled liquid obtained from overnight autolysis at 40 °C. After elution from the Hg-Sepharose column with β-mercaptoethanol, the cathepsin B was dialyzed twice against 50 mM sodium acetate, 0.2 M NaCl, 1 mM EDTA, pH 5.5, buffer and once against 0.025 M sodium acetate, 0.2 M KCl, 5 mM $HgCl_2$, pH 4.5, buffer. The resultant mercurated enzyme was concentrated from 65 to 10 ml in an Amicon 8200 concentrator and then applied to a Bio-Gel P-60 column (75 × 2.5 cm) and eluted with 0.025 M sodium acetate, 0.2 M KCl, pH 4.5, buffer. A typical column profile is shown in Fig. 1. The first peak, identified by reaction with glycyl-L-phenylalanine β-naphthylamide (23), is due to cathepsin C. The third peak is cathepsin B. The cathepsin B was assayed routinely using N^{α}-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester. With this substrate, specific activity is defined as the change in absorbance at 325 nm/min for a protein concentration of 1 A_{280} unit at 20 °C in pH 5.1, 25 mM sodium acetate buffer. The concentration of the active enzyme was derived by titrating the active-site —SH with 2,2′-dipyridyl disulfide, which provides a highly specific titer for active site over nonactive site thiols (24). Six preparations gave a range of 0.40–0.65 active-site thiols/molecule of enzyme, assuming identity with Bar-

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[1] The abbreviation used is: RR, resonance Raman.

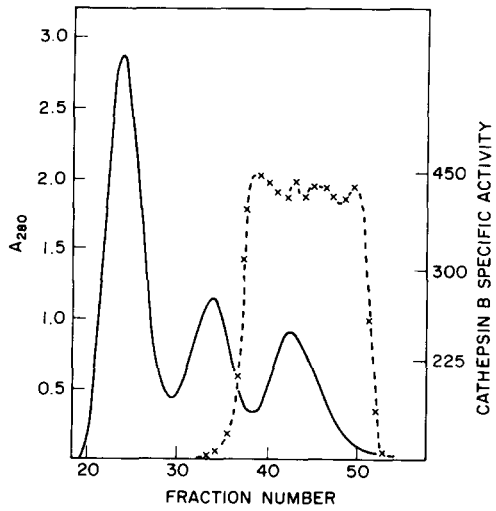

FIG. 1. **Elution profile from the P-60 column.** 3-ml fractions were collected, and the column was run at 20 ml/h. Fractions with a specific activity greater than 300 were pooled. The specific activity is measured with benzyloxycarbonyllysine *p*-nitrophenyl ester.

rett and Kirschke's extinction coefficient of 5.2×10^4 for human cathepsin B from liver (25). This finding is in accord with the conclusion of Barrett (25) that approximately half of "purified" cathepsin B is active material. Using the enzyme concentration derived from the active-site —SH titration, our enzyme gave a k_{cat}/K_m of 3.7×10^6 s^{-1} M^{-1} (pH 6.8, 20 °C) with the fluorometric substrate benzyloxycarbonyl-Phe-Arg-4-methyl-7-coumarylamide. The kinetic constants for the thionoester substrates used in this study were also calculated with respect to the active-site —SH concentration. Typically, 12 mg of cathepsin B were obtained from 1 kg of pig spleen, and this was sufficient for six RR experiments.

Papain was purified from Sigma twice crystallized material by Hg-Sepharose affinity chromatography (26). The concentration of active papain was taken as equal to the active-site —SH titer obtained using 5,5′-dithiobis-(2-nitrobenzoic acid) (27) or 2,2′-dipyridyl disulfide. Both titrants gave the same value of —SH for papain, usually in the range 0.90–0.99/molecule of enzyme.

N-Methyloxycarbonylphenylalanine was prepared in 80% yield by the reaction of L-phenylalanine with an equimolar, ethereal solution of methyl chloroformate added in aliquots at 0 °C while maintaining a pH of 10–11. After the reaction was completed, the aqueous solution was extracted with ether and then acidified. The oily material produced was extracted with CH_2Cl_2 washed with H_2O and then with saturated NaCl solution and dried-over Na_2SO_4. The CH_2Cl_2 was removed, and the oily residue was crystallized on standing. The purity of the compound was checked by NMR and used without further purification.

N-Methyloxycarbonylphenylalanylaminoacetonitrile was prepared by the method of Lowe and Yuthavong (28) with the following modifications. Isobutylchloroformate and *N*-methylmorpholine were substituted for ethyl chloroformate and triethylamine, respectively, to decrease racemization (29). The purity of the nitrile was confirmed by NMR, and the compound was used without further purification.

N-Methyloxycarbonylphenylalanylglycine methyl thionoester was prepared by modifying previously described procedures (20, 30). The nitrile (2.61 g, 0.01 mol) was added to a 250-ml round-bottomed flask with 50 ml of CH_2CL_2 and 0.52 ml of MeOH and cooled to 0 °C. HCl (g) was bubbled into the mixture for 20 min. The resultant cloudy solution was treated with an excess (100–150 ml) of ether which produced a sticky, white precipitate. The ethereal fraction was decanted, and the solids were dried under vacuum for 1 h. The dried solids were then dissolved with MeOH:pyridine (5:1, 18 ml) and treated with H_2S (g) at 0 °C for 25 min. The mixture (solids formed during H_2S treatment) was stoppered for 5 min and then titrated to pH 1–2 with 2 N HCl. The water mixture was extracted with CH_2Cl_2, and the organic solution was washed with 1 N HCl, saturated NaCl solution, and dried-over Na_2SO_4. After the CH_2Cl_2 solution was filtered and the solvent was removed, the white solid residue was purified by passage down a silica gel column using CH_2Cl_2:MeOH (9:1) as solvent. Yield was 2.1 g (68%); ^1H NMR (d_6-acetone): δ 3.05 (m, 2), 3.52 (s, 3), 4.11 (s, 3), 4.13 (d, 2), 4.48 (m, 1), 6.18 (br, 1), 7.25 (s, 5), 7.78 (br, 1).

$C_{14}H_{18}N_2O_4S$

Calculated: C 54.19 H 5.80 N 9.03 S 10.3
Found: C 54.24 H 5.78 N 8.95 S 10.45

N-Methyloxycarbonylphenylalanylglycine ethyl dithioester was prepared as described above except ethanethiol was used in 3-fold excess in place of methanol, and ice-cold pyridine saturated with H_2S (g) for 10 min was used in place of the MeOH:pyridine mixture. Also, the silica gel column chromatography purification step used CH_2Cl_2:acetone (9:1) as eluant. Yield was 2.51 g (74%); ^1H NMR (d_6-acetone): δ 1.28 (t, 3), 3.15 (m, 2), 3.28 (q, 2), 3.51 (s, 3), 4.52 (d, 2), 4.55 (m, 1), 6.42 (br, 1), 7.32 (s, 5), 8.1 (br, 1).

$C_{15}H_{20}N_2O_3S_2$

Calculated: C 52.94 H 5.88 N 8.23 S 18.82
Found: C 52.83 H 5.97 N 8.09 S 18.66

Methods

RR Spectroscopy—RR spectra were obtained using approximately 80 milliwatts of 324 nm excitation from a Coherent Radiation 2000K krypton laser, a Spex triplemate triple monochromator, and a Tracor Northern TN-6132 diode array-based photon detection system (31). A reaction mixture consisted of 300 μl of enzyme (A_{280} = 10–14), pH 6.0, in phosphate buffer, plus 30 μl of 50 mM $CH_3OC(=O)$-Phe-$NHCH_2C(=S)OCH_3$ in CH_3CN. The organic solvent (CH_3CN) was added in order to increase substrate solubility. Prior to the reaction, either enzyme, in its mercurated form, was activated by stirring with β-mercaptoethanol (3 μl/ml) for 30 min and passing the solution down a Sephadex G-15 column (14 × 1 cm). The reaction mixture was contained in a quartz cuvette (5 × 5 × 20 mm), stirred by a small Teflon-coated magnetic stirring bar, and examined by the usual 90° illumination-detection geometry for Raman spectroscopy. Spectral accumulation was begun a few seconds after mixing, and, typically, 10 2-s exposures were accumulated. To obtain the RR spectrum of $CH_3OC(=O)$-Phe-$NHCH_2C(=S)SC_2H_5$ in its solid form, the powder was used to coat the inside of a cylindrical quartz tube which was then spun in an NMR spinner and examined in the back-scattering geometry.

pH-stat Kinetics—All pH-stat measurements were performed at 20 °C. Papain and cathepsin B were activated and assayed as described above, except that deionized distilled H_2O containing 1 mM EDTA was used for the G-15 column elution. For $CH_3OC(=O)$-Phe-$NHCH_2C(=S)OCH_3$, initial rates were measured on a Radiometer RTS822 recording titration system. The reaction medium had a final concentration of 0.3 M NaCl, 1 mM EDTA, and 20% acetonitrile for papain or 10% acetonitrile for cathepsin B in a final volume of 20 or 5 ml, respectively. The rate was determined by monitoring the addition of a standardized NaOH solution by a pH-stat apparatus. Initial rates were measured at a range of substrate concentrations of 0.01–0.15 mM for papain and 0.04–1.0 mM for cathepsin B. Plots of s/v *versus* s were linear, and kinetic parameters were determined by a linear least-squares fit of the data using weights proportional to the initial velocities. This weighting scheme was determined to be appropriate in a separate study from this laboratory.[2] The equations used are those given by Cornish-Bowden (32).

RESULTS AND DISCUSSION

The RR spectra of $CH_3OC(=O)$-Phe-$NHCH_2C(=S)SC_2H_5$ in solution and in the solid phase (Fig. 2) demonstrate that the conformations of this molecule with regard to the glycinic and dithioester linkages are very similar to those found for dithioesters of glycine derivatives $RNHCH_2C(=S)SC_2H_5$ where R is an acyl group (20–22, 33). In hydrogen-bonding solvents, the latter compounds assume two major conformational states designated A and B (33). Conformer B is characterized by a small (≃20 °) $NHCH_2$-CS(thiol) torsional angle which results from a HOMO-LUMO type attraction between the N and thiol S atoms (22). In conformer A, this torsional angle is close to 150°, and there is contact between the N

[2] P. R. Carey, R. H. Angus, H.-H. Lee, and A. C. Storer, unpublished observation.

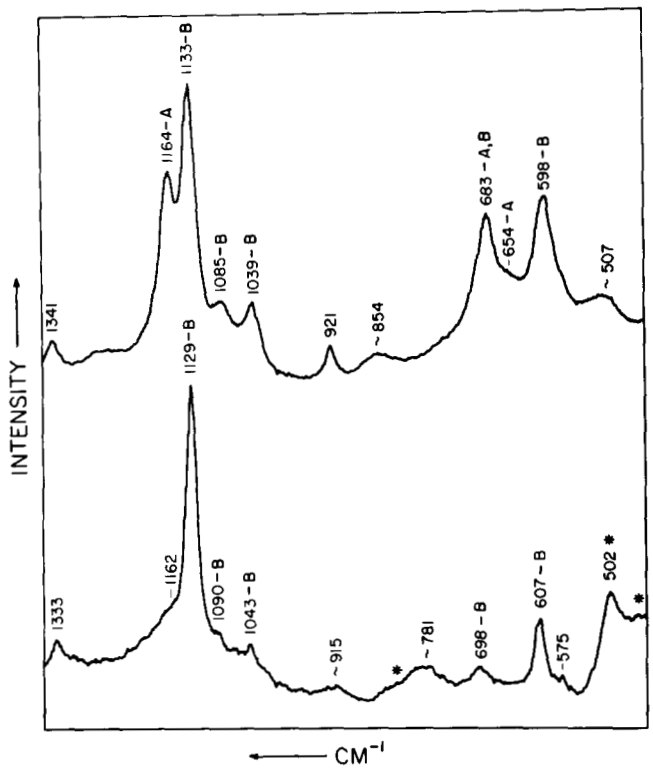

FIG. 2. **RR spectra of $CH_3OC(=O)$-Phe-$NHCH_2C(=S)SC_2H_5$ (5×10^{-4} M) in H_2O containing 1% CH_3CN (*top*) and of the powdered solid (*bottom*).** The aqueous solution was prepared by diluting a solution of the dithioester in CH_3CN into H_2O. The major A and B conformer features (see text) are labeled. The feature in the *top spectrum* at 921 cm^{-1} is due to CH_3CN. The *asterisks* in the *bottom spectrum* indicate regions where quartz makes a substantial contribution. Each spectrum is the result of 30 2-s accumulations with ~60 milliwatts of 324 nm excitation and a 12-cm^{-1} spectral slit.

Conformer B Conformer A

atom and the thiono(=S) sulfur (21). The difference in the $NHCH_2$-CS torsional angle between conformers A and B changes the vibrational coupling within the two forms, and, as a consequence, conformers A and B have separate and characteristic RR signatures. These have been studied in detail for $RNHCH_2C(=S)SC_2H_5$ type molecules (20–22, 33) and have been used to follow conformational events in dithioacyl-enzymes, $RNHCH_2C(=S)S$-enzyme, involving plant cysteine proteinases (18, 19, 30, 34). Based on these earlier studies, all the major features in the RR spectrum of $CH_3OC(=O)$-Phe-$NHCH_2C(=S)SC_2H_5$ in H_2O (Fig. 2, *top*) can be assigned to modes from conformer A or B. The peak at 1164 cm^{-1} is due to conformer A and probably contains substantial contributions from $\nu_{C=S}$ and ν_{C-C}. The 1133-, 1085-, and 1039-cm^{-1} bands are characteristic conformer B features, and the intense 1133-cm^{-1} peak is an important B conformer marker. The peak at 598 cm^{-1} is due to conformer B, while conformer A makes a contribution to the spectral intensity near 655 cm^{-1} and makes the major contribution to the 683-cm^{-1} profile. In general, conformer B is the thermodynamically more stable form and is usually the conformer found in the solid state. This occurs in the present case as evidenced by Fig. 2. In the RR spectrum of the solid form, the characteristic markers of conformer A have disappeared, leaving the markers due to conformer B, principally the intense feature at 1129 cm^{-1} and the medium feature at 609 cm^{-1}. The peak positions for the solid and solution forms do not correspond exactly because crystal-packing forces perturb the torsional angles in the $C(=O)$-NH-CH_2-C-S-CH_2 bonds from the value found in solution (22).

The substrate $CH_3OC(=O)$-Phe-$NHCH_2C(=S)OCH_3$ was chosen on the basis of its anticipated high specificity for both papain (28) and cathepsin B (14). The RR spectra of the reaction intermediates formed between the substrate and cathepsin B and papain are shown in Fig. 3. The RR spectrum for the dithioacylcathepsin B is typical of a dithioester intermediate involving a glycine-to-cysteine proteinase linkage (17) and thus provides proof of the existence of the dithioacylcathepsin B species. The *bottom trace* in Fig. 3 is for the cathepsin B reaction mixture 3 min after mixing; the substrate is depleted, and the peaks due to the dithioacylcathepsin intermediate have disappeared. The identity of the *top* and *middle traces* in Fig. 3 demonstrates that the conformations in the $-C(=O)NHCH_2C(=S)SCH_2-$ portions of the two dithioacyl-enzymes take up the same B-type forms. The $NHCH_2$-CS(thiol) torsional angles lie between ±20°, the dithioester groups are s-*cis* and planar, and the CS-CH_2C torsional angles are probably close to ±90°, and in each case the

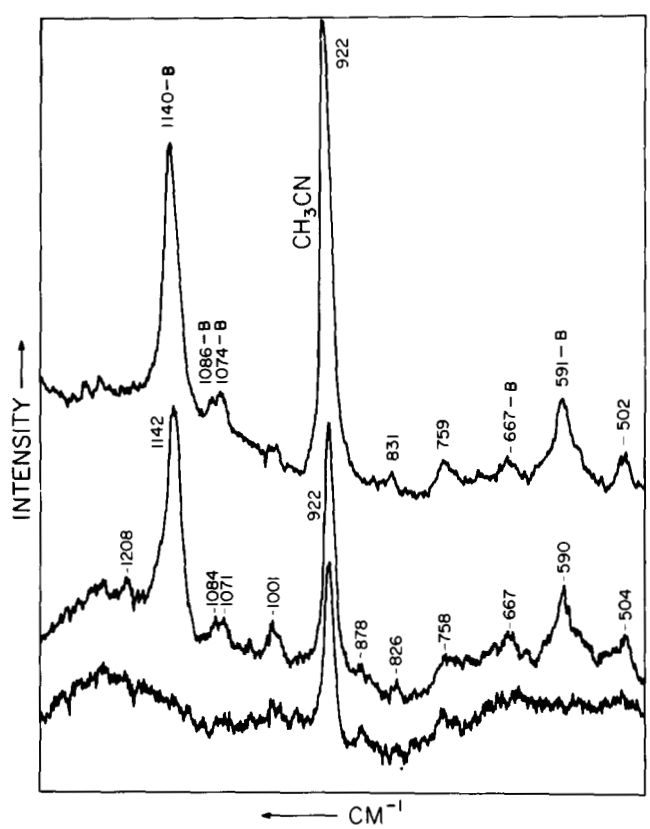

FIG. 3. **RR spectra of $CH_3OC(=O)$-Phe-$NHCH_2C(=S)S$-papain (*top*) and $CH_3OC(=O)$-Phe-$NHCH_2C(=S)S$-cathepsin B, (*center*).** Conditions are given under "Experimental Procedures." The *bottom trace* is the cathepsin B reaction mixture 3 min after mixing, when the substrate supply has been exhausted and the dithioacyl-enzyme RR peaks have disappeared. The major B conformer features are labeled, and the peaks at 922 and 760 cm^{-1} are due to CH_3CN.

values are the same or very similar for both dithioacyl-enzyme intermediates. There is no evidence for any feature in the RR spectra in Fig. 3 which is due to a species other than conformer B. This indicates that at the signal-to-noise level of the traces in Fig. 3, any other species, if present, must comprise less than 8% of the total dithioacyl-enzyme population. Thus, it seems that for the present substrate, both the plant and mammalian enzymes exert conformational selection and bind the acyl group in just one of the available states. For the critical -NH-CH_2-C(=S)-S-CH_2- linkages, the conformations are found to be the same within the limits of detection, with the N and thiol S atoms coming into close contact. Based on the earlier studies quoted above, it is possible to place tentative limits on the level at which structural differences would be detected. For example, we know that the position of the intense mode near 1130 cm^{-1} is sensitive to the $NHCH_2$-CS(thiol) torsional angle. A variation of 5° in this angle would probably be undetected; 10° is probably at the limit where differences would begin to be seen, and differences of 25° would almost certainly be detected. When considering these values, it is helpful to remember that torsional angles such as the $NHCH_2$-CS usually vary by ±10° in proteins due to dynamic fluctuations (35). It is likely that the RR spectrum is insensitive to changes of conformation in the phenylalanine portion of the substrate; therefore, no conclusions can be drawn regarding this moiety.

The conclusions of Bajkowski and Frankfater (2, 15), based on kinetic studies, that there are substantial mechanistic homologies between papain and cathepsin B find further support in the present work. For the reactions of $CH_3O(=O)$-Phe-$NHCH_2C(=S)OCH_3$ with papain and cathepsin B, the k_{cat}/K_m values are 44.1 ± 2.4 and 6.67 ± 0.14 mM^{-1} s^{-1}, and the k_{cat} values are 0.53 ± 0.01 and 1.15 ± 0.02 s^{-1}, respectively. For thionoester substrates of this type, $k_{cat} = k_3$, the rate constant for deacylation (18), and thus the papain and cathepsin B intermediates have similar deacylation rates. Taken together, the identical geometries of a critical portion of the dithioacylpapain and -cathepsin B and the similarity in k_3 values are evidence for a highly conserved deacylation process. A similar striking correspondence among dithioacyl-enzyme RR spectra and deacylation parameters has been found for several plant cysteine proteinases (18), and it is now apparent that the characteristic chemical features of conformer B have been retained for at least one mammalian cysteine proteinase.

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