RESONANCE RAMAN SPECTRA OF CHYMOTRYPSIN ACYL ENZYMES*

by

P. R. Carey and Henry Schneider

Division of Biological Sciences

National Research Council of Canada

Ottawa, Ontario, Canada K1A 0R6

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ABSTRACT

Resonance Raman spectra of cinnamoyl and α-toluyl acyl enzymes of α-chymotrypsin have been obtained. Bands associated with the aromatic portion of the acylating groups were identified and could be distinguished in a cinnamoyl derivative from those associated with the ethylenic residue. Spectral differences in the acyl enzyme relative to substrate and product were observed. These differences, which represent changes in vibrational modes of substrate bonds due to specific interaction with the active site, provide a novel approach to the study of the catalytic mechanisms of enzymes.

INTRODUCTION: Events occurring to the substrate bonds being altered during enzyme action are crucial to the catalytic process. Resonance Raman spectroscopy offers a novel way to obtaining information about such events. It provides extensive and detailed information about vibrational modes of suitable ligands bound to proteins (1,2) and therefore, could provide information about proximity, orientation and deformation effects involving the key bonds in acyl enzymes. The present paper describes for the first time the resonance spectra of acyl enzymes of α-chymotrypsin.

The present study depended largely on the use of novel ester substrates with properties allowing facile obtention of resonance Raman spectra. These properties are absorption above ~385 nm, photostability, non-fluorescence and resonance enhancement from bonds of critical interest (2). Results are described using two

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of these substrates, 4-amino-3-nitro-trans-cinnamic acid methyl ester and 4-hydroxy-3-nitro-trans-cinnamic acid ethyl ester. Data are also reported for a chromophoric substrate described by other workers (3).

MATERIALS AND METHODS: The acyl enzyme 4-amino-3-nitro-trans-cinnamoyl-α-chymotrypsin (II) was prepared by mixing for 30 seconds at room temperature 1 volume of 5×10^{-2} M I in dimethylformamide with 99 volumes of 2×10^{-4} M α-chymotrypsin in water at pH 6.5-7.0 and then rapidly lowering the pH to 3.0. Unreacted I and the product III were removed by dialysis at 4°C (7 to 12 hours). The acyl enzyme after dialysis did not release amounts of III detectable by resonance Raman measurements during 16 hours storage at pH 3.0 at 5°C. About 30% of the enzyme was acylated as determined from the amount of III produced on raising the pH to 7.5.

The absorption spectrum of I has two features above 300 nm at pH 3.0, 30% CH_3CN: λ_{max} = 323 nm, $\varepsilon = 3.29 \times 10^4$ lm^{-1} cm^{-1}; λ_{max} = 425 nm, $\varepsilon = 6.78 \times 10^3$ lm^{-1} cm^{-1}. The absorption maxima in the acyl enzyme are red shifted to 336 and 429 nm respectively. Details of synthesis of I and kinetics of hydrolysis will be presented elsewhere (4).

The acyl enzyme 2-hydroxy-3-nitro-α-toluyl-α-chymotrypsin was prepared in a flow system (4) by mixing an aqueous solution of α-chymotrypsin (2×10^{-4} M) at pH 3.0 containing IV (2×10^{-4} M) and acetonitrile (1% v/v) with a tris buffer, pH 8.6: final pH = 8.5. Raman measurements were made 15 seconds after mixing. During

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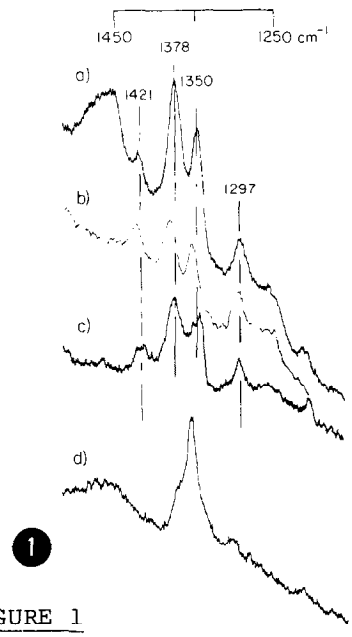
Abbreviations: I = 4-amino-3-nitro-trans-cinnamic acid methyl ester. II = 4-amino-3-nitro-trans-cinnamoyl-α-chymotrypsin. III = 4-amino-3-nitro-trans-cinnamic acid. IV = 5-nitro-2-benzofuranone. V = 2-hydroxy-5-nitro-α-toluyl-α-chymotrypsin. VI = 2-hydroxy-5-nitro-α-toluic acid.



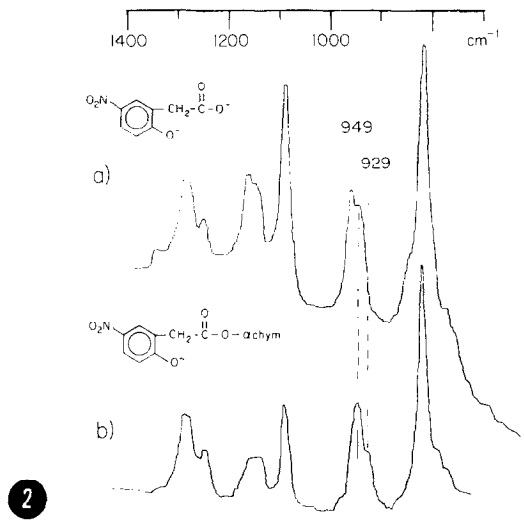


FIGURE 1

Resonance Raman spectra of:

a) 2.5 x 10^{-3} M 4-NH_2-3-NO_2-trans-cinnamic acid methyl ester (I) in MeOH.

b) 4-NH_2-3-NO_2-trans-cinnamoyl-α-chymotrypsin (II). Protein concentration 4 mg/ml (~1.6 x 10^{-4} M), active site occupancy ~30%.

c) Approximately 10^{-4} M 4-NH_2-3-NO_2-trans-cinnamic acid (III) in H_2O pH 3.0.

d) 2.5 x 10^{-3} M 4-NH_2-3-NO_2-trans-cinnamic acids methyl ester (I) in MeOD (90%); D_2O (10%, v/v).

Spectral conditions: 4416 Å excitation 20-70 mW power, 1,000 or 2,000 counts/second full scale, time constant 5 or 2 secs, spectral slit width 8 cm^{-1}.

The apparent splitting in the 1421 and 1350 cm^{-1} peaks of (c) is not reproduced in repeated spectra.

FIGURE 2

a) 2-OH-5-NO_2-α-toluic acid (VI) (doubly ionised form). 10^{-4} M in H_2O, borate buffer pH 10.2.

b) 2-OH-5-NO_2-α-chymotrypsin (V), 10^{-4} M, in H_2O, tris buffer pH 8.5, 1% CH_3CN, 15 secs after formation of acyl enzyme.

Spectral conditions 4579 Å excitation 50 mW power, 8,000-20,000 counts/sec full scale, time constant 2 secs, spectral slit width 8 cm^{-1}.

this time indetectable amounts of product VI would be produced by enzymic or base catalyzed hydrolysis (3).

Raman spectra were obtained with a Jarell Ash 25-100 spectrometer and argon, krypton or helium-cadmium lasers.

RESULTS AND DISCUSSION: Details of the interaction can be elicited through differences in the spectra of the substrate, acyl enzyme and products. A number of such differences occur (Figures 1 and 2) They consist of small shifts in frequency and substantial changes in relative intensity. Since the resonance Raman spectrum from the acylating group is so much more intense than the ordinary Raman spectrum of the protein, the protein contribution to the acyl enzyme spectrum can be ignored.

A spectral feature will be particularly valuable if it can be associated with a group frequency, if solvent effects are understood and if effects resulting from interactions at the aromatic binding site can be separated from those near the ester linkage. In spectra of I, II and III two bands have been identified meeting these criteria. The symmetric $-NO_2$ stretch at 1350 cm^{-1} is associated with the aromatic nucleus and a feature at 1625 cm^{-1} is sensitive to changes about the $-CH=CH-\overset{O}{\overset{\|}{C}}-$residue.

The peak at 1350 cm^{-1} in I, assigned to the NO_2 symmetric stretching frequency (2), is insensitive to solvent, hydrolysis to III, and ionization of the carboxyl group (pH 3.0 → 8.3) (Table 1). The shift to 1355 ± 1 cm in the acyl enzyme therefore probably represents slight twisting about the $C-NO_2$ bond (2). Preliminary studies with the substrate 4-hydroxy-3-nitro-trans-cinnamoyl-ethyl ester reveal that the symmetric NO_2 frequency in this analogue does not change on acylation by more than 5 cm^{-1}.

The band at 1625 cm^{-1} is associated with the -CH=CH-stretching vibration and probably contains a contribution from an aromatic

TABLE 1

Major Features in Raman Spectra of 4-NH_2-3-NO_2 Cinnamic Acid Derivatives

methyl ester 2 x 10^{-3} M in dimethyl sulphoxide	1623 (2.5)	a	1379 (10)	1351 (9)	1295 (2.5)	-----	818 (6)
methyl ester 3 x 10^{-3} M in CH_3CN	1627[c] (2.5)	1420	a	1351 (5)	1292 (2.5)	1260 (br)	815 (5)
methyl ester 2 x 10^{-3} M in $CHCl_3$	d	d	1375[c] (10)	1350[c] (sh)	1300[c] (2.5)	-----	825 (5)
methyl ester 10^{-3} M in MeOH	1625 (3.5)	1423 (2)	1378 (10)	1350 (7)	1296 (4)	1260 (br)	820 (5.5)
methyl ester 10^{-3} M in 90% MeOH, 10% H_2O (acidified)	1625 (3)	a	1378 (10)	1353 (7)	1296 (4)	1258 (br) (2)	822 (6)
methyl ester 10^{-3} M in 90% MeOD, 10% D_2O	1625 (2.5)	≥1430	1368 (sh)	1353[h] (10)	1297 (1.5)	-----	820 (3)
methyl ester 10^{-3} M in 90% MeOD, 10% D_2O (acidified)	1624 (2)	≥1425	1370 (sh)	1356[h] (10)	g	-----	820 (4)
4-NH_2-3-NO_2 cinnamic acid ~10^{-4}M H_2O, pH 3.0	a	1421 (13)	1378 (10)	1350 (8)	1297 (4)	-----	
4-NH_2-3-NO_2 cinnamic acid ~10^{-4}M D_2O, pD 3.0	1628 (2)	1436 (4)	1361 (10)	f	g	-----	
acyl-enzyme pH 3.0, H_2O	1625[e]	1426 (6)	1382 (10)	1355 (8)	1396 (8)	1260 (br)	825 (10)

a solvent peak
c on luminescent background
d obscured by background
e partly obscured by solvent; position confirmed in D_2O
f unresolved shoulder
g undetected
h NO_2 peak masked
br broad
sh shoulder
Approximate peak intensities are given in parenthesis.

ring mode. It is insensitive to dielectric effects (Table 1). However, it also reflects changes about the -C(=O)-O-residue. When the carboxyl group of III is protonated (pD = 3.0) it is at 1628 cm^{-1} and when ionized (pH 8.3) it is at 1638 cm^{-1}. In the acyl enzyme at pH 3.0, the band remains at 1625 cm^{-1} (Table 1).

The constancy of the ethylenic band at 1625 cm^{-1} in both

substrate and acyl enzyme indicate that the conformation about this linkage is the same in both compounds, namely trans with the phenyl and ethylenic residues essentially coplanar. This result provides for possible resolution of an ambiguity in the x-ray data for indoleacryloyl-α-chymotrypsin (5). The electron-density map for this acyl enzyme is consistent with two orientations for the indole residue relative to ethylenic residues; (1) they are planar or (2) the dihedral angle is 50°. Because of steric similarities, the 4-NH_2-3NO_2 cinnamoyl group probably adopts a conformation in the acyl enzyme very similar to the indoleacryloyl group. Thus, coplanarity in the 4-NH_2 analogue suggests this is probably true also for indoleacryloyl-α-chymotrypsin.

Resonance Raman spectra of the acyl enzyme 2-OH-5-NO_2-α-toluyl-α-chymotrypsin and the product acid are shown in Figure 2. Three major differences can be seen. The feature near 1160 cm^{-1} drops in relative intensity, a shoulder appears at 929 cm^{-1}, and there is a reduction in relative intensity of the unresolved peak at 962 cm^{-1}. These changes probably represent interactions between the substrate aromatic residue and the enzyme active site, since the π electron conjugation does not extend to the carboxyl group. Resonance Raman spectra of the substrate IV do not complicate the spectra since, unlike the acyl enzyme or product, it does not absorb at the excitation wavelength.

CONCLUSIONS: The results substantiate the view that resonance Raman spectroscopy is a useful tool for studying the catalytic mechanism of enzymes. Many spectral perturbations in substrate groupings can be detected when the acyl enzyme is formed. Effects involving the aromatic residue are readily detectable. Detailed studies with I and IV as well as with the many possible analogues will be useful in delineating interactions about the

aromatic residue. However, of greater interest is the location of a spectral region in a cinnamoyl derivative sensitive to changes involving the -C(=O)-O- grouping, the critical grouping in the chymotryptic hydrolysis of cinnamate esters. Although the carbonyl group in the acyl enzyme cannot be seen directly in the spectra, effects where it is involved can be monitored in the -CH=CH- bond because of conjugation.

The present results with cinnamoyl acyl enzymes are restricted to pH 3.0, where the acyl enzyme is essentially stable. Key questions concern the spectral changes, particularly those involving the acryloyl residue, when the acyl enzyme is converted to an active form by raising pH. Such changes have been observed and are under active study.

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