The Use of Resonance Raman Spectroscopy to Monitor Catalytically Important Bonds during Enzymic Catalysis

APPLICATION TO THE HYDROLYSIS OF METHYL THIONOHIPPURATE BY PAPAIN

(Received for publication, December 7, 1978, and in revised form, February 20, 1979)

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SUMMARY

Resonance Raman spectra were obtained of the dithio-acyl-enzyme intermediate produced during the papain-catalyzed hydrolysis of methyl thionohippurate. Intense resonance Raman features were observed in the C=S and C=S stretching regions from the intermediate's -C-S- chromophore. These results demonstrate that by using a single atom replacement, i.e. sulfur for oxygen, the catalytically crucial bonds in the ester moiety can be monitored during enzymolysis via the resonance Raman spectrum. The method can be extended to other enzymes whose catalytic mechanisms involve the formation of a thiol-acyl intermediate.

Resonance Raman spectroscopy holds considerable promise for the delineation of enzymic reactions at the molecular level (see Ref. 1 and references therein). By recording the RR spectra of a chromophoric substrate, it is possible to monitor the vibrational spectrum of the substrate throughout enzymolysis. Since vibrational spectra are sensitive to many of the effects thought to be important in enzymic reactions, new insights into mechanisms and, in general, forces in proteins, are to be expected. At the moment, however, the method suffers from two drawbacks. First, to achieve the selectivity and specificity obtainable from RR spectroscopy, chromophoric substrates with extended π electron chains have been used. In particular, the esterolysis of substrates based on the cinnamyl or furlacryloyl skeletons by chymotrypsin and papain have been studied (2-5). These are not natural substrates for the enzymes in question with the result that the turnover of the enzymes is relatively slow. Any interpretation of the data obtained must take into account this low reactivity and hence any possible differences in mechanistic detail between these and more specific substrates. The second drawback is that the intense features observed in the resonance Raman spectra of the substrates and the acyl-enzyme intermediates are associated with those parts of the cinnamoyl or furlacryloyl skeletons that are spatially removed from the bonds undergoing catalytic transformation. Thus, intense RR modes from the ester grouping: -C-X-, X = S or O, are not observed. The present communication outlines a means whereby both drawbacks are overcome and intense RR features associated with the key catalytic bonds in specific substrates can be obtained. The method relies on the use of laser sources in the near-ultraviolet, which have become available recently, and the recognition that the dithioester chromophore -C-S-, with intense absorption bands in the near-ultraviolet, may provide a suitable RR label for probing the chemistry of the ester group during enzymolysis.

The catalytic hydrolysis of ester and amide substrates by papain proceeds through the formation of an acyl-enzyme intermediate in which a covalent linkage is formed to an active site cysteine residue (6, 7). Using absorption spectroscopy, Lowe and Williams (6) were able to monitor this process during the reaction of papain with methyl thionohippurate. They observed the transient appearance of a chromophoric intermediate with a λmax at 313 nm. Using kinetic evidence and spectral comparisons with model compounds, they were able to show that this intermediate was a dithioacyl-enzyme:

Methyl thionohippurate differs from methyl hippurate, a commonly used substrate for papain, in the replacement of an oxygen atom by a sulfur atom. Thus, by a single atom replacement in a substrate, it is possible to produce a chromophoric acyl-enzyme intermediate. This obviously can be extended from methyl hippurate, to even more specific substrates, i.e. short peptide sequences. Moreover, provided the necessary thiono- substrates can be synthesized, chromophoric intermediates can also be obtained with other enzymes whose mechanisms involve the formation of a thiol-acyl intermediate.

We now report that RR spectra from the dithio-intermediate (I) can be obtained and that these spectra exhibit intense features in the C=S and C=S stretching region. Thus, the way is open for monitoring vibrational modes associated with those bonds undergoing catalytic transformation in specific substrates.

EXPERIMENTAL PROCEDURES

Materials—Papain, twice crystallized, was purchased from the Sigma Chemical Co. as a suspension in sodium acetate. The papain was further purified by the method of Humberg et al. (8) using affinity chromatography. After elution from the column, the enzyme...
was converted to inactive mercuripapain by the addition of 1 eq of mercuric chloride. The inactive enzyme was then concentrated to approximately 10 mg/ml by the partial dehydration of dialysis bags containing the enzyme, using Ficoll 400 (Pharmacia Fine Chemicals). The concentrated papain was reactivated as required by the method of Soejima and Shimura (9). The thiol content of the enzyme was determined using 5,5'-dithiobis(2-nitrobenzoic acid) as described by Ellman (10). The enzyme prepared in this way was found to contain 0.99 ± 0.02 mol of active cysteine per mol of protein.

Ethyl dithioacetate was synthesized as described by Marvel et al. (11) and was obtained as an orange oil.

Calculated: C 39.96 H 6.71 S 53.3
Found: C 40.2 H 6.75 S 53.0

Methyl thionohippurate was synthesized as described by Lowe and Williams (6). Crystals were obtained, m.p. 80-81°C.

Calculated: C 57.5 H 5.3 N 6.7 S 15.3
Found: C 57.4 H 5.4 N 6.7 S 15.5

Methods—UV absorption spectra were obtained using a Cary 118 spectrophotometer. The apparatus used to obtain the resonance Raman spectra consisted of a Coherent Radiation 3000 K krypton ion laser with a Spex 0.5-m double spectrometer and direct current detection. The near-UV laser lines were separated using a Pellin-Broca prism and plasma lines were removed from the selected laser line using a tunable grating filter F100 (PTR Optics Corp., Waltham, MA). The samples (1 ml) were contained in a 3-ml quartz fluorescence cuvette and stirred using a micromagnetic stirrer to prevent photo-degradation of the sample in the laser beam. All samples were prepared at room temperature in a buffer solution containing 20% acetonitrile, 0.3 M NaCl, 5 mM EDTA, and 50 mM sodium phosphate, pH 6.

RESULTS

Fig. 1 contains Raman spectra of solutions of papain, methyl thionohippurate, and ethyl dithioacetate obtained using 337.5 nm excitation. The Raman peaks at 758, 878, 926, 1046, 1079, and 1375 cm⁻¹ in all the spectra are due to buffer components and acetanilide. The rising baseline in the enzyme spectrum (Fig. 1a) is caused by protein luminescence. The spectrum of methyl thionohippurate (Fig. 1b) has features at 623, 707, 1006, 1157, 1207, and 1330 cm⁻¹. This spectrum is probably not resonance-enhanced to a significant extent because the major absorption band of the substrate (230 nm) is at a much shorter wavelength than the excitation wavelength. The weak absorption peak (log ε 1.4) of the substrate at 340 nm (Fig. 2) is probably a n → π⁺ transition and as such does not contribute significantly to resonance enhancement. Ethyl dithioacetate was chosen as a model for the dithioacetyl-enzyme and has a UV absorption band with λ_max at 307 nm (log ε 4.08) (6). The resonance Raman spectrum of this ester has intense features at 587 cm⁻¹ and 1192 cm⁻¹ (Fig. 1c). The peak at 1192 cm⁻¹ has been assigned in infrared spectra to the stretching vibration of the C=S bond (11-13). We tentatively assign the peak at 587 cm⁻¹ to the symmetric stretching vibration of the C=S bonds since C−S stretching frequencies are generally found in the range 570 to 720 cm⁻¹ (14-16).

An absorption spectrum of the dithioacyl-enzyme obtained using a mixture of methyl thionohippurate and papain at pH 6 is shown in Fig. 2. This spectrum was obtained within 2 min of mixing, during which time the concentration of the intermediate varies only slightly (see inset, Fig. 2). The dithioacyl-enzyme has a λ_max at 315 nm due to the dithioester group and the variation of optical density with time at this wavelength is shown in the inset to Fig. 2. After 30 min, the residual density at 315 nm is due to a side product of the reaction (P₃₀₂) which has a λ_max of 332 nm. The absorption spectrum of P₃₀₂ is shown in Fig. 2. This side product can be separated from the dithioacyl-enzyme by chromatography on Bio-Gel P₄ or by Sephadex G-25 columns.
the enzyme by passage of the reaction mixture down a Sephadex G-15 column. The amount of P_{32} produced during the reaction is dependent upon the amount of substrate used and also the pH of the reaction mixture, more is produced at lower pH. The spectrum of thiohippuric acid, the acid product of the hydrolysis of methyl thion hippurate does not contain a peak at 332 nm.

The resonance Raman spectrum of the dithioacyl-enzyme intermediate was obtained in the spectral regions where C=S and C==S stretching vibrations are expected, at various times after mixing substrate and enzyme. Each partial spectrum shown in Fig. 3 was obtained using a fresh reaction mixture, thus keeping the exposure of the samples to the laser beam to a minimum and avoiding possible complications from photo-isomerization or photodecomposition. From Fig. 3, it can be seen that an intense peak is present in the spectrum of the dithioacyl-enzyme intermediate at 1130 cm^{-1} and by comparison with the model compound this is assigned to a mode possessing C==S stretching character. Several peaks are seen in the C—S stretching region. The peak at 623 cm^{-1} is a substrate peak (see Fig. 1b) and the transient peaks at 560 cm^{-1} and 598 cm^{-1} are tentatively assigned to C—S stretching vibrations. These spectra have been reproduced on several occasions and with several preparations of enzyme and substrate. The transient peak at 1130 cm^{-1} was also observed using 334.5 nm excitation with a Coherent Radiation CR12 argon ion laser and a Spex 1401 double spectrometer.

**DISCUSSION**

A comparison of Figs. 2 and 3 shows that the time dependences of the intensities of the peaks in the absorption and resonance Raman spectra due to the dithioacyl-enzyme intermediate can be correlated, thus reinforcing the assignment of enzyme and SH by the mechanism shown in Scheme 2.

The side product, P_{32}, is thought to be dithiohippuric acid. The deacylation step in the papain-catalyzed hydrolysis of cetero and amidase is reversible (17-19), thus the thiohippuric acid product can react with the papain to give a thioacyl-enzyme and ^{35}SH by the mechanism shown in Scheme 2.

\[
\begin{align*}
\text{S} & \quad \text{OH} \\
\text{R—C} & \quad \text{C} \\
\text{S—PAPAIN} & \quad \text{O} \\
+ & \quad \text{HS—PAPAIN} \\
\text{R—C} & \quad \text{S—PAPAIN} \\
\text{SH}
\end{align*}
\]

**Scheme 2**

The ^{35}SH produced is readily detectable in the form of H_{2}S. Nucleophilic attack by the ^{35}SH ions on both the substrate and acyl enzyme would then produce dithiohippuric acid. Two further pieces of evidence for this are that dithioacetic acid has an absorption peak at 332 nm and that on treating a solution of the substrate with H_{2}S a compound is produced with a \lambda_{max} at 332 nm. Moreover, the very weak features near 625 and 1125 cm^{-1} in the spectrum after 30 min (Fig. 3b) may be due to the —CSS— chromophore of dithiohippuric acid since the RR spectrum of this moiety in ionized dithioacetic acid has intense features near 625 and 1125 cm^{-1}. In any event, the 31-min spectrum in Fig. 3b demonstrates that the stable side product does not significantly contribute to the RR spectra at shorter times. Furthermore, the low intensity RR peaks observed from the side product suggest that this species is present in very small amounts and thus any side reaction intermediate en route to the side product must be at an even lower concentration and undetectable by RR spectroscopy.

The resonance Raman spectrum of the dithioacylpapain differs markedly from that of the model compound, ethyl dithioacetate. A comparison of Fig. 3, a and b shows that in the acyl-enzyme spectrum the peak possessing C==S stretching character is shifted relative to that of the model compound by 62 cm^{-1}. Moreover, in the C—S stretching region, two peaks appear in the intermediate spectrum compared to one in that of the model. The two peaks could be due to a vibrational splitting of a band from a single chemical species, or due to more than one dithio-intermediate on the reaction pathway. Since the spectroscopic properties of the dithioester group have not been thoroughly investigated, nor time-resolved studies completed, it is premature to discuss the nature of any of these spectral differences. However, it is likely that the differences reflect the lability of the dithioester linkages in the enzyme's active site. Support for this hypothesis comes from the fact that the 62 cm^{-1} shift in the C==S stretching peak cannot be accounted for by the changes in macroscopic dielectric constant. The position of the C==S peak only varies from 1199 cm^{-1} in hexane (13) to 1192 cm^{-1} in 20% CH_{2}CN 80% H_{2}O (this paper).

A full investigation of the vibrational and electronic spectroscopy of several model compounds, together with studies on related substrate and enzyme systems, is being undertaken to provide insight into the nature of the observed spectral changes. In this way, some progress may be made on the basic problem of relating differences in kinetic rate constants to chemical changes occurring in the key substrate linkages in active sites.

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The use of resonance Raman spectroscopy to monitor catalytically important bonds during enzymic catalysis. Application to the hydrolysis of methyl thionohippurate by papain.
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J. Biol. Chem. 1979, 254:3163-3165.

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