RESONANCE RAMAN INVESTIGATION OF β-(2-FURYL)-ACRYLOYL-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

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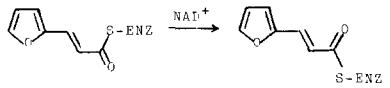
1. Introduction

Addition of NAD^+ to FA-GPD* has been shown [1] to activate furylacyl enzyme for nucleophilic attack by phosphate; concurrently, a visible spectral red shift (λ_{max} 344 nm to λ_{max} 360 nm) is observed [2]. Based upon the latter observation, it has been suggested that the chemical activation results from the following conformational change from s-*trans* to s-*cis* in the FA chromophore. The conformational change is thought to be associated with the activation of the acyl group to nucleophilic attack by phosphate:



s-*trans* s-*cis*

Similarly the formation of FA-chymotrypsin yields a visible spectrum red-shifted (λ_{max} 309 nm to λ_{max} 320 nm) compared to that for model furylacyl esters [3]. Again, s-*trans* to s-*cis* isomerization has been suggested as the basis for the visible spectral shift. However, an X-ray crystallographic study of indole-acryloyl-α-chymotrypsin has indicated that the indole-acryloyl group is in the *trans*, s-*trans* configuration [4]. Similarly a recent resonance Raman (RR) study of a substituted cinnamoyl-α-chymotrypsin shows no vibrational spectral change upon formation of acyl enzyme [5].

In contrast, a resonance Raman study of cinnamoyl-papain shows major changes of ν(C=C) accompanying the red visible spectral shift upon acylation of papain [6].

In order to further investigate the events accompanying covalent acyl enzyme formation in chymotrypsin and the ligand-induced activation of FA-GPD, we report the RR spectrum of FA-α-chymotrypsin, FA-GPD and its NAD^+ complex and model compounds of FA-thio and oxygen esters.

* For a list of abbreviations used in this paper see table 1

2. Materials and methods

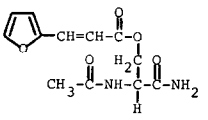
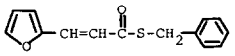
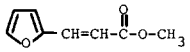
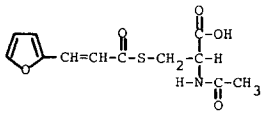
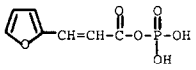
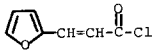
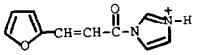
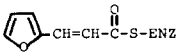
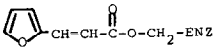
2.1. *Isolation of GPD*

GPD was prepared from sturgeon muscle by the method in [7]. Protein concentration was determined from A_{280} using $\epsilon_{280} = 1.29 \times 10^5\ M^{-1}\ cm^{-1}$. Specific activity was determined by the method in [8]. Typical enzyme preparations had spec. act. 290 μM NADH/mg/min; this is somewhat lower than the 330 μM NADH/mg/min reported [7]. The enzyme was also assayed for 'active' SH groups using Ellman's reagent (DTNB); typical spectrophotometric determination yielded 90% of the expected 4 SH groups/tetramer. Enzyme was precipitated with ammonium sulfate and stored as a suspension in the presence of NAD^+ at 5°C; in this form the enzyme could be stored for several weeks without loss of activity.

2.2. *Acylation of GPD*

Before acylation, enzyme was centrifuged at 31 000 rev./min for 40 min; the pellet was suspended in pH(D) 7.0, 0.01 M ethylenediamine buffer containing 0.1 M KCl and 10^{-3} M EDTA. Excess

Table 1

Compound	Structure	Abbreviation
N-acetyl-*O*-β-(2-furyl)-acryloyl-serinamide[a] λ_{max} 309 nm		FA-serine
β-(2-furyl)acryl-oyl-thiobenzyl-ester[b] λ_{max} 337 nm		FA-thiobenzylester
β-(2-furyl)acryl-oyl-methyl ester[c] λ_{max} 306 nm		FA-methyl ester
N-acetyl-S-β-(2-furyl)-acryloyl-cysteine[d] λ_{max} 338 nm		FA-cysteine
β-(2-furyl)acryl-oyl-phosphate[b] λ_{max} 307 nm ϵ = 26 200		FAP
β-(2-furyl)acryl-oyl-chloride[c]		FA-chloride
β-(2-furyl)acryl-oyl-imidazole[c] λ_{max} 353 nm ϵ = 27 000		FAI
β-(2-furyl)acryl-oyl-glyceraldehyde-3-phosphate dehydrogenase λ_{max} 344 nm ϵ = 30 000		FA-GPD
β-(2-furyl)acryl-oyl-α-chymotrypsin λ_{max} 320 nm		FA-chymotrypsin

[a] [3], [b] [1], [c] [9], [d] [10]

ammonium sulfate was removed by gel filtration on P-30 Biogel. NAD^+ was added to bring the ratio of NAD^+/tetramer to 0.5; this procedure was necessary because NAD^+ is an effector needed for acylation. Determination of the NAD/enzyme ratio was carried out on the basis of A_{280}/A_{260}. Acylation was achieved by incubation of 5×10^{-3} M FAP with enzyme for 15 min at 25°C. Excess FAP was removed by gel filtration on Biogel P-30. The number of FA groups/enzyme tetramer was assigned from A_{345} ($\epsilon = 3.0 \times 10^4$ M^{-1} cm^{-1}) and A_{280} ($\epsilon = 1.29 \times 10^5$ M^{-1} cm^{-1}). Typically acylation produced enzyme which contained 1.0–2.2 acyl groups/tetramer. The concentration of FA-GPD after Biogel filtration was insufficient to allow determination of the RR spectrum, so enzyme was concentrated in a collodion bag dialysis apparatus by application of vacuum to produce reverse osmosis. Final concentration of FA-GPD of 350–500 μM allowed determination of the RR spectrum.

α-Chymotrypsin was 3 × crystallized purchased from Worthington Biochemical Co. Solutions of 1 mM α-chymotrypsin in 0.1 M acetate buffer (pH(D) 4.0) were acylated with FAI in acetonitrile. The reaction of FAI is quantitative; therefore we carried out acylation with an excess of α-chymotrypsin sufficient to avoid an RR signal from free FAI.

2.3. *Model compounds and substrates*

Furylacrylic acid was obtained from Aldrich Chemical and recrystallized from ethanol–water. For preparation of other model compounds see references in table 1.

2.4. *Raman spectroscopy*

RR* spectra were recorded at room temperature on a Spex 1401 laser Raman spectrometer with a Coherent Radiation Laboratories Model 52 Argon ion laser using the excitation line at 488 nm. Spectral resolution was 5 cm^{-1}. Samples were introduced into

* Raman spectra using 488 nm excitation are exciting into the low ν tail of the FA electronic transition and are more properly termed 'pre-resonance' rather than RR spectra. Enhancement factors between 10^2 and 10^3 were obtained

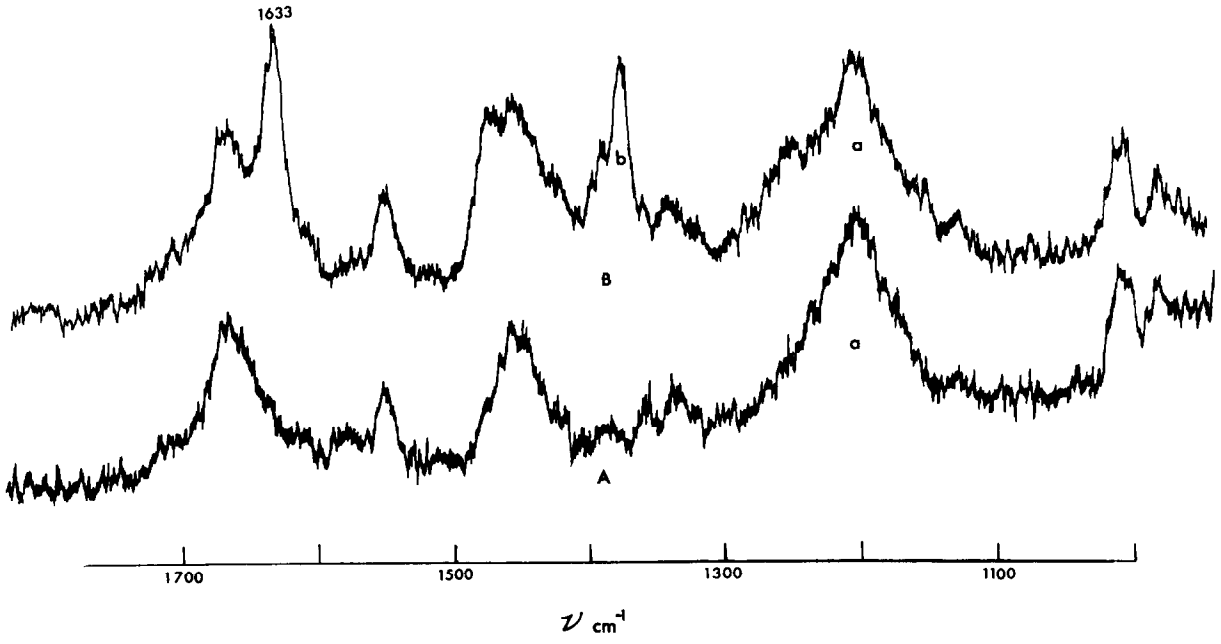


Fig.1.(A) RR spectrum of α-chymotrypsin in pD 4.0, 0.1 M acetate buffer. [α-chymotrypsin] = 1.0×10^{-3} M. (B) RR spectrum of FA-α-chymotrypsin in pD 4.0, 0.1 M acetate buffer. The peak labeled (a) is the result of ν(O–D) for D_2O while (b) is a peak associated with acetonitrile.

the Raman spectrometer in capillary tubes and the Raman scattered light was detected at 90° to the exciting laser beam. Samples were stable over the course of the experiments with no degradation of the Raman spectral bands. SO_4^{-2} (0.05 M) was employed as an internal standard in GPD spectral experiments.

3. Results and discussion

Figure 1(B) shows the RR spectrum of FA-chymotrypsin in D_2O; the Raman spectrum of chymotrypsin at the same instrument settings is shown in fig.1(A). A recent isotopic labeling study of cinnamoylpapain has indicated that a band at 1640–1570 cm^{-1} in cinnamoyl derivatives represents the ν(C=C) vibrational mode [6]. One of the major spectral bands in FA-chymotrypsin occurs at 1633 cm^{-1} and is assigned to ν(C=C). This vibrational mode has been shown to be sensitive to the s-*trans*–s-*cis* conformational change [$\Delta\nu$(C=C) = 10–20 cm^{-1} lower frequency in s-*cis* compounds] [11,12] and is one of the major peaks in intensity as expected for a ν(C=C) band. The position of this band in the spectra of each of the several species studied is given in table 2.

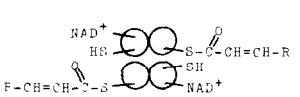
As may be seen, the ν(C=C) band occurs at the same frequency in FA-chymotrypsin and in the model systems, FA-serine and FA-methyl ester. This indicates that no major change in bonding or conformation occurs in the furylacryloyl group covalently bound to the active site at α-chymotrypsin. There are no significant differences between model and FA-chymotrypsin occurring in other spectral bands. These data suggest that the changes in the visible spectrum occurring during acylation of chymotrypsin by FAI may be associated with bonding changes which result in an energetically lowered excited electronic state rather than bonding changes in the ground state.

Table 2

	ν(C=C), (cm^{-1})
FA-chymotrypsin	1633
FA-methyl ester	1635
FA-serine	1637
FA-GPD	1600
FA-GPD–NAD^+	1587
FA-thiobenzylester	1614
FA-cysteine	1609

The RR spectrum of FA-GPD is shown in fig.2(B) along with the spectrum of unacylated enzyme, fig.2(A). As mentioned previously, a large visible spectral change accompanies binding of NAD^+ to FA-GPD; fig.2(C) indicates that the ν(C=C) frequency also shows a marked change upon NAD^+ binding. Besides the frequency change, addition of NAD^+ also produces a large increase of intensity in ν(C=C) bond relative to the SO_4^{-2} internal standard shown as (a) in fig.2. Table 2 also shows that ν(C=C) of the model compounds, FA-cysteine and FA-thiobenzylester occurs at ~1612 cm^{-1} while NAD^+-liganded FA-GPD has the ν(C=C) band at 1587 cm^{-1}. The NAD^+-induced RR spectral change of FA-GPD is quite similar to that observed for the change in ν(C=C) on formation of FA-papain and cinnamoylpapain [6]. In all these cases the intensity of ν(C=C) increases and there is a shift to lower frequency.

This contrasts with FA-chymotrypsin where no vibrational spectral change accompanies the red visible spectral shift observed upon formation of FA-enzyme. Correspondingly, X-ray analysis has indicated that the conformation of indoleacryloyl-α-chymotrypsin is *trans*, s-*trans* [4], the same conformation as that observed in solutions of the free chromophores. Therefore, conformational changes do not account for the visible spectral red shift in FA-chymotrypsin. We suggest that the Raman spectral changes observed in the NAD^+ complex of FA-GPD and FA-papain result from a conformational change from *trans*, s-*trans* in solution of FA derivatives to *trans*, s-*cis* on the enzyme surface. Studies using Raman spectroscopy indicated that such a conformational change would be expected to cause a decrease of 10–15 cm^{-1} in ν(C=C) [11,12]. Such a conformational change might also be expected to cause increased reactivity of the carbonyl group to nucleophilic attack as discussed elsewhere [3]. If this suggestion is correct, the binding of two additional NAD^+ molecules to the following FA-GPD is responsible for a conformational change in the FA chromophore:



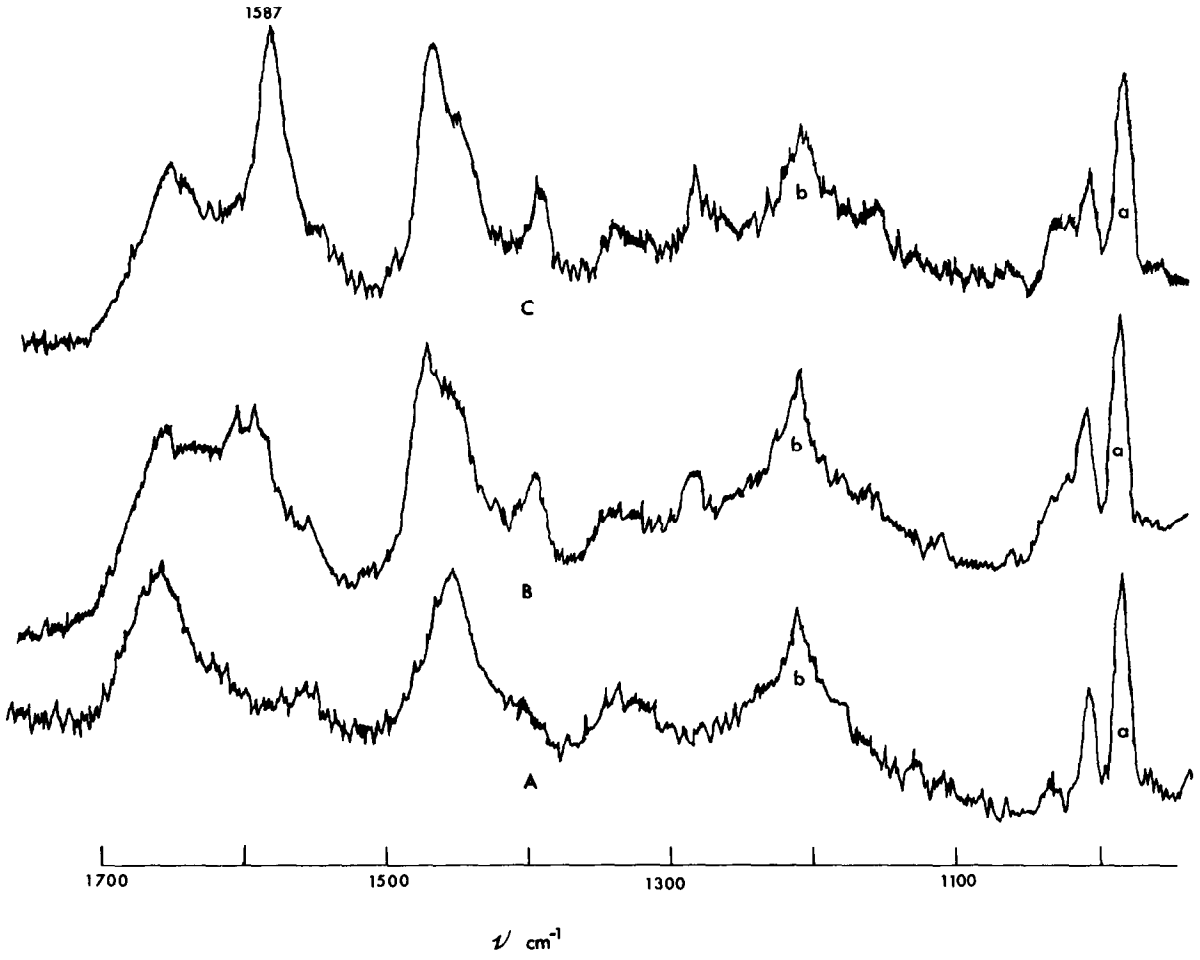


Fig.2.(A) RR spectrum of GPD in D_2O, pD 7.0, 0.1 M ethylene diamine buffer containing 0.1 M KCl and 10^{-3} M EDTA. [GPD] = 400 μM (tetramer). (B) RR spectrum of FA-GPD, same conditions as (A). (C) RR spectrum of FA-GPD–NAD^+. Final concentration of [NAD^+] = 1.0×10^{-3} M. The peak labeled (a) is ν(O–S) for 0.05 M SO_4^{-2} added as an internal standard. The peak labeled (b) is ν(O–D) for D_2O.

Thus, addition of NAD^+ to the site of an FA group would cause the s-*trans*, s-*cis* conformational change accompanied by a red visible spectral shift and a shift to lower energy of ν(C=C). Accompanying the conformational change, the acyl group would be activated for reaction with phosphate.

Acknowledgements

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