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Resonance Raman and Electronic Absorption Spectral Studies of Some β-(2-Furyl)acryloylglyceraldehyde-3-phosphate Dehydrogenases†

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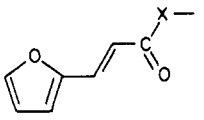
ABSTRACT: Electronic absorption and resonance Raman spectra, together with rates of arsenolysis, are used to show that the acylated subunits of the rabbit furylacryloylglyceraldehyde-3-phosphate dehydrogenase (FA-rabbit enzyme), but not the FA-sturgeon enzyme, exist as a mixed population of at least two forms. One form of the FA-rabbit enzyme has a high rate of arsenolysis, and its near-UV absorption maximum (λ_{max}) is red shifted on binding NAD^+, while the other has a lower rate of arsenolysis, and its absorption spectrum appears to be unaffected by excess NAD^+. The FA-sturgeon enzyme exists as a single population that has a high rate of arsenolysis, and its λ_{max} is red shifted on binding NAD^+. This red shift and the concomitant position of $\nu_{C=C}$, the band due to the ethylenic double bond stretching vibration in the resonance Raman spectrum, indicate that in the presence of NAD^+ the π electrons of the FA chromophore are polarized, i.e., a permanent dipole has been set up along the long axis of the FA group. It is argued that this does not result in activation of the carbonyl group by setting up polarization (i.e., $^{\delta+}C-O^{\delta-}$) within that group alone. A comparison of the $\nu_{C=C}$'s and λ_{max}'s for a series of FA derivatives of the type



where X = H, N, or O, shows a clear correlation. However, when X = S as, e.g., in thiol esters, this correlation breaks down. This is explained in terms of a possible through-space $d\pi-p\pi$ overlap between the ethylenic bond π orbitals and the empty sulfur 3d orbitals.

D-Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12; GDH)[1] catalyzes the NAD^+-dependent reversible oxidative phosphorylation of D-glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate, via the formation of a covalent acyl enzyme intermediate in which the acyl group is linked to the sulfur of an active-site cysteine (Harris & Waters, 1976). The enzyme, composed of four chemically identical subunits, exhibits cooperativity in the equilibrium binding of NAD^+ and "half-of-the-sites reactivity" toward some active-site-specific acylating and alkylating agents (Seydoux et al., 1974b). To investigate the elementary steps of the enzyme reaction, Malhotra & Bernhard (1968, 1973) used the substrate analogue β-(2-furyl)acryloyl (FA) phosphate and obtained the chromophoric acyl enzyme furylacryloylglyceraldehyde-3-phosphate dehydrogenase with catalytic properties similar to those of the physiological intermediate. The absorption spectrum of the FA group was used to monitor the catalytic steps in the enzyme reaction (Malhotra & Bernhard, 1968, 1973; Berni et al., 1977; Vars et al., 1979).

The FA group is also a suitable resonance Raman (RR) probe of the enzyme reaction allowing the vibrational spectrum of the substrate to be recorded during enzymolysis (Carey, 1978). In this paper we report kinetic data, together with absorption and resonance Raman spectroscopic studies, on the FA enzymes obtained by using GDH from rabbit and sturgeon muscle. The interpretation of the resonance Raman and some of the absorption data uses, as a framework, the ideas developed in the two preceding papers (MacClement et al., 1981; Phelps et al., 1981). The first area considered in the present work concerns the heterogeneity of furylacryloyl binding in

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[1] Abbreviations used: FA, β-(2-furyl)acryloyl; FA-P, FA-phosphate; FA-S, thiol ester or thiol acyl enzyme of FA; FA-rabbit enzyme and FA-sturgeon enzyme, respectively, rabbit and sturgeon muscle FA-glyceraldehyde-3-phosphate dehydrogenases; GDH, glyceraldehyde-3-phosphate dehydrogenase; FA-GDH, FA-glyceraldehyde-3-phosphate dehydrogenase; RR, resonance Raman; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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the active sites. Whereas half-of-the-sites reactivity has hitherto been discussed in terms of the differences between acylated and nonacylated sites, a finding presented here is that for the FA-rabbit enzyme the acylated subunits exist as a mixed population of at least two forms. The second area dealt with concerns the activation of the FA group by the addition of excess NAD^+ to the acyl enzyme. Other workers have indicated that a red shift of the FA absorption maximum seen upon the addition of NAD^+ signifies activation of the FA group (Bernhard & Lau, 1972), i.e., the C=O group becomes more C^+–O^- like and thus more prone to nucleophilic attack. However, the absorption and RR data taken together argue against such an activation for the FA substrate, although activation may well occur for the natural substrate. Another proposal for activation of the FA group in the acyl enzyme toward catalytic attack concerns possible *s*-trans to *s*-cis isomerism promoted by NAD^+ binding prior to nucleophilic attack [Schmidt et al. (1978) and references therein]. The present spectroscopic data are unable to confirm or refute this hypothesis. Evidence is presented however which suggests that such a conformational change would not make the carbonyl more prone to catalytic attack. The last area to be discussed in this work concerns the unexpected and atypical spectroscopic properties of furylacryloyl thiol esters and acyl enzymes. The d orbitals of the sulfur atom are proposed as a possible source of this anomalous behavior.

Materials and Methods

The barium salt of β-(2-furyl)acryloyl phosphate (FA-P) was synthesized by the method of Malhotra & Bernhard (1968). β-(2-Furyl)acryloyl thioethyl ester was obtained by treating furylacrylic acid (Aldrich, recrystallized and decolorized; 1.38 g, 10 mmol) and triethylamine (1.1 g; 10 mmol) dissolved in tetrahydrofuran (50 mL) with ethyl chloroformate (Aldrich; 1.08 g, 10 mmol). After 10 min the reaction mixture was filtered by suction to remove triethylamine hydrochloride, and the resulting solution was treated with 5 drops of triethylamine and 2 mL of ethanethiol. After 19 h the mixture was partitioned between an aqueous solution of 5% $NaHCO_3$ and ethyl ether. The ether extract was dried over anhydrous Na_2SO_4 and the ether removed by rotary evaporation to give a pale brown liquid (yield 93%). The NMR spectrum was in excellent agreement with that expected for this compound. FA-imidazole and the methyl ester were prepared according to MacClement et al. (1981).

Glyceraldehyde-3-phosphate dehydrogenase was obtained from rabbit skeletal muscle (sp act. = 147 units/mg) by the method of Ferdinand (1964) and from sturgeon muscle (sp act. = 320 units/mg) by the method of Seydoux et al. (1973). Both enzymes were stored as crystalline suspensions of the holoenzyme in solutions of saturated ammonium sulfate.

Enzyme from both sources was acylated with FA-P by the method of Malhotra & Bernhard (1968). The enzyme was first dissolved in a minimum of buffer containing 0.01 M ethylenediamine hydrochloride, 0.1 M KCl, and 0.001 M EDTA at pH 7.0 and the ammonium sulfate removed by passage down a Sephadex G-15 column. The enzyme was then allowed to react with 5 mM FA-P for 15 min at room temperature in the presence of 1 enzyme equiv of added NAD^+. The acyl enzyme produced was then separated from unreacted substrate by passage down a second Sephadex G-15 column.

Denatured enzyme samples were prepared by the precipitation of the enzyme with 0.07 M perchloric acid followed by dialysis overnight against distilled water. The precipitated protein was then redissolved by carefully adding it to an equal volume of a 2% $NaDodSO_4$ solution.

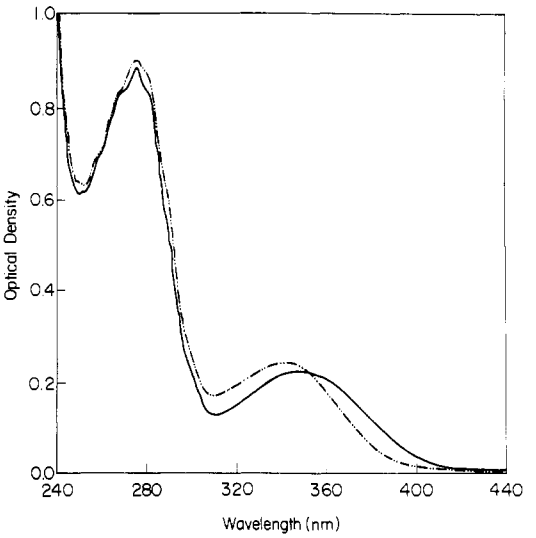


FIGURE 1: Electronic absorption spectra of (—) sturgeon muscle and (–··–) rabbit muscle FA-GDH. The number of acyl groups per tetramer in both cases is ~1.3.

Absorption spectra were obtained by using a Cary 118 spectrophotometer. The apparatus used to obtain the resonance Raman spectra consisted of a Coherent Radiation 3000K krypton ion laser, a Spex 0.5m double spectrometer, and direct-current detection. The near-UV laser lines were separated by using a Pellin-Broca prism. The thiol ester and thiolacyl enzyme analogues of furylacryloyl were found to be more photolabile than their oxygen counterparts. In a sample cell consisting of a stationary quartz cuvette photodegradation occurred rapidly with 350.7-nm excitation. Thus, a flow system (using a syringe pump) was used in which the sample made a single pass through a quartz capillary tube arranged at right angles to the laser beam. A pump speed was chosen such that photodegradation of the sample was negligible. All spectral measurements were made at room temperature in a buffer solution containing 0.01 M ethylenediamine hydrochloride, 0.1 M KCl, and 0.001 M EDTA at pH 7.0. Raman peak positions were reproducible to ±1 cm^{-1}; calibration using noble gas discharge lines showed that the absolute values were accurate to better than ±2 cm^{-1}.

Results

Typical absorption spectra of both rabbit and sturgeon muscle furylacryloylglyceraldehyde-3-phosphate dehydrogenase are shown in Figure 1. The numbers of acyl groups incorporated per tetramer [calculated as described in Malhotra & Bernhard (1968)] were consistently in the ranges 1.2–1.5 and 1.4–1.7 for the rabbit and sturgeon enzymes, respectively. The acyl enzyme contained NAD^+ bound at the nonacylated active sites (Malhotra & Bernhard, 1968). Excess NAD^+ caused a red shift in the FA portion of the acyl enzyme absorption spectra (Figure 2) In the presence of 1 mM NAD^+ the λ_{max} at 342 nm of the FA-rabbit enzyme is shifted to 350 nm with an ~15% reduction in ϵ_{max}, whereas with the sturgeon enzyme the peak at 348 nm is shifted to 364 nm with no reduction in ϵ_{max}. On denaturation of the rabbit acyl enzyme, the absorption peak at 342 nm blue shifts to 334 nm (Figure 2).

The difference spectra of the acyl enzymes in the presence and absence of 1 mM NAD^+ both have maxima at 380 nm and minima at 332 nm (Figure 2). The changes in optical density at 380 nm plotted as a function of the added NAD^+ concentration for both enzymes (Figure 4) also show similar behavior. However, the magniture of the changes seen in

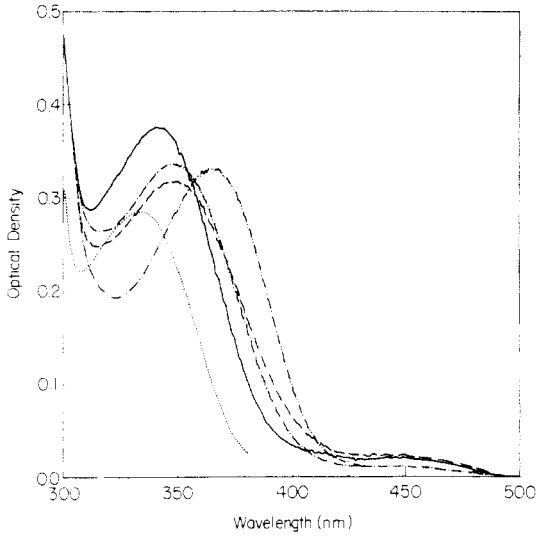


FIGURE 2: Electronic absorption spectra of FA-rabbit enzyme in the absence (—) and presence (– – –) of 1 mM NAD^+; denatured FA-rabbit enzyme in the absence of added NAD^+ (···); FA-sturgeon enzyme in the absence (–·–) and presence (–··–) of 1 mM NAD^+.

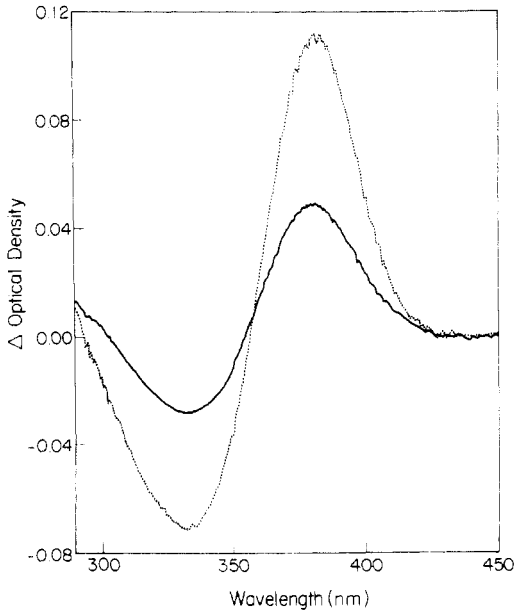


FIGURE 3: Difference spectra of FA-rabbit enzyme (—) and FA-sturgeon enzyme (···) in the presence and absence of 1 mM NAD^+. The acyl group concentrations were 11 and 13 μM, respectively.

Figures 3 and 4 for the rabbit acyl enzyme is half that observed for the sturgeon acyl enzyme. This difference is discussed below.

Both acyl enzymes undergo phosphorolysis and arsenolysis in the presence of NAD^+ and inorganic phosphate or arsenate, respectively. The arsenolysis reaction was monitored by observing the change, with time, in the acyl enzyme absorption at 350 nm in the presence of 2 mM arsenate. The reaction of arsenate with the FA-rabbit enzyme was found to be biphasic (Table I). Under the same conditions the sturgeon enzyme reacts with a single rate constant. In the absence of added phosphate or arsenate there is a slow rate of hydrolysis of the acyl enzymes. For the rabbit enzyme this hydrolytic rate increases slightly upon addition of NAD^+ whereas the hydrolytic rate for the sturgeon enzyme is unaffected by NAD^+ (Table II).

The resonance Raman (RR) spectra obtained in the range 1200–1670 cm^{-1} for both the rabbit and sturgeon acyl enzymes in the absence and presence of 1 mM NAD^+ are shown in

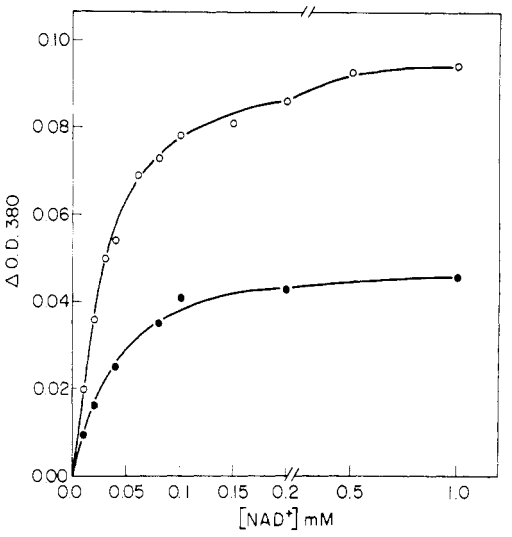


FIGURE 4: Plots of ΔOD_{380} obtained for FA-rabbit (●) and FA-sturgeon (○) enzymes as a function of added NAD^+. The acyl group concentrations were 9 and 11 μM, and the normalized limiting values, at ∞ $[NAD^+]$, for ΔOD_{380} were 0.0042 and 0.0085 $\Delta OD_{380}/\mu M$ acyl group, respectively.

Table I

acyl enzyme	acyl group concn (μM)	% acyl enzyme reacting with rate k	pseudo-first-order rate constant of arsenolysis, k[a] (s^{-1})
FA-rabbit	30	50–70[b]	0.078
		30–50[b]	<0.001
FA-sturgeon	30	>96	~0.27

[a] Measured in the presence of 2 mM sodium arsenate and 1 mM NAD^+ at room temperature. Rate constants were determined from the data by the method of Swinbourne (1960). [b] Variations within these limits were observed between different acyl enzyme preparations.

Table II

acyl enzyme	hydrolytic rate[a] no NAD^+	hydrolytic rate[a] 1 mM NAD^+
FA-rabbit	0.09×10^{-3} (3)[b]	0.2×10^{-3} (3)
FA-sturgeon	0.17×10^{-3} (4)[b]	0.17×10^{-3} (4)

[a] Units are the percent change in OD_{355} per second at room temperature; 355 nm is the isosbestic point for the acyl enzyme in the presence and absence of 1 mM NAD^+ (see Figure 3). [b] Values given are the averages of the number of determinations shown in parentheses.

Figure 5. For the enzyme from sturgeon the value for $\nu_{C=C}$, the band due to the ethylenic double bond stretching frequency, is seen in Figure 5 to be 1597 and 1592 cm^{-1} in the absence and presence of NAD^+, respectively. These values using 350.7-nm excitation compare to those reported under preresonance Raman conditions (using 488.0-nm excitation) of 1600 and 1587 cm^{-1} (Schmidt et al., 1978). The discrepancies could be due to the improved quality of the present data or, possibly for the spectra taken in the presence of NAD^+, that excitation at 488.0 nm preferentially enhances the Raman spectrum of a minor population of acyl enzymes having an absorbance maximum to the red of that due to the majority of acyl enzymes. The RR spectrum of the denatured FA-rabbit enzyme is shown in Figure 6. Because of the intensity-enhanced RR spectrum from the FA chromophore, the protein makes no contribution to the spectra shown in Figures 5 and 6. A weak broad feature due to the O–H bend of water is seen at 1645 cm^{-1}.

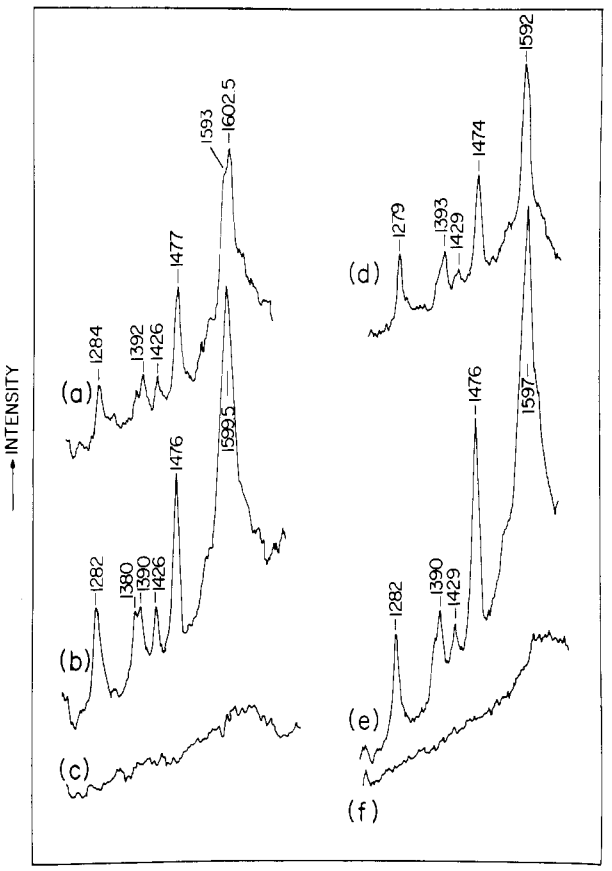


FIGURE 5: Resonance Raman spectra of (a) FA-rabbit enzyme + 1 mM NAD^+, (b) FA-rabbit enzyme, (c) nonacylated rabbit enzyme + 1 mM NAD^+, (d) FA-sturgeon enzyme + 1 mM NAD^+, (e) FA-sturgeon enzyme, and (f) nonacylated sturgeon enzyme + 1 mM NAD^+. The acyl group concentrations were in the range 30–40 μM, and the concentration of enzyme tetramers was 25–30 μM. The spectra were obtained by using 100 mW of 350.7-nm excitation light, a spectral split of 8 cm^{-1}, and a scan speed of 1.5 cm^{-1}/s.

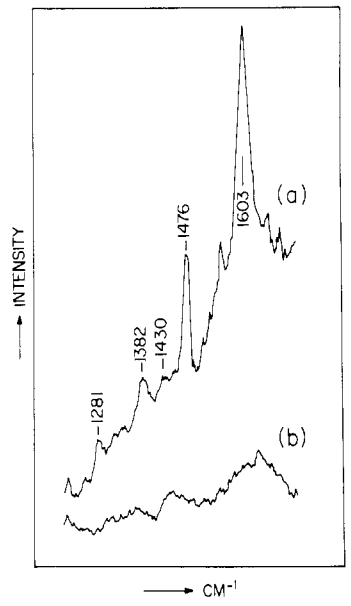


FIGURE 6: (a) Resonance Raman spectrum of denatured FA-rabbit enzyme in 1% $NaDodSO_4$ in the absence of added NAD^+ and (b) Raman spectrum of a ($NaDodSO_4$) 1% solution. Conditions were as described for Figure 5.

The assignments of the peaks in resonance Raman spectra of FA derivatives are discussed by MacClement et al. (1981). They assign the peaks near 1475 and 1390 cm^{-1} to furan ring modes, the peak near 1280 cm^{-1} to an unspecified acryloyl mode, and the peak near 1600 cm^{-1} to the acryloyl ethylenic

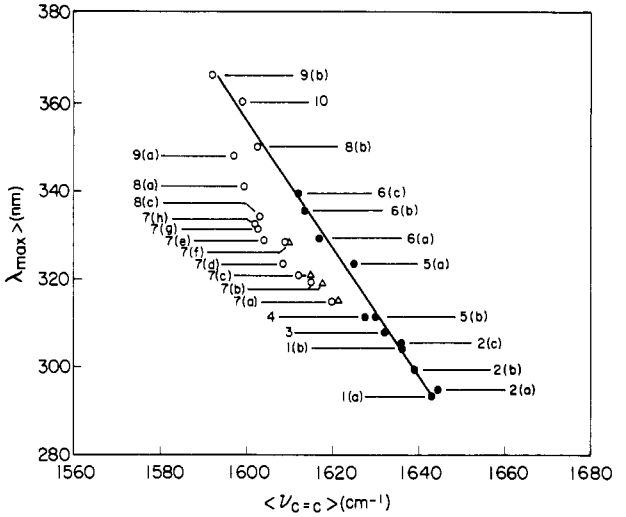


FIGURE 7: Plot of $\langle \nu_{C=C} \rangle$ against $\langle \lambda_{max} \rangle$ for a variety of FA derivatives: (1) furylacrylic acid (a) ionized in H_2O and (b) protonated in H_2O; (2) methyl ester of furylacrylic acid (a) in hexane, (b) in CH_3CN and (c) in H_2O; (3) furylacryloyl phosphate at pH 7.0 in H_2O; (4) FA-CHO (furylacrolein) in CH_3CN; (5) FA-chymotrypsin (a) at pH 3.0 and (b) denatured; (6) FA-imidazole (a) in 100% CH_3CN, (b) in CH_3CN–H_2O (1:1), and (c) in CH_3CN–H_4O (1:9); (7) FA-SCH_2CH_3 (a) in hexane, (b) in CCl_4, (c) in CH_3CN, (d) in CH_3-CN–H_2O (3:1), (e) in CH_3CN–H_2O (1:4), (f) in Me_2SO, (g) in Me_2SO–H_2O (1:4), and (h) in H_2O; (8) FA-rabbit enzyme (a) – NAD^+, (b) + 1 mM NAD^+, and (c) denatured; (9) FA-sturgeon enzyme (a) – NAD^+ and (b) + 1 mM NAD^+; (10) FA-papain at pH 3. The open symbols represent the data points obtained for FA thiol derivatives by using 350.7-nm (○) and 647.1-nm (△) excitation, and the closed symbols represent the data points for the other FA derivatives obtained by using 350.7-nm (●) excitation.

stretching vibration, $\nu_{C=C}$. The assignments of the peaks near 1380 and 1426 cm^{-1} are not discussed. The exact positions of these peaks for an aqueous solution of furylacryloyl thioethyl ester are 1284, 1377 (shoulder), 1390, 1428, 1479, and 1602 cm^{-1}. No Raman peaks were observed for the acyl enzymes above 1650 cm^{-1}, and therefore the carbonyl moiety could not be monitored.

The $\nu_{C=C}$ values for a wide range of FA derivatives in a variety of solvent systems are plotted in Figure 7 as a function of the λ_{max} of the near-UV FA electronic transition. The $\nu_{C=C}$ and λ_{max} values for FA-papain and FA-chymotrypsin were taken from Carey et al. (1978)[2] and MacClement et al. (1981), respectively.

Discussion

Heterogeneity of FA Binding. The kinetics of arsenolysis (Table I), the RR data (Figure 5), and the absorption data (Figures 3 and 4) all bear on the problem of heterogeneous binding of the FA-acyl groups in the active site. In the foregoing papers (MacClement et al. 1981; Phelps et al., 1981) on acylchymotrypsins only one kind of heterogeneity had to be considered, viz., that due to different acyl conformers sitting in a population of identical active sites. For FA-GDH complexes we must consider, in addition, that the active sites themselves are capable of assuming different conformations; this we term active-site heterogeneity whereas the different conformers of FA bound to identical active sites will be called substrate or FA heterogeneity.

The arsenolysis results given in Table I indicate that in the presence of NAD^+ the FA-rabbit enzyme contains two pop-

[2] Figure 2 in this reference is correct. However, the value of $\nu_{C=C}$ for furylacryloyl-papain and furylacryloylimidazole (in 5% dimethylformamide, 95% H_2O) in Table I of this reference should read 1599 and 1612 cm^{-1}, respectively.

Table III: Ratio of Bandwidths at Half-Height of Ethylenic Mode at ~1600 cm^{-1} to That of Furyl Ring Mode at ~1476 cm^{-1}, Taken from Figures 5 and 6

	−NAD$^+$	+NAD$^+$
FA-rabbit enzyme (denatured)	2.3	
FA-rabbit enzyme (native)	2.4	2.9
FA-sturgeon enzyme (native)	2.6	1.6

ulations of acyl groups with differing rates of arsenolysis but that the arsenolysis of the FA-sturgeon enzyme proceeds with a single rate constant suggesting a single population of acyl groups for this enzyme. Consonant with this result, the RR data provide evidence for a homogeneous population (in both substrate and enzyme conformation) of FA-sturgeon enzyme in the presence of NAD$^+$, whereas heterogeneity is apparent under the same conditions for the derivative from the rabbit enzyme. In the preceding papers (MacClement et al., 1981; Phelps et al., 1981) it is argued that a broad ethylenic stretching frequency $\nu_{C=C}$ is evidence for a heterogeneous population of FA groups. If the bandwidths at half-height of the FA-GDH complexes are taken as a measure, both FA-rabbit and FA-sturgeon enzymes, in the absence of NAD$^+$, show broad $\nu_{C=C}$ features (Figure 5 and Table III) compared to the furyl ring mode at 1476 cm^{-1}. This broadening could be due to enzyme or substrate heterogeneity, or both. Upon addition of NAD$^+$, the band shape of $\nu_{C=C}$ for FA-sturgeon enzyme narrows and becomes similar to that of the furyl mode at 1477 cm^{-1} (Table III). This is evidence that the FA group in the sturgeon enzyme, in the presence of NAD$^+$, is primarily in a single conformation in a single population of active sites. However, this is not the case for the rabbit enzyme, after addition of NAD$^+$, $\nu_{C=C}$ for the FA-GDH broadens slightly compared to the 1477-cm^{-1} ring mode (Table III). In fact, a shoulder near 1593 cm^{-1} is seen on the main 1602.5-cm^{-1} peak for each acyl rabbit enzyme preparation. Thus, for the rabbit acyl enzyme + NAD$^+$ the FA group may be in more than one conformation in a single population of active sites or in conformationally distinct active sites. It is not likely, however, that substrate heterogeneity alone can account for the kinetic results since this would require interconversion between the substrate conformers to occur at a slower rate than the second stage of the biphasic deacylation. It is also possible that both forms of heterogeneity coexist for the acyl rabbit enzyme.

The absorption data can now be discussed in the light of the above findings. Both rabbit and sturgeon hologlyceraldehyde-3-phosphate dehydrogenases contain four tightly bound NAD$^+$ molecules per tetramer [for a dicussion of the properties of glyceraldehyde-3-phosphate dehydrogenases from a variety of sources see Harris & Waters (1976)]. The bound NAD$^+$ is essential for both the acylation and deacylation reactions to occur; however, upon acylation the NAD$^+$ molecules are expelled from the acylated subunits (Malhotra & Bernhard, 1968, 1973). The expelled NAD$^+$ molecules can be forced back into the active sites by exposing the acyl enzyme to high concentrations of NAD$^+$ (Malhotra & Bernhard, 1973). The binding of NAD$^+$ to the acylated subunits is accompanied by a red shift in the absorption spectrum of the bound FA moiety. The shift for the acyl sturgeon enzyme is from 348 to 364 nm with no reduction in intensity of the peak. However, for the acyl rabbit enzyme the shift is smaller, from 342 to 350 nm, and there is a 15% loss in intensity for the peak (Figure 2). Crucially, Figure 3 demonstrates that the underlying *changes*, bringing about the apparent shifts in λ_{max} in Figure 2, are the same. For both acyl enzymes the addition of NAD$^+$ causes an increase in absorbance at 380 nm and a decrease at 332 nm. The magnitude of these changes is different for the rabbit and sturgeon preparations, and an explanation can be advanced on the basis of the heterogeneity of FA binding. After addition of NAD$^+$, the increase in OD at 380 nm per acyl enzyme is approximately twice as large for the sturgeon compared to the rabbit enzyme (Figures 3 and 4). Given the heterogeneity of the FA sites in the rabbit enzyme in the presence of NAD$^+$ and the homogeneity of the sites in the sturgeon enzyme, we propose that with excess NAD$^+$ approximately half the FA-rabbit enzyme population possesses an environment about the FA group identical with the environment about the FA group in the homogeneous FA-sturgeon enzyme population. The main evidence is that the absorption changes shown in Figure 3 take place in identical positions for both acyl enzymes but, in magnitude, are half as great for the "rabbit" compared to the "sturgeon" acyl enzyme. The model also accounts for the shoulder at 1593 cm^{-1} in the RR spectrum of FA-rabbit enzyme + NAD$^+$ which is in the same position as the sharp 1592-cm^{-1} peak in the RR spectrum (Figure 5) of FA-sturgeon enzyme + NAD$^+$. On this basis, it also seems likely that the ~50% of the FA-rabbit enzyme that red shifts on binding NAD$^+$ is the same 50–70% that reacts with arsenate at the faster rate (see Table I). At 1 mM NAD$^+$ the rabbit enzyme appears to be saturated with NAD$^+$ (Figure 4), i.e., there is no evidence for the binding of NAD$^+$ to a second set of sites with a lower affinity; therefore, either only ~50% of the rabbit enzyme will bind NAD$^+$, in the range of NAD$^+$ concentrations used, or the binding of the NAD$^+$ to the remaining 50% of the acylated subunits has no effect on the absorption spectra of the FA groups at these sites. Since bound NAD$^+$ is essential for the acylation step (it is unlikely that a fraction of the enzyme that is acylated cannot bind NAD$^+$ unless this binding capacity has been lost after the acylation step.

The heterogeneity of FA-rabbit enzyme preparations was also noted by Malhotra & Bernhard (1968), who reported that in the presence of excess arsenate, phosphate, or NADH the disappearance of the 342-nm absorption band did not follow first-order kinetics. The same authors provide strong evidence that this heterogeneity is not due to nonspecific acylation, and they demonstrate the specific acylation by FA-P of the active-site thiol of cysteine-149 (Malhotra & Bernhard, 1968).

The existence of isozymes or tetramer asymmetry are other possible sources of heterogeneity. However, no evidence has been found to suggest that rabbit muscle glyceraldehyde-3-phosphate dehydrogenase is composed of a mixture of isozymes. The enzyme has been obtained by several different purification procedures in many laboratories, and in each case no evidence was found for a heterogenic enzyme preparation (Ferdinand, 1964; Allison & Kaplan, 1964; Bloch et al., 1971; Scheek & Slater, 1978). The possibility of the glyceraldehyde-3-phosphate dehydrogenase tetramers being asymmetric has been suggested by several workers who supply ample evidence to support their claims (Malhotra & Bernhard, 1968, 1973; Stallcup & Koshland, 1973a–c; Watson et al., 1972; Buehner et al., 1973; Bode et al., 1975; Moras et al., 1975; Garavito et al., 1977; Kellershohn & Seydoux, 1979). However, this asymmetry was proposed to account for the difference between subunits that are acylated by FA-P (two per tetramer) and subunits that are not acylated by FA-P. Asymmetry was not proposed to account for any differences between populations of acylated subunits.

The differences between the two acyl rabbit enzyme populations could be explained by a chemical modification of ap-

proximately half the acylated subunits. That only half of the subunits are affected is possibly evidence against such an explanation, although due to the complex nature of this enzyme it cannot be excluded entirely. Since the rabbit enzyme reacts with excess FA-P in the presence of NAD^+ with a single pseudo-first-order rate constant (Malhotra & Bernhard, 1968; this study, data not presented), it seems likely that any modification would occur after acylation. In this regard Bode et al. (1975) reported that two alkylatable sites, not at the active centers, are created by acylation of the rabbit muscle enzyme with FA-P, and these authors concluded that the pseudosubstrate causes an intramolecular rearrangement that exposes two sulfhydryl groups other than those of the nonacylated active-site cysteines. These exposed thiols could be possible candidates for a chemical modification, e.g., oxidation, that abolishes the red shift and alters the kinetics of the acyl enzyme.

The favored explanation for the two populations of the acyl rabbit enzyme is that acylation of two identical sites occurs, followed by a conformational change for each tetramer which leads to the two acylated sites becoming dissimilar. MacQuarrie & Bernhard (1971) proposed an intramolecular rearrangement of the subunits in the tetramer to explain a mixed population of subunits obtained after deacylation of an alkylated sample of FA-rabbit enzyme. They produced FA enzyme containing two acyl groups per tetramer and then alkylated the two remaining active-site thiols by using iodoacetic acid. Deacylation of this enzyme (the deacylation kinetics were very similar to those of the nonalkylated acyl enzyme, i.e., complex) produced a dialkylated enzyme of which only one active-site thiol (per tetramer) could be reacylated by FA-P. They suggest that this nonequivalence of the two subunit populations is induced after deacylation; however, the possibility exists that it could occur before deacylation. This would account for the complex deacylation kinetics and also the heterogenic composition of the acyl enzyme described above.

The conformational change following the acylation of two sites on each tetramer could be a manifestation of the half-of-the-sites reactivity exhibited by the enzyme (Seydoux et al., 1974b). NAD^+ binding to the rabbit muscle enzyme has been shown to be negatively cooperative in the presence and absence of acylating agents (Boers & Slater, 1973). The coenzyme binds very tightly to two of the subunits with equal affinities; however, the dissociation constants for NAD^+ to the two remaining sites are weaker and dissimilar, i.e., $K_1 = K_2 < K_3 < K_4$ (Boers & Slater, 1973). In the absence of acylating agents, the sturgeon enzyme also exhibits negative cooperativity in the binding of NAD^+ with, in this case, two classes of sites, i.e., $K_1 = K_2 < K_3 = K_4$ (Kelemen et al., 1975). The 3-phosphoglycerol sturgeon acyl enzyme exhibits a hyperbolic binding function for NAD^+, i.e., all four sites bind NAD^+ with the same affinity (Seydoux et al., 1974a). By use of the theory of half-of-the-sites reactivity (Seydoux et al., 1974b), it can be argued that acylation of GDH by FA-P occurs on the two subunits that bind NAD^+ the most tightly and that after acylation a conformational rearrangement occurs such that the acylated subunits are now equivalent to the two subunits with lower affinities for NAD^+. This would explain the heterogeneity of the FA-rabbit enzyme preparations (the two acylated subunits have different affinities for NAD^+) and the homogeneity of the FA-sturgeon enzyme preparations (the two acylated subunits have the same affinity for NAD^+).

Evidence for Through-Space $d\pi$–$p\pi$ Interaction between Sulfur and Ethylenic Orbitals in FA–Sulfur Compounds. In the first paper in this series (MacClement et al., 1981) it was shown that a broad RR band due to the ethylenic stretching mode was evidence for the coexistence of a number of conformers involve *s*-cis–*s*-trans isomerizations about the ethylenic single bonds, and since each conformer gives rise to a slightly different $\nu_{C=C}$, the measured band $\langle\nu_{C=C}\rangle$ contains more than one unresolved contribution within its band profile. This situation probably pertains to the RR spectrum of denatured FA-GDH shown in Figure 6. The ring modes, e.g., at 1476 cm^{-1}, are little perturbed by conformers involving the acryloyl moiety and are sharp compared to $\langle\nu_{C=C}\rangle$ at 1603 cm^{-1} (Table III).

The second generalization discussed in MacClement et al. (1981) was that for any substituted furylacryloyl or thienylacryloyl derivative a plot of the measured absorption maximum $\langle\lambda_{max}\rangle$ against $\langle\nu_{C=C}\rangle$ gave a straight line. Each chromophore generates its own line, e.g., in Figure 10 of MacClement et al. (1981) the imidazole, acid, ester, and chymotrypsin derivatives of thienylacryloyl lie on a line separate to that generated by the same derivatives of 5-methylfurylacryloyl. Points can be shifted along the line, e.g., by a change in the equilibrium among the isomers involving the acryloyl group, by inductive effects within the molecules (e.g., $-COOCH_3$ going to –CO–imidazole), or by changes in the dielectric and hydrogen bonding properties of the solvent. Exceptions to the $\langle\nu_{C=C}\rangle$ vs. $\langle\lambda_{max}\rangle$ correlation are found when the laser beam used in the Raman experiment sets up a new photochemically generated equilibrium among the isomers of the acryloyl group (MacClement et al., 1981) or when steric crowding occurs about the acryloyl carbonyl (Phelps et al., 1981). Remarkably, most sulfur esters of the furylacryloyl group lie off the $\langle\nu_{C=C}\rangle$ vs. $\langle\lambda_{max}\rangle$ plot generated by other derivatives of the FA group (Figure 7). Three sulfur derivatives do, however, lie on the correlation, viz., FA-papain (at pH 3.0) and FA-GDH for both the rabbit and sturgeon enzymes in the presence of NAD^+. Although the point for the rabbit enzyme is a measured value, it likely represents a blend resulting from two different situations (vide infra), and it may be coincidental that this point falls on the line. Care was taken to ensure that the reproducibility of the $\langle\nu_{C=C}\rangle$'s in Figure 7 was ± 1 cm^{-1}. Moreover, the fact that $\langle\nu_{C=C}\rangle$'s for the thiol ester, measured by using 647.1- and 350.7-nm excitation, were the same within experimental error (Figure 7) demonstrates that artifacts due to photoisomerization were absent.

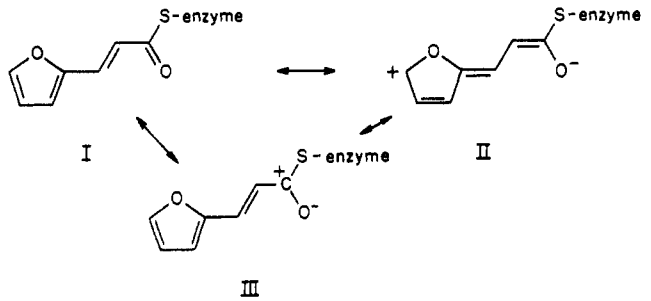
The anomalous behavior of the thiol compounds can be explained on the basis of the properties of the $d\pi$ orbitals of sulfur. Sulfur d orbital overlap with ethylenic π orbitals has been suggested to cause the bathochromic shifts seen for β,γ-unsaturated sulfides (Jaffe & Orchin, 1962; Fehnel & Carmack, 1949; Koch, 1949,) and, in the present work, sulfur is the only element substituted on the –C=O group in Figure 7 possessing empty and accessible d orbitals. It is therefore proposed that through-space $d\pi$–$p\pi$ overlap between the sulfur d orbitals and the $p\pi$ orbitals associated with the ethylenic double bond occurs and that this interaction gives rise to a chromophore which is different from the furylacryloyl chromophore in the absence of sulfur. Since different chromophores lie on different $\langle\nu_{C=C}\rangle$ vs. $\langle\lambda_{max}\rangle$ plots, the S compounds possessing $d\pi$–$p\pi$ overlap no longer lie on the "furylacryloyl" plot. Conformational changes completely destroying the $d\pi$–$p\pi$ interaction will cause the FA-S points to revert to the FA plot, and, thus, on this basis, the FA-sturgeon enzyme + NAD^+ and FA-papain points lying on the main FA line in Figure 7 have no $d\pi$–$p\pi$ interactions. The FA-S points lying off the main correlation in Figure 7 may

come from spectral data that represent $\nu_{C=C}$ and λ_{max} from a single conformer, or, more likely, the measured spectral parameters may come from a blend of isomers each having quantitatively different $d\pi$–$p\pi$ interactions. The existence of a multiplicity of isomers, each with a slightly different $d\pi$–$p\pi$ interaction, accounts for the fact that the FA-S points lying off the main correlation do not form a second straight line since, in this case, the number of chromophores can increase with the number of possible isomers.

Two possible effects, involving conformational change of the partial charge residing on the sulfur atom, could disrupt the $d\pi$–$p\pi$ interactions in FA–thiol compounds. Thus, in FA-papain and the FA-sturgeon enzyme in the presence of NAD^+, the FA group may be forced into a conformation in which $d\pi$–$p\pi$ overlap is minimal. For example, it appears from space-filling models that the *s*-cis conformation about the =C–C=O single bond has poor $d\pi$–$p\pi$ overlap compared to the *s*-trans conformer. The second possible factor which could minimize $d\pi$–$p\pi$ interaction concerns the fractional positive charge on the sulfur atom. In valence bond terms resonance structures of the type

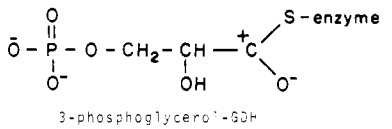
result in a partial positive charge on the sulfur (Nyquist & Potts, 1959). Such a charge may be necessary for optimum $d\pi$–$p\pi$ overlap (Jaffe & Orchin, 1962) since in the absence of a fractional positive charge (δ^+) the d orbitals are extremely diffuse and poor $d\pi$–$p\pi$ overlap will result. The δ^+ charge causes the d orbitals to contract increasing charge density at the point of overlap with the ethylenic π orbitals. Clearly, on this basis, any effect reducing the amount of positive charge on the S atom will reduce the $d\pi$–$p\pi$ overlap. Reduction of the δ^+ may be brought about by placing another positively charged group near the sulfur. In the FA-GDH enzymes in the presence of NAD^+, an arginine residue that is uncovered on NAD^+ binding (Nagradova et al., 1978) or the NAD^+ itself (Biesecker et al., 1977) is a good candidate. In the acylpapain the protonated imidazole of histidine-159 may be responsible since it is proposed that this positively charge residue donates a proton to the sulfur atom during hydrolysis of the acyl enzyme (Lowe, 1976). On the basis of the present data, however, we cannot favor either the conformatonal change or the charge explanation.

"Activation" of the Carbonyl Group. Since FA-GDH from sturgeon is homogeneous in the presence of NAD^+ and since this species lies on the $\langle\nu_{C=C}\rangle$ vs. $\langle\lambda_{max}\rangle$ curve (Figure 7), complications due to possible $d\pi$–$p\pi$ overlap (above) do not occur. As discussed in the foregoing papers (MacClement et al., 1981; Phelps et al., 1981), a low $\langle\nu_{C=C}\rangle$ and a high $\langle\lambda_{max}\rangle$ for an acyl enzyme are good evidence that the entire π-electron system is polarized, i.e., it possesses a permanent dipole moment due to the importance to the true structure of resonance (canonical) structures of types II and III.



It is this polarization which causes the red shift in λ_{max} along the line in Figure 7 compared to the other compounds obeying the $\langle\nu_{C=C}\rangle$–$\langle\lambda_{max}\rangle$ correlation. There are several possible causes of polarization (MacClement et al., 1981), one or more of which may occur in the present systems. Importantly, it can be argued that in compounds of the type FA enzyme, a red shift resulting from polarization throughout the FA chromophore causes a deactivation of the C=O toward nucleophilic attack (Phelps et al., 1981). This is because the favored resonance form for attack is III whereas forms of the type II, which are important when polarization is found, reduce the contribution of III to the true structure. A second factor adding to the overall stability of a red-shifted acyl enzyme is that the drop in $\langle\nu_{C=C}\rangle$ shows that the energy of the electronic ground state is lowered and thus the activation energy to the transition state may be increased.

It has been suggested that the red shift in λ_{max} of FA-GDH on addition of NAD^+ reflects an activation of the acyl group by NAD^+ (Malhotra & Bernhard, 1973). Although complicated by $d\pi$–$p\pi$ effects, it is likely that this red shift involves some increased electron polarization in the FA-S chromophore, and, on the basis of the above argument, we would not expect a marked increase in the C^+–O^- character of the carbonyl. In fact, although the addition of NAD^+ causes a marked red shift for FA-GDH for both the sturgeon and rabbit enzymes, the rate of hydrolysis is unaffected for the former and only slightly increased for the latter (Table III). This result, although supporting our ideas on electron polarization in the entire FA-S system, is therefore inconsistent with the hypothesis that the red shift is a direct result of an activation of the acyl group, e.g., by an increase in the polarization of the carbonyl group. Further support for this conclusion comes from the fact that FA-GDH, from the yeast enzyme, does not red shift upon the addition of NAD^+, although the enzyme is active in all respects (Byers & Koshland, 1973). For the complexes from the rabbit and sturgeon sources considered here, the rates of both arsenolysis and phosphorolysis of the acyl enzyme are greatly increased in the presence of NAD^+. In the FA-GDH, therefore, it is likely that the NAD^+ aids arsenolysis and phosphorolysis by bringing about a conformational change (Bolotina et al., 1967; Schlessinger & Levitzky, 1974; Nagradova et al., 1978) which uncovers the inorganic phosphate (or arsenate) binding site near the carbonyl. This site formation may also occur for the natural 3-phosphoglycerol acyl enzyme with the added effect that carbonyl activation *can* occur for the natural substrate. This is because for FA-GDH, binding NAD^+ near the acyl group sets up polarization throughout the chromophore (e.g., structure II) at the expense of polarization ($-C^+-O^-$) of the carbonyl. In the natural substrate, which does not possess and extended π-electron system, the induced partial positive charge remains on the carbonyl; it cannot be further delocalized. Thus



As an additional source of activation of the FA-GDH molecules by NAD^+, it has been proposed that, upon binding, NAD^+ causes an *s*-trans to *s*-cis isomerization about the =C—C=O single bond in the FA group thereby removing the α hydrogen from the proposed path of the attacking ^-OH nucleophile (Charney & Bernhard, 1967; Schmidt et al., 1978). The 13-cm^{-1} shift to lower wavenumbers reported for the preresonance Raman spectrum of the FA-sturgeon enzyme

after addition of excess NAD^+ (Schmidt et al., 1978) was cited as evidence for *s*-trans to *s*-cis isomerization. A smaller shift of 5 cm^{-1} is noted in the present work. However, as described in MacClement et al. (1981), the possible causes of such a shift and concomitant changes in the absorption spectra include charge effects, hydrogen bonding, changes in dielectric constant, and local and extended dipoles, in addition to isomerizations of the acryloyl moiety. The present spectroscopic evidence, while firmly identifying the occurrence of electron polarization in the FA ligand, cannot precisely define the causes of that effect. It may be argued, however, that the *s*-trans to *s*-cis isomerism would not on steric grounds make the C=O more accessible since nucleophilic attack does not take place along the line of the C=O bond but an an angle of ~107° to it (Bürgi et al., 1973). Thus, the α hydrogen in the *s*-trans compound is not expected to sterically hinder nucleophilic attack.

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