Role of Nicotinamide Adenine Dinucleotide as an Effector in Formation and Reactions of Acylglyceraldehyde-3-phosphate Dehydrogenase†

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ABSTRACT: The equilibrium spectral and reactivity properties of a chromophoric acylglyceraldehyde-3-phosphate dehydrogenase (FA-GPDH) have been previously reported. Transient studies of these properties are reported herein. As with true 3-phosphoglyceroyl-enzyme these properties depend on the presence of bound coenzyme (NAD^+). The reactivity of the acyl-enzyme toward acceptors (phosphate and arsenate) parallels the extent of its NAD^+-induced spectral change [Malhotra, O. P., & Bernhard, S. A. (1973) *Proc. Natl. Acad. Sci U.S.A. 70*, 2077–2081]. The transcient deacylation of FA-GPDH, preincubated with NAD^+, is kinetically biphasic. The relative amplitudes of the fast vs. the slow phase depend on NAD^+ concentration but are independent of the nature and concentration of the acyl acceptor. At saturating NAD^+ and acceptor concentrations, kinetic biphasicity persists. Perturbation of the acyl-apoenzyme spectrum by NAD^+ is also kinetically biphasic. Evidence is presented that the NAD^+-requiring acylation of the enzyme results in a protein conformation in which the acyl group is both spectrally perturbed and reactive toward acyl transfer. This acyl-enzyme undergoes a relatively slow isomerization to a conformation in which the acyl spectrum is unperturbed and unreactive in acyl transfer. These two acyl-enzyme conformations are also distinguished by their relative affinities for NAD^+; hence, NAD^+ is an effector of the conformational equilibrium. Kinetic biphasicity, wherever observed, can be accounted for in terms of two processes: (1) reactivity of the "active" acyl conformation and (2) slow isomerization of the inactive to the active conformation. The two acyl-enzyme conformers are present in finite albeit variable amounts dependent on the extent of NAD^+ ligation. Evidence is presented suggesting that each of these conformers has a unique function.

Glyceralde-3-phosphate dehydrogenase (GPDH)[1] is special among the *allosteric* enzymes in that the chemical properties of a catalytically competent covalent acyl-enzyme intermediate (eq 1) are modulated by the noncovalent interaction with the

$$\underset{\text{D-glyceraldehyde 3-phosphate}}{\text{RCHO}} + NAD^+ + \underset{\text{enzyme}}{\text{E–SH}} \rightleftarrows \underset{\text{acyl-enzyme}}{\text{E–S–CO–R}} + NADH + H^+ \quad (1a)$$

$$\text{E–S–CO–R} + HPO_4^{2-} \underset{NAD^+}{\overset{NAD^+}{\rightleftharpoons}} \text{E–SH} + \text{RCO–}OPO_3^{2-} \quad (1b)$$

effector NAD^+ (Racker & Krimsky, 1952; Harting & Velick, 1954; Hilvers & Weenen, 1962; Malhotra & Bernhard, 1968). In resting muscle the concentration of *acyl*-enzyme accounts for an appreciable fraction of the total concentration of the glycolytic intermediates from fructose 1,6-diphosphate to phosphoenolpyruvate (Bloch et al., 1971). The extent of acylation affects the avidity of NAD^+ binding at the enzyme site. Since the concentration of active sites is comparable to the total NAD^+ concentration, the equilibrium free energy of formation of NAD^+ in situ is affected by this acylation–deacylation equilibrium.

Models of allosteric regulation of enzyme activity usually share the assumption that a redistribution between active and inactive enzyme conformational states occurs upon interaction with an effector (Monod et al., 1965; Koshland et al., 1966; Atkinson et al., 1965; Stadtman, 1970). In a previous paper, we drew attention to the necessity of considering, in addition, the role of the effector in the properties of covalent enzyme–substrate intermediates (Malhotra & Bernhard, 1973). Earlier, we described the reaction of a chromophoric pseudosubstrate, β-(2-furyl)acryloyl phosphate (FAP), with GPDH giving rise to furylacryloyl-GPDH (FA-GPDH which is an analogue of the 3-phosphoglyceroyl-enzyme intermediate in its chemical properties and in the dependence of these properties on bound NAD^+ (Malhotra & Bernhard, 1968, 1973; Seydoux et al., 1973). This acyl chromophore has advantages over the true acyl substrate for mechanistic studies since changes of bond hybridization (and hence of reactivity) and changes in covalent ligation are readily measured and subject to chemical interpretation. As has already been shown, FA-GPDH undergoes all of the reactions of the "true" acyl-enzyme and shows similar affinity and reactivity dependence on effector ligands. Ligand-dependent changes in the acyl-bond structure, for the various reactions of eq 1 are directly discernable in the FA-enzyme, whereas similar ligand dependence is only inferable from the kinetic data with 3-phosphoglyceroyl-enzyme (Schwendimann et al., 1976). Reaction of the holoenzyme with excess FAP yields only a *di*-FA-enzyme tetramer. This stoichiometric limitation to two out of four reactive thiols is consistent with a variety of anticooperative properties observed with this enzyme. Anticooperative NAD^+ binding (Conway & Koshland, 1968; Boers et al., 1971; Seydoux et al., 1973; Scheek & Slater, 1978), anticooperative chemical and thermal inactivation of GPDH (Givol, 1969; Malhotra et al., 1978), and the anticooperative kinetics of reduction and deacylation of tetra-3-phosphoglyceroyl-enzyme (Seydoux & Bernhard, 1974; Kellershohn & Seydoux, 1979) demonstrate the functional nonequivalence of sites in the tetrameric enzyme made up of four covalently identical polypeptides (Harris et al., 1963; Perham & Harris, 1963; Seydoux et al., 1973). Thus, the chromophoric acyl-enzyme is analogous to 3-phosphoglyceroyl-enzyme in regard

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[1] Abbreviations used: GPDH, glyceraldehyde-3-phosphate dehydrogenase; NAD, nicotinamide adenine dinucleotide; EDTA ethylenediaminetetraacetic acid; ATP, adenosine 5′-triphosphate.

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to protein conformational interactions as well as chemistry of catalysis. We have therefore chosen to investigate chemical and physical properties of the diacyl-enzyme (FA-GPDH). We are less hesitant in drawing physiologically relevant conclusions from such experiments because of the following.

All of the reactions indicated in eq 1 are not only observable with the pseudosubstrate (furylacryloyl) but also the observable catalytic processes are dependent on NAD^+ and/or NADH concentrations in a manner quantitatively identical with that found with the true substrate (Kellershohn & Seydoux, 1979; Kelemen et al., 1975; Seydoux et al., 1976). This is especially significant since the avidity of interaction of the enzyme with NAD is very highly dependent on whether or not the active-site sulfhydryl group is acylated, whereas NADH ligation is virtually unaffected by acylation. Since the furylacryloyl and the 3-phosphoglyceroyl residues are structurally quite distinct, the common affinity for coenzyme in the two derivatives must reflect a common binding site for the coenzyme whose structure is dependent on acylation but is independent of the nature of the acyl group.

The chromophoric properties and the reactive properties of FA-GPDH are related. The binding of NAD^+ to the acylated sites results in a large spectral perturbation of the furylacryloylacyl-enzyme. The thioester electronic transition is shifted to the red, from 345 to 360 nm (~5 kcal/mol), upon binding of NAD^+. This large shift of spectrum is obligatory for catalytic reaction with acyl acceptors (phosphate and arsenate) (Malhotra & Bernhard, 1973).

These convenient and qualitatively revealing properties of the furylacryloyl group permit us to perform three types of experiments, all relevant to the physiological reaction system and all dealing with the interrelationship between acylation, nucleotide binding, and reactivity. These experiments all deal with the effect of NAD^+ on the kinetic pathway and on the properties at equilibrium; they involve the formation of acyl-enzyme from acyl phosphate, the spectral perturbation of the acyl-enzyme upon interaction with nucleotide, and the kinetics of the acyl-transfer process. The significance of these results for the modulation of the physiological reaction and for the glycolytic metabolism is discussed.

Experimental Procedures

Materials. Sturgeon muscle GPDH was isolated by the method of Seydoux et al. (1973). NAD^+ and glyceraldehyde 3-phosphate diethyl acetal barium salt were purchased from Sigma Chemical Co. An aqueous solution of DL-glyceraldehyde 3-phosphate was prepared as described by Furfine & Velick (1965). NAD^+ and glyceraldehyde 3-phosphate were assayed enzymatically by the procedure of Ferdinand (1964). β-(2-Furyl)acryloyl phosphate barium salt was prepared and solubilized as described earlier (Malhotra & Bernhard, 1968). Its concentration was assayed spectrophotometrically ($\epsilon_{307nm} = 2.62 \times 10^4\ M^{-1}\ cm^{-1}$). All other reagents were of analytical grade.

Protein and Enzyme-Bound NAD^+ Assay. Protein and enzyme-bound NAD^+ were assayed from A_{280} and A_{280}/A_{260} values, respectively, by using the calibration data described earlier (Seydoux et al., 1973).

Preparation of FA-GPDH. The holoenzyme (10–30 μM) was treated with excess FAP (~5 mM) in ethylenediamine buffer (10 mM ethylenediamine, 0.1 M KCl, and 1 mM EDTA), pH 7.0, at 25 °C. After ~5 min the solution was chilled to 0 °C and passed through a Bio-Gel P-30 column (1 × 20 cm) equilibrated with the ethylenediamine buffer. The spectrum of the protein fraction was recorded immediately on a Cary 14 spectrophotometer from which the concentrations of protein, bound NAD^+, and FA groups ($\epsilon_{345nm} = 3.0 \times 10^4\ M^{-1}\ cm^{-1}$) were calculated. Apo-FA-GPDH was prepared by passing the above FA-enzyme through a small column of charcoal [washed according to Krimsky & Racker (1963)] in a Pasteur pipet (charcoal/protein ratio was in the range of 5–10 w/w).

Preparation of Carboxymethylated FA-GPDH. Isolated FA-GPDH without charcoal treatment (~20 μM) was treated with excess iodoacetate (~0.4 mM) for a few minutes at 25 °C and the excess reagent removed by gel filtration (Bio-Gel P-30). Carboxymethylated FA-GPDH was then passed through a charcoal column as above to obtain carboxymethylated apo-FA-GPDH. Completion of the carboxymethylation reaction was checked by an indirect procedure. The carboxymethylated FA-GPDH was deacylated (1–2 mM arsenate, pH 7.0), and the free SH groups in the resulting protein were assayed by using Elman's reagent [5,5′-dithiobis(2-nitrobenzoate)] at 412 nm on a Cary 14 spectrophotometer ($\Delta\epsilon_{412nm} = 13.6 \times 10^3\ M^{-1}\ cm^{-1}$) (Ellman, 1959). The number of such groups was invariably equal to the number of FA groups that were initially present, indicating that all the remaining SH groups had reacted with iodoacetate.

Titration of Apo-FA-GPDH with NAD^+. Solutions of apo-FA-GPDH or its carboxymethylated derivative (3 mL) in ethylenediamine buffer were treated with small volumes (10 μL at a time) of concentrated NAD^+ solution, and the absorbance at the desired wavelength was recorded with a Cary 16 spectrophotometer (after the absorbance reached a constant value). Appropriate corrections were applied for the small changes in volume upon addition of aliquots of the NAD^+ solution.

Kinetics of Deacylation. FA-GPDH (or its carboxymethylated derivative) and NAD^+ were incubated for 5–7 min at desired concentrations in ethylenediamine buffer (0.79–mL total volume) to allow completion of spectral perturbation. The reaction was started by adding 10 μL of acceptor solution. Absorbance was monitored at 360 nm on a Cary 14 spectrophotometer.

Results

Transient Kinetics of the Interaction of Acyl-Apoenzyme with NAD^+. The effect of bound NAD^+ on the *acyl*-enzyme equilibrium spectrum of FA-GPDH has been described previously (Malhotra & Bernhard, 1973). Two discrete FA-enzyme spectra components (isosbestic point at 360 nm) contribute to the observed spectrum, depending on the extent of bound NAD^+ at the site of acylation. When NAD^+ is added to acyl-apoenzyme and the time-dependent absorbance is monitored at a fixed wavelength, other than at the isobestic point, the kinetics are markedly biphasic. There is both a fast "burst" and a "slow" change (Figure 1). In the results which follow we make reference to a variety of distinctive parameters which we can calculate from these measurements. These are the specific first-order rate constants for the slow phase, k_{slow}, and the amplitudes of the absorbance change, in each phase, A_{fast} and A_{slow}. Since the magnitudes of these amplitudes exhibit Michaelian saturation in their [NAD^+] dependence, we can also calculate apparent dissociation constants for enzyme·NAD^+ complexes in these two phases.

The kinetics of the fast burst process cannot be monitored by conventional spectrophotometry. The amplitudes of the two kinetic processes can be resolved, however, by extrapolation of the slow change to zero time. The amplitude of the fast phase absorbance change is a consequence of both the FA spectral perturbation, and the "Racker band", which is formed instantaneously upon the binding of NAD^+ to the nonacylated

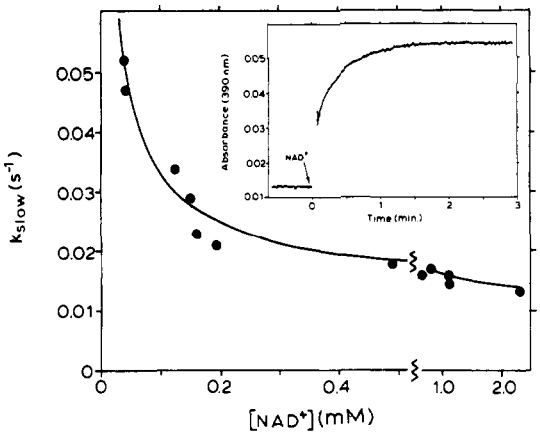


FIGURE 1: Kinetics of furylacryloyl spectral perturbation of apo-FA-GPDH by NAD^+ as measured by changes of absorbance at 390 nm. The protein concentration was 2.62 μM with 1.7 FA groups/tetramer. The initial A_{390} was 0.0135. A small aliquot of NAD^+ (20 μL of 10 mM) was added as indicated. The amplitude of the fast component contains a calculable contribution from the weakly absorbing Racker band formed due to the binding of NAD^+ to the nonacylated sites. (Insert) NAD^+ concentration dependence of the first-order rate constant of the slow phase of spectral perturbation.

Table I: Percent Amplitude of the Fast Process in the Spectral Perturbation of FA-GPDH with Different Initial and Final [NAD^+]

initial [NAD^+] (μM)	final [NAD^+] (μM)	$A_{fast} \times 100/(A_{fast} + A_{slow})$
0	196.1	23[b]
0	49.0	33-36[b]
49.0	96.2	64-78
96.2	141.5	100
141.5	185.2	100
185.2	363.6	100

[a] Protein concentration was 2.12 μM with 1.59 FA groups/mol of enzyme. The absorbance of the solution was monitored at 390 nm before and after addition of NAD^+. [b] Corrected for Racker band formation due to NAD^+ binding to the nonacylated sites.

sites (Bloch, 1970). Since the Racker band absorption spectrum is known, it can be subtracted from the extrapolated total absorbance change in the fast process. This correction is readily applied since the concentrations of NAD^+ utilized here are always sufficient to saturate the nonacylated sites (Malhotra & Bernhard, 1973). The racker band contribution can also be eliminated experimentally by carboxymethylation of these nonacylated but alkylation-reactive SH sites of the FA-enzyme prior to the additionof NAD^+ (MacQuarrie & Bernhard, 1971; Malhotra & Bernhard, 1973).

The relative amplitudes of the two kinetic phases of acyl spectral perturbation vary with both the initial and the final NAD^+ concentrations as is shown in Table I. The slow component of spectral perturbation invariably displays single-exponential kinetic behavior. Its first-order specific rate is independent of the FA-GPDH concentration and is inversely related to the NAD^+ concentration, approaching a constant *minimal* specific rate of $\sim$0.013 s^{-1} at high NAD^+ concentrations (Figure 1).

Transient Kinetics of Deacylation. A molecular pathway as described by eq 1 requires that in the presence of excess acceptor (arsenate or phosphate) the disappearance of acyl group should be pseudo first order (single exponential). We have shown earlier with rabbit muscle FA-enzyme that the deacylation reaction (with excess arsenate, phosphate, or NADH) does not exhibit the first-order kinetics (Malhotra & Bernhard, 1968). The same is true of sturgeon muscle

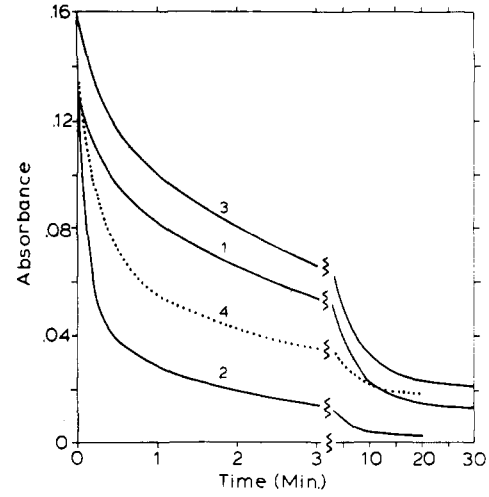


FIGURE 2: Kinetics of deacylation of FA-GPDH. In each case, the reaction was started with a small volume of acceptor and monitored at 360 nm. Protein and NAD^+ were preincubated for equilibration. Concentration conditions were as follows: (1) 2.4 μM protein with 2.08 FA groups/mol of enzyme, 25 μM NAD^+, and 0.5 mM arsentate; (2) 2.4 μM protein with 2.08 FA groups/mol of enzyme, 250 μM NAD^+, and 0.5 mM arsenate; (3) 4.1 μM protein with 2.03 FA and 2 carboxymethyl groups per mol of enzyme, 65 μM NAD^+, and 0.5 mM arsenate; (4) 3.1 μM protein with 1.83 FA groups/mol of enzyme, 131 μM NAD^+, and 0.5 mM phosphate. (Some curves have been shifted along the vertical scale for clarity.)

FA-enzyme (Figure 2). The time-dependent disappearance of the acyl group can be quantitatively treated as the sum of two first-order processes (eq 2)

$$A_t - A_\infty = A_{fast}e^{-k_{fast}t} + A_{slow}e^{-k_{slow}t} \quad (2)$$

where A_t and A_∞ are the absorbance values at times equal to t and infinity, respectively, k_{fast} and k_{slow} are the two rate constants, and A_{fast} and A_{slow} are the amplitudes of the two rate processes. Since NAD^+ is required for the deacylation reaction (Malhotra & Bernhard, 1968, 1973) and the coenzyme brings about a time-dependent spectral change in the FA-enzyme, FA-enzyme was preincubated with NAD^+ before starting the reaction with the acceptor. The deacylation reaction was monitored at 360 nm, which is the isosbestic point for the NAD^+-perturbed vs. unperturbed acyl-enzyme spectrum. Thus, the observed deviation from first-order kinetics cannot be attributed to any spectral perturbation per se. The parameters k_{fast}, k_{slow}, A_{fast}, and A_{slow} vary with the concentrations of coenzyme and acceptor. The effects of these variables are discussed below.

Effect of NAD^+ Concentration on the Kinetics of Deacylation. The kinetic parameters for deacylation of FA-GPDH in the presence of arsenate or phosphate at various concentrations of NAD^+ are summarized in Figure 3. The two rate constants (k_{fast} and k_{slow}) exhibit parallel variation with NAD^+ concentration and extrapolate to 0 in the absence of NAD^+. As in equilibrium spectral perturbation experiments (Malhotra & Bernhard, 1973) carboxymethylation of SH groups at the nonacylated sites has no effect on the kinetics of deacylation and its dependence on NAD^+ concentration. The NAD^+ concentration dependence of the amplitude of the fast process is shown in Figure 3C. Note that both below 25 μM NAD^+ and above $\sim$0.5 mM this amplitude is finite and independent of [NAD^+].

Michaelian dependence of k_{fast} and k_{slow} on NAD^+ concentration is apparent in both the phosphorolysis and the arsenolysis reaction at NAD^+ concentrations up to 0.6 mM (Figure 3A,B). Note the similarity of these K_m values to the NAD^+ dissociation constants calculated from the equilibrium

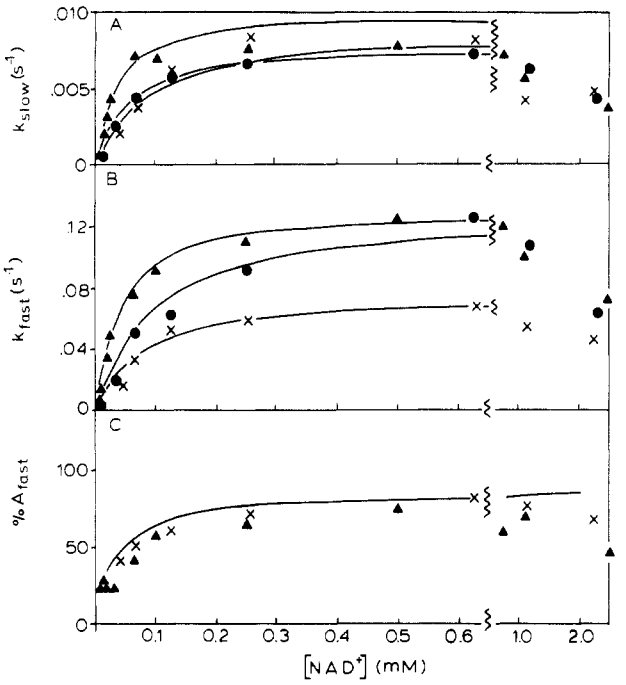


FIGURE 3: NAD^+ concentration dependence of the kinetics of deacylation. The kinetics kinetics were monitored at 360 nm as described in Figure 2. The three rate parameters k_{slow} (A), k_{fast} (B), and % A_{fast} (C) are plotted. Concentrations were as follows: (▲) 0.5 mM arsenate and 2.4 μM protein having 2.08 FA groups/enzyme; (●) 0.5 mM arsenate and 4.1 μM protein having 2.03 FA and 1.9 are carboxymethyl groups per enzyme; (×) 0.5 mM phosphate and 3.1 μM protein having 1.83 FA groups/enzyme. In (A) and (B) the theoretical curves were calculated for a Michaelian behavior with the constants of Table II. The theoretical curve in (C) has been calculated as explained under Discussion.

Table II: Kinetic and Equilibrium Parameters for the Effect of NAD^+ on Various Reactions of FA-GPDH

(A) Kinetic Parameters

pretreatment of FA-GPDH	reaction	fast phase K_M	fast phase k_f[a] (s^{-1})	slow phase K_M	slow phase k_f[a] (s^{-1})
none	arsenolysis[b]	40	0.133	31	0.010
carboxymethylation	arsenolysis[b]	100	0.133	51	0.008
none	phosphorolysis[b]	76	0.077	87	0.009

(B) Equilibrium Spectral Perturbation Parameters

pretreatment of FA-GPDH	K_d (μM)[c]
none	65 ± 15
carboxymethylation	71

[a] At [NAD^+] saturation. [b] The arsenate or phosphate concentration was 0.5 mM. [c] Calculated from the data of Figure 2.

spectral perturbation data (Table II). Although k_{fast} shows some acceptor-dependent variation (see next section), the magnitudeof k_{slow} appears to approach the same limiting value (~0.01 s^{-1}) with both acceptors.

Further increases in the NAD^+ concentration above 1 mM lead to gradual decreases in both the fast and the slow rates of deacylation. These results at very high NAD^+ concentration are consistent with the effect of high NAD^+ concentrations on the spectral perturbation (Figure 4). The spectrally perturbed acyl form also decreases with increasing NAD^+ concentration above 1 mM. Increasing NAD^+ concentration in this range also leads to a reduction in the relative amplitude of the fast phase of spectral perturbation. Since there are quantitative and discrete signals for the binding of NAD^+ to both acylated sites (the "red shift") and the nonacylated sites (the Racker band), we have readily ascertained that four

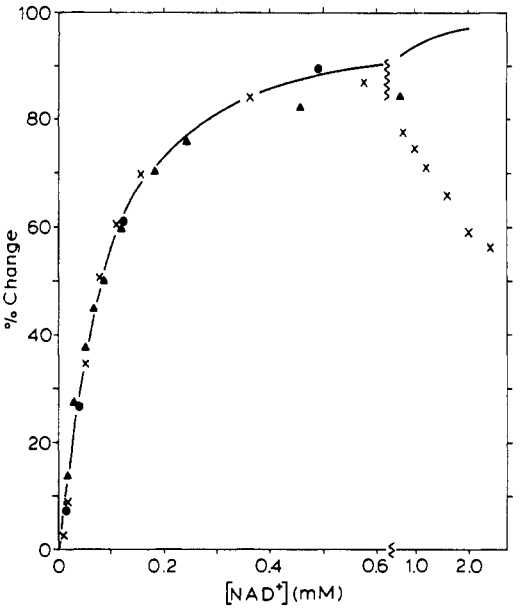


FIGURE 4: Equilibirum spectral perturbation titration of FA-GPDH and carboxymethylated FA-GPDH with NAD^+. A solution of FA-GPDH (●, ×) or its carboxymethyl derivative (▲) was treated with small aliquots of NAD^+, and the absorbance at 390 nm was noted before and after each addition had equilibrated. Protein concentration was as follows: (●) 1.7 μM, having 2.1 FA groups and 1.35 NAD^+ per enzyme molecule; (×) 4.1 μM, having 1.9 FA groups and practically no bound NAD^+; (▲) 2.25 μM having 1.5 FA and 2.5 carboxymethyl groups per mol of enzyme (A_{280}/A_{260} = 2.06). In the titration of apo-FA-GPDH (×), both [NAD^+] and change in absorbance values were corrected for the binding of NAD^+ to the nonacylated sites and for the absorbance on the resultant Racker band. These corrections are significant only at low [NAD^+]. The data are plotted as the percent of the maximum possible change in absorbance. The theoretical curve (solid line) has been calculated as explained in Appendix I.

NAD^+ molecules bind to the diacyl-enzyme tetramer at NAD^+ concentrations of ~0.5 mM (Malhotra & Bernhard, 1973). Although we cannot at this time describe the detailed molecular events responsible for this reversal, it is of interest to note that the distribution between rapidly and slowly reacting acyl groups is determined exclusively by the extent of spectral perturbation throughout the NAD^+ concentration range, suggesting that the spectral reversal at high [NAD^+] represents a return to the same enzyme conformational states as were present at low [NAD^+].

Effect of Phosphate and Arsentate Concentration on the Kinetics of Deacylation. In these experiments, the NAD^+ concentration was held constant at 65–70 μM. At this concentration the fast and the slow amplitudes are almost equal; hence, differential effect of the acceptor on the biphasic rates and their amplitudes is likely to be more easily detectable. The results are shown in Figure 5. The amplitudes of the fast and slow processes are independent of the nature and concentration of the acceptor. k_{fast} shows a strictly linear (first-order) dependence on the acceptor concentration. At the same acceptor concentration, k_{fast} for arsenolysis is ~2.5 times faster than k_{fast} for phosphorolysis. k_{slow} shows an apparent Michaelian variation with acceptor concentration. The limiting value of k_{slow} at high acceptor concentrations observed here is close to that expected at the NAD^+ concentration employed (compare with Table II). At very low arsenate concentration (10 μM) where the time-dependent changes in absorbance are far slower than that governed by k_{slow}, the irreversible deacylation reaction exhibits simple (monophasic) pseudo-first-order kinetics.

Kinetics of the Acylation Reaction and Its Dependence on NAD^+ Concentration. Since NAD^+ affects the spectrum of

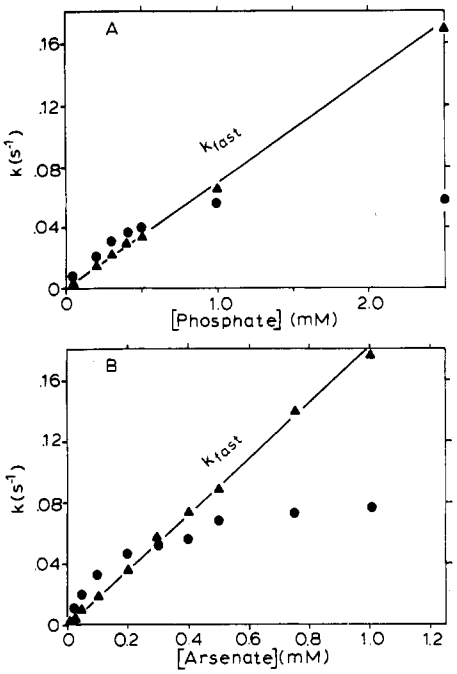


FIGURE 5: Acceptor concentration dependence of the kinetics of deacylation of FA-GPDH. (A) Phosphorolysis; (B) arsenolysis. The kinetics were monitored as described in Figure 20. Concentration conditions were as follows: (A) 4.0 μM protein having 1.75 FA groups/enzyme and 68.5 μM NAD+; (B) 4.2 μM protein having 1.63 FA groups/enzyme and 72 μM NAD+. (▲) $k_f a_{st}$; (●) 10 × k_{slow}.

the FA groups covalently bound to the active site, the most convenient wavelength for studying the kinetics of acylation via spectral differences between FAP and FAE is at the isobestic point for apo- vs. holo-FA-GPDH (λ 360 nm; Malhotra & Bernhard, 1973). At this wavelength, the reaction between GPDH and excess FAP obeys simple pseudo-first-order kinetics; the pseudo-first-order rate constant is linearly dependent on the FAP concentration (up to 5 mM FAP). At all FAP concentrations, the reaction comes to an end after the introduction of 2 FA groups/mol of enzyme.

The pseudo-first-order $k_{acylation}$ (360 nm) extrapolates to 0 at 0 NAD+ concentration, thus demonstrating the absolute requirement for bound NAD+ in acylation as is the case in the rabbit muscle enzyme (Malhotra & Bernhard, 1968). The requirement for bound NAD+ in the acyl-transfer step has long been established (Racker & Krimsky, 1952; Harting & Velick, 1954; Hilvers & Weenen, 1962; Malhotra & Bernhard, 1968). All of these NAD+-dependent reaction rates saturate at high NAD+ concentrations.

The kinetics of reaction, as monitored at wavelengths other than 360 nm, are more complex. At these wavelengths, the time-dependent absorbance changes can be fit to the biphasic expression of eq 2. A_{fast} is positive at all wavelengths. A_{slow} is positive at wavelengths below 330 nm and negative at higher wavelengths (Figure 6). The biphasicity is much more pronounced at lower NAD+ concentrations. The effect of NAD+ concentration on the kinetics of the acylation reaction, as monitored at 390 nm, is shown in Figure 7. The derived kinetic parameters are listed in Table III. Under these conditions the two rate constants are nearly invariant with changes in NAD+ concentration. A_{slow} is very profoundly affected by the NAD+ concentration. Its numerical value is very small at 0.5–1 mM NAD+ and increases at lower concentrations. The data suggest that the reaction of enzyme with FAP leads initially to the spectrally perturbed acyl-enzyme species and the latter "relaxes" slowly to an equilibrium mixture of perturbed and unperturbed acyl-enzyme.

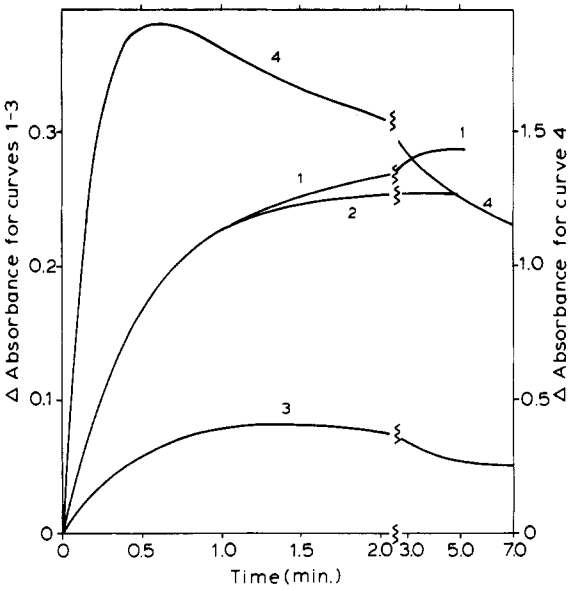


FIGURE 6: Time-dependent absorbance changes in the reaction of FAP and GPDH monitored at 350 (curve 1), 360 (curve 2), and 390 nm (curves 3 and 4). For curves 1–3, the concentrations were 3.8 μM protein, 65 μM NAD+, and 1.2 mM FAP. Curve 4 is for 82 μM holoenzyme (with 4 NAD+ bound/enzyme) and 4.4 mM FAP. The reaction was started by adding enzyme to a solution of FAP.

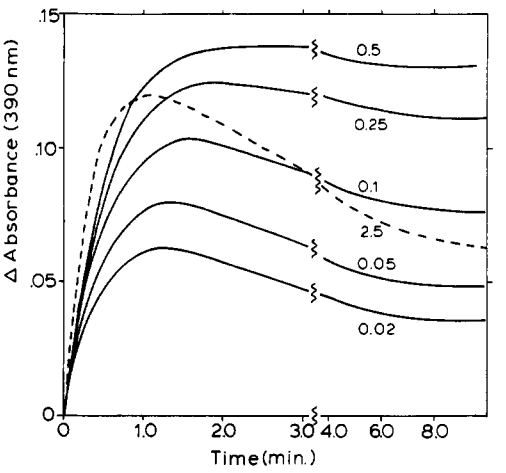


FIGURE 7: Time-dependent absorbance changes at 390 nm in the reaction of FAP and GPDH at different NAD+ concentrations. The protein and FAP concentrations were 3.8 μM and 1.15 mM, respectively. The NAD+ concentrations (mM) are indicated. The reaction was started by adding enzyme to a solution of FAP.

Table III: Effect of NAD+ Concentration on the Kinetics of Reaction of FAP and GPDH Monitored at 390 nm[a]

[NAD+] (mM)	fast phase k (s^{-1})	fast phase amplitude (A_{390})	slow phase k (s^{-1})	slow phase amplitude (A_{390})	total amplitude (A_{390})
0.05	0.026	0.169	0.010	−0.120	0.049
0.12	0.028	0.160	0.0086	−0.082	0.078
0.27	0.028	0.164	0.010	−0.047	0.117
0.52	0.030	0.159	0.0089	−0.025	0.134
1.27	0.038	0.153	0.0051	−0.015	0.138
2.52	0.043	0.167	0.0068	−0.104	0.063

[a] The reaction was started by adding holoenzyme to a solution of FAP and NAD+, and the time-dependent absorbance changes at 390 nm (Figure 7) were analyzed according to the equation $A_\infty - A_t = A_{fast}e^{-k_{fast}t} + A_{slow}e^{-k_{slow}t}$. Concentrations of protein and FAP were 3.82 μM and 1.14 mM, respectively.

Time-Dependent NAD+-Induced Changes in the Absorption Spectrum. Previously, we have presented evidence that there

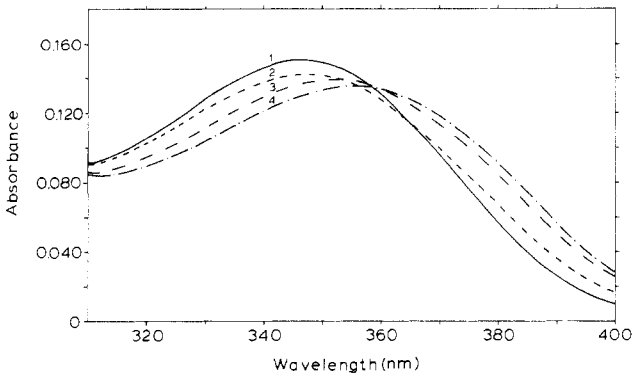


FIGURE 8: Time-dependent changes in the acyl absorption spectrum of FA-GPDH on the addition of NAD^+. FA-GPDH was 2.8 μM having 1.9 FA groups and 1.2 NAD^+ per enzymes (curve 1). A small aliquot of NAD^+ (final concentration 0.59 mM) was then added and the solution scanned at rapid intervals. (Curve 2) 2 s; (curve 3) 17 s; (curve 4) 47 s. A Hewlett-Packard spectrophotometer (HP 8450 A) was utilized, and the scans were corrected for dilution.

are only two spectrally discrete species in the equilibrium mixture during the titration of the furylacryloyl-enzyme with NAD^+. This is evident from the single isobestic point at 360 nm regardless of the NAD^+ concentration. Since in the present experiments, we utilize kinetic, as well as equilibrium, data it was of interest to examine for the possibility of additional spectral components during the time course of approach to equilibrium following perturbation of the NAD^+ concentration. Such an experiment is shown in the rapid repetitive spectral scans of Figure 8. It is clear from these scans that during this kinetic time course additional spectral components contribute to the overall absorption spectrum. These contributions are small but readily detectable at particular wavelengths. They introduce complications at wavelengths where the spectral change due to changes in the distribution of major components is small (near the isobestic point) and where the spectral contributions of these transient components are near maximal. For the discussion which follows, we restrict ourselves to wavelengths where the observed kinetic spectral changes are attributable overwhelmingly to components which are responsible for the equilibrium spectral changes.

Discussion

(1) Rate-Limiting Factor in Acyl-Enzyme Reactions Is a Slow Protein Isomerization. The results presented herein add further insights into the relationship between the protein conformation and the electronic structure of reactive centers along the catalytic pathway. Previously, we had shown that NAD^+-dependent changes in the furylacryloyl-enzyme spectrum were essential for subsequent catalytic reactivity in the acyl transfer. These new results show that a slow spectral change subsequent to NAD^+ ligation correlates directly with slow changes in reactivity of the acyl-enzyme. At excessive substrate and NAD^+ concentrations, fast and slow reaction components are evident in all of the processes we have observed. The first-order rate constants for the slow processes are similar in magnitude. Since the same limiting rate is reached in acyl-transfer reactions, independent of the participating acceptor, it is likely that the limiting slow rate arises from an isomerization of the protein, di-FA-GPDH.

The fact that all processes (spectral perturbation, phosphorolysis, arsenolysis, and acylation) occur, in part, with a velocity which is much faster than this common slow isomerization indicates that the fast reacting conformation must preexist prior to the addition of NAD^+ to the acyl-apoenzyme.

Table IV: Apparent Equilibrium Constant between the Fast and Slow Reacting Species (A_{fast}/A_{slow}) for Spectral Perturbation and Deacylation Reactions at Different NAD^+ Concentrations

	A_{fast}/A_{slow}	
[NAD^+] (mM)	spectral perturbation[a]	deacylation
<0.03	0.33	0.3–0.35
0.04		0.72
0.048	1.0	
0.10	1.1	1.3
0.125		1.5
0.15	2.0	
0.25		2.6
0.63		4.5

[a] For spectral perturbation experiments, the NAD^+ concentration was increased from that shown here to near saturation (0.5 mM), and the amplitudes of the fast and slow phases were monitored at 390 nm.

A gross estimate of this conformational isomerization equilibrium (eq 3) can be obtained from the data presented.

$$EAc \rightleftarrows E'Ac \quad (3)$$

Although the slow rates of arsenolysis and phosphorolysis are common, the faster kinetic components of these two processes differ (and are linearly dependent on the concentration of the particular acceptor). If the entire kinetic course of these reactions is due to one slow and one fast phase of reaction, we can estimate the preexistent acyl-enzyme conformational equilibrium constant (eq 3) by measuring the relative amplitudes of chemical transformation in the fast phase compared to the total amplitude change in the fast plus slow phases of deacylation, assuming that the two acyl conformations (EAc and E'Ac) have the same extinction coefficient. To enhance the plausibility of this assumption, we have chosen to measure the deacylation amplitude change at the isosbestic point for the NAD^+-dependent spectral perturbation at equilibrium (360 nm). An alternative estimate of this conformational equilibrium constant can be obtained from the ratio of the fast phase to the total amplitude change upon the addition of saturating NAD^+ to the acyl-apoenzyme. The plausibility of this latter method rests on the assumption that there are only two furylacryloyl spectral components: one for the NAD^+-bound and conformationally isomerized acyl-enzyme (E'Ac·NAD^+) and one for all other acyl-enzyme species.

In the presence of saturating NAD^+, we can still detect a slow as well as a fast rate of phosphorolysis and arsenolysis. Thus, we can estimate the preexistent conformational equilibrium constant at NAD^+ concentrations approaching saturation, as well as that for the acyl-apoenzyme. The apparent equilibrium constants so determined are given in Table IV. As is evident, the comparative values are in qualitative agreement with the simple conformational isomerization considered in eq 3. These results, as well as those presented earlier, demonstrate that a major furylacryloyl-enzyme spectral change is dependent on the ligation of NAD^+ to the acyl-enzyme. The present results further demonstrate that the conformation required for NAD^+-dependent spectral perturbation is itself preferentially stabilized by the binding of NAD^+. From the large difference in NAD^+ affinity between acylated and nonacylated sites (Malhotra & Bernhard, 1973), it is safe to conclude that all NAD^+-dependent phenomena reported herein relate to ligation at the acylated sites.

(2) Color Change and Acyl Reactivity Require NAD^+-Bound Active Conformer. An examination of the acyl group reactivity in phosphorolysis and arsenolysis, both in the slow and the fast kinetic phases, shows that NAD^+ binding is ob-

ligatory for acyl group reactivity (Figure 3). Since the amplitudes of the individual phases of reaction correlate well with the amplitudes of the two phases of [NAD$^+$]-dependent spectral perturbations (as discussed above), a unique perturbed acyl-enzyme·NAD$^+$ complex appears to be responsible for the major color change and for the reactivity observed. However, minor components are detectable during the transient phase of spectral perturbation by NAD$^+$. The stringency of the restriction of reactivity to a unique spectrally perturbed acyl-enzyme·NAD$^+$ conformation is considered in further detail below. Since NAD$^+$ influences the acyl-enzyme conformational equilibrium and since the acyl-enzyme is oligomeric with four NAD$^+$ binding sites of at least two nonequivalent types, the [NAD$^+$]-dependent isomerization pathway is not, a priori, clearly defined. The remainder of this discussion deals with an analysis of the microscopic details of this pathway and its relevance to glycolysis.

(*3*) *Binding of NAD$^+$ to the Active Conformer Is Preferential.* We must take into account the fact that the conformational isomerization equilibrium is influenced by ligation with NAD$^+$. The simplest model which takes explicit account of this NAD$^+$-dependent equilibrium is that of eq 4. As-

$$\begin{array}{ccc} \text{EAc} & \overset{K_0}{\rightleftharpoons} & \text{E'Ac} \\ {\scriptstyle K_{NAD}}\downarrow\uparrow & & \downarrow\uparrow{\scriptstyle K'_{NAD}} \\ \text{EAc·NAD}^+ & \rightleftharpoons & \text{E'Ac·NAD}^+ \end{array} \qquad (4)$$

suming this model, the data demand the following. The species E'Ac must have a higher NAD$^+$ affintiy than the unreactive and spectrally unperturable form (EAc). The ratio of the two equilibrium isomerization constants (K_1/K_0) must be the ratio of the two ligand dissociation constants (K_{NAD}/K_{NAD}'). This ratio is calculated to be ~15 (Table IV). Two questions immediately arise. (i) Is there a preferential pathway in the overall NAD$^+$-induced isomerization? (ii) Is the model of eq 4 adequate to quantitate the effects of ligand concentration on the properties of the acyl-enzyme?

(*4*) *A Substantial Pathway for Isomerization Equilibrium Involves Isomerization Followed by Ligation.* Assuming the NAD$^+$ binding to either conformation is a rapid, diffusion-controlled process, an exclusive pathway of ligand binding *followed by* isomerization leads to a prediction of Michaelian behavior for the dependence of the slow specific rate of spectral perturbation (k_{slow}) on NAD$^+$ concentration (eq 5). On the other hand, an exclusive pathway involving isomerization *followed by* rapid ligation predicts an inverse dependence of the macroscopic specific rate on ligand concentration (eq 6).

$$\text{EAc} \overset{\text{NAD}^+}{\rightleftharpoons} \text{EAc·NAD}^+ \overset{k_{1,slow}}{\rightleftharpoons} \text{E'Ac·NAD}^+$$

$$k_{slow} = \frac{k_1[\text{NAD}^+]}{K_{NAD} + [\text{NAD}^+]} \qquad (5)$$

$$\text{EAc} \underset{k_{-0}}{\overset{k_0}{\rightleftharpoons}} \text{E'Ac} \overset{K_{NAD'}}{\rightleftharpoons} \text{E'Ac·NAD}^+$$

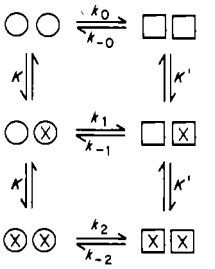
$$k_{slow} = k_0 + \frac{k_{-0}K_{NAD'}}{K_{NAD'} + [\text{NAD}^+]} \qquad (6)$$

As is shown in Figure 1, there is a substantial contribution from the ordered pathway of eq 6. However, since the enzyme is oligomeric, there may be further [NAD$^+$]-dependent contributions to the spectral perturbation kinetics due to the effect of partially ligated species on the rate of isomerization at adjacent acyl sites.

(*5*) *NAD$^+$ Ligation at One Acyl Site Affects Isomerization Equilibrium at an Adjacent Acyl Site.* We assume the model of eq 4 and that isomerizations are the only slow processes. Accordingly, it follows that upon addition of NAD$^+$ to acyl-enzyme, there should be a fast phase in which already isomerized enzyme reacts rapidly with ligand. Residual activity is evident in a slow phase in which EAc and/or EAc·NAD$^+$ are converted to the conformationally altered E'Ac and E'Ac·NAD$^+$ species, respectively. Since the spectral probe we utilize measures *only* the formation of E'Ac·NAD$^+$, the spectrally unperturbed species are EAc, EAc·NAD$^+$, and E'Ac. Further, since the ratios [EAc]/[E'Ac] and [EAc·NAD$^+$]/[E'Ac·NAD$^+$] are fixed, it can be shown that the relative amplitudes of the fast and slow phases of spectral perturbation should be independent of the starting fractional saturation with NAD$^+$: In an independent site model (i.e., eq 4) NAD$^+$ ligation cannot exert an influence on subsequent further NAD$^+$ ligation. Hence, the total fast phase amplitude following a series of small additions of NAD$^+$ should be the same as the fast phase amplitude observed with a single more concentrated aliquot of NAD$^+$ so as to reach the same final concentration.

The experimental results (Table I) are inconsistent with this prediction from eq 4: The total amplitude of the fast phase is much greater when NAD$^+$ is added in several small aliquots as compared to that for a single addition to reach the same final NAD$^+$ concentration. The relative amplitude of the fast phase increases progressively with increasing initial NAD$^+$ concentration. Hence, the binding of NAD$^+$ to one acyl-enzyme site enhances the affinity for subsequent NAD$^+$ ligation to the adjacent acyl site, a phenomenon we are at a loss to explain other than by NAD$^+$-induced changes in the interdimer interactions.

(*6*) *Results Are Generally Consistent with a Simple Allosteric Model.* Our kinetic data indicate that in the absence of any NAD$^+$, ~25% of the acyl-enzyme is already in the isomerized (E'Ac) form. Moreover, in the presence of saturating NAD$^+$ concentrations, approximately 15–20% of the acyl-enzyme exists as the EAC·NAD$^+$ conformer (as indicated by the slow rate process in deacylation). This preexistence of both conformers regardless of the effector ligand obviates the functional need for alternative intermediate conformers. Hence, we have considered immediately the simplest allosteric model, namely, the concerted model illustrated by eq 7



(7)

In this simplest model, we assume only one differently colored and reactive acyl-enzyme (E'Ac·NAD$^+$) which can exist at either of the two acyl sites of the E' tetramer.

For the deacylation reaction, our assumptions are that (i) only the spectrally perturbed species (AcE'·NAD) is reactive with the acyl acceptor and (ii) the chemical reaction of this species with the acceptor is faster than the rate of isomerization of Ac-E or AcE·NAD$^+$. Such a model would explain the biphasic kinetics of the reaction of FA-GPDH with phosphate or arsenate. If the reaction of the acceptor with AcE'·NAD$^+$ is bimolecular, k_{fast} should be proportional to both the acceptor concentration (see Figure 5) and the fraction of NAD$^+$-ligated, spectrally perturbed sites. Therefore, k_{fast} should show the same NAD$^+$ concentration dependence as is observed in spectral perturbation. The experimental data (Table II) are

Table V: Derived Parameters for the Allosteric Model of Equation 7

Equilibrium Constants			
allosteric constant $E'Ac_2/EAc_2$ (L)		0.3	
NAD^+ binding constant to EAc_2 (K)		7 mM^{-1}	
NAD^+ binding constant to $E'Ac_2$ (K')		33 mM^{-1}	
Rate Constants			
k_0	0.02–0.03 s^{-1}	k_0	0.067–0.10 s^{-1}
k_1	0.01–0.02 s^{-1}	k_{-1}	0.007–0.014 s^{-1}
k_2	0.011 s^{-1}	k_{-2}	0.0017 s^{-1}

consistent with this requirement of the model of eq 7.

Those parameters which can be calculated from the quantitative data presented are listed in Table V. The analysis of the data according to this allosteric model and the appropriate derivations are contained in the Appendix.

(*7*) *There Are Some Complicating Considerations in Regard to Kinetic Predictions of the Simple Allosteric Model.* Unlike the kinetic and equilibrium parameters discussed above, the dependence of k_{slow} (deacylation) on NAD^+ concentration can only be qualitatively explained on the basis of the allosteric model and the derived parameters (Table V). The model predicts that the K_{m,NAD^+} for this rate constant should be equal to $1/K'$. This conclusion is based on the expectation that the slow rate is due exclusively to the *isomerization* of E to E'. The absolute magnitude of the slow rate of deacylation is determined by fractional ligation with NAD^+ of the E'Ac species. Hence, the slow rate shoudl be [NAD^+]-dependent according to the dissociation constant, $1/K'$ (30 μM). The observed values (31 and 51 μM for arsenolysis and 87 μM for phosphorylysis) are somewhat higher. In this discussion, we have not considered any specific effect of the acceptor on the rate of isomerization or on the affinity of acyl-enzyme for NAD^+. Even minor acceptor-induced changes in any of these parameters might be adequate to bring about a 2-fold change in the K_m.

In the discussion about spectral perturbation, we have assumed that only the isomerized and NAD^+-liganded sites ($E'Ac{\cdot}NAD^+$) are spectrally distinct from all other sites. However, the data of Figure 8 suggest that $EAc{\cdot}NAD^+$ sites may be spectrally distinct from EAc sites. Spectra recorded at varying times after the addition of NAD^+ to FA-GPDH do not share a unique isobestic point. However, at 390 nm, where the spectral perturbation has been monitored, the spectral change due to the ligation of NAD^+ with EAc yielding $EAc{\cdot}NAD^+$ is virtually negligible as compared to the spectral change due to the $E'Ac \rightarrow E'Ac{\cdot}NAD^+$ ligation. Consequently, we invariably observe a fast burst phase followed by a slow, single-exponential phase of absorbance change, the amplitudes in each phase being dependent on the prior EAc $\rightleftarrows$ E'Ac conformational equilibrium.

(*8*) *Conformation and Electronic Isomerizations We Observe Are Physiologically Relevant.* *he conformational change, which we observe by its effect on the NAD^+-dependent color of the acyl-enzyme, is functional. Only the electronically perturbed acyl group participates in the chemical reactions involving acyl transfer. Previously, it has been shown that the same chromophoric acyl-enzyme interacts with NADH to produce a uniquely "blue-shifted" acyl-enzyme spectrum (Bernhard & Malhotra, 1974; Schwendimann et al., 1976). The extent of the spectral shift to the blue with NADH is comparable to the extent of the conformationally induced shift to the red with NAD^+. Hence it would appear plausible from the largeness of this shift that a conformational alternative to the red-shifted acyl-enzyme conformation exists in the blue-shifted NADH·acyl-enzyme complex. It is known that the blue-shifted NADH complex is the only acyl-enzyme capable of undergoing reduction (Schwendimann et al., 1976). Hence, it can be concluded that conformational isomerizations are involved directly in the overall complex catalytic pathway of eq 1. Elsewhere, we present detailed evidence that the species EAc_2 and the species $EAc_n{\cdot}ATP$(n = 1 or 2) preferentially bind NADH over NAD^+. These complexes with NADH give rise to the blue-shifted acyl spectrum. Assuming that protein isomerizations are the only processes giving rise to the very slow unimolecular rates which we observe, there should be a direct correspondence between the fractional *slow* reaction of the acyl-enzyme with NADH (blue shift) and the fractional *rapid* reaction with NAD (red shift). These fractional amplitudes are variable with acyl content as is illustrated in Table VI. As is evident from the table, there is a direct correspondence between fractional slow blue shift and fractional rapid red shift, regardless of the value of the allosteric equilibrium constant. Since the affinities for dinucleotides are the same for FA- and 3-phosphoglycerolylacyl-enzymes and since there is a large difference in affinity for NAD^+ between enzyme and acyl-enzyme, it seems reasonable to conclude that the same or similar conformational changes are involved in the reaction of "true substrate" as have been noted with the FA-enzyme. In the formation of the ATP precursor 1,3-diphosphoglycerate from D-glyceraldehyde 3-phosphate and NAD^+, both desorption of NADH from EAc and isomerization of EAc to E'Ac are required steps. It would thus appear that the very slow allosteric isomerization rates which we report for the FA-enzyme are very much more rapid in the case of the true (substrate) acyl-enzyme. Consequently, the 3-phosphoglyceroyl thioester linkage "catalyzes" the rate of protein isomerization. This isomerization process is involved in rate limitation with the true substrate, and this rate limitation is apparently operative in vivo (Bloch et al., 1971).

Table VI: Percent of the Total Amplitude of Spectral Perturbation of the FA-Enzyme by NAD^+ and by NADH at Different Extents of Acylation

FA groups/ mol of enzyme	% amplitude obsd in fast phase: NAD^+ (0.47 mM)	NADH (0.06 mM)
0.4–0.46	70 ± 10	30 ± 10
1.2	40 ± 5	55 ± 5
1.4	35 ± 5	60–70, 65 ± 5
1.9	20 ± 5	75 ± 5

Acknowledgments

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Appendix

(*I*) *Calculation of Allosteric Equilibria.* The extent of cooperativity evident from the deviation from Michaelian behavior in a binding isotherm depends on the value of the Hill coefficient (n_H). This coefficient depends on both the allosteric isomerization constant (L) and the two binding constants for NAD^+ (K and K'). It can be shown by the method of F. W. Dahlquist (personal communication) that the Hill coefficient, n_H, is related to these constants by eq 8.

$$n_H = \frac{4}{(\psi_1/\psi_2^{1/2}) + 2} \qquad (8)$$

where $\psi_1 = 2(K + LK')/(L + 1)$ and $\psi_2 = (K^2 + LK'^2)/(L + 1)$. The allosteric constant (L) can be readily obtained as follows: (1) by measurement of the fractional amplitude in the fast phase of spectral perturbation upon the addition of a saturating concentration of NAD$^+$ to acyl-apoenzyme; (2) by measuring the fractional amplitude in the fast phase of deacylation of FA-GPDH at very low [NAD$^+$]. According to our assumption of a uniquely reactive NAD$^+$-bound acyl-enzyme conformation, both these amplitudes depend on the concentration of Ac_2E' in the absence of NAD$^+$. From both these measurements (Table IV), we calculate the value of 0.3 for $L = [Ac_2E']/[Ac_2E]$. The proportion of the E′ species increases with NAD$^+$ concentration as is indicated by the greater relative amplitude in the fact phase (Table IV). At high NAD$^+$ concentration a more precise estimate of the relative proportion of the E′ species (ligated and unligated) is obtainable from the deacylation amplitudes. At nearly saturating [NAD$^+$] (0.63 mM) 82% of the total phosphorolysis reaction proceeds in the fast phase. Hence, at this concentration the $[E'/E]$ ratio is equal to 4.5 (Table IV). This represents a 15-fold increase from the [E′/[E] value of 0.3 at 0 NAD$^+$. According to the model of eq 7, it has been shown that the conformational equilibrium for NAD$^+$-saturated species is given by eq 9. This gives a K'/K ratio of ~4. Since

$$[E'Ac_2(NAD^+)_2]/[EAc_2(NAD^+)_2] = L(K'/K)^2 \quad (9)$$

saturation with NAD$^+$ is not complete, the actual value may be somewhat higher than 4. For a K'/K ratio of 4, the Hill coefficient, n_H, calculated from eq 8 is found to be 1.11. Such a small value would not be sufficient to distinguish the equilibrium titration results from that predicted on the basis of a noncooperative independent site model.

Initial estimates of K and K' were obtained from the above ratio and the consideration that affinity constants obtained from analysis of the data according to the noninteracting site model (eq 4) must lie between K and K'. Accordingly, iterative procedures gave calculated values of $K = 7$ mM^{-1} and $K' = 33$ mM^{-1}. According to the model of eq 7, the mole fraction of NAD$^+$-ligated sites in the E′ state should be directly related to the fraction of the total possible spectral perturbation (eq 10), where X is the total concentration of [NAD$^+$] assuming

$$\frac{\text{ligated E' sites at [NAD}^+\text{]} = X}{\text{ligated E' sites at saturating [NAD}^+\text{]}} = \frac{X(1 + K'X)(K^2 + LK^2)}{K'[(1 + KX)^2 + L(1 + K'X)^2]} \quad (10)$$

$[NAD^+] \gg [E]_0$. In figure 4, the predicted curve is calculated accordingly. It agrees very well with the experimental points.

(II) Kinetic Analysis of the Allosteric Model (Eq 7). The fractional amplitude in the fast phase of acylation is equal to the fraction of enzyme present in the E′ state. This amplitude is given by eq 11. The calculated fractional amplitudes as

$$\text{mole fraction of all the E' species} = \frac{L(1 + K'X)^2}{(1 + KX)^2 + L(1 + K'X)^2} \quad (11)$$

a function of all the concentration are in agreement with the experimental amplitudes (Figure 3C).

In addition to its effect on the amplitudes and the isomerization equilibrium constants, the effect of NAD$^+$ on the three kinetic parameters, k_{slow} for spectral perturbation and k_{fast} and k_{slow} for deacylation, are observable. The relaxation time for spectral perturbation by NAD$^+$ is given by $1/k_{slow}$. From the allosteric model of eq 7, k_{slow} is given by eq 12

$$k_{slow} = \frac{k_0 + 2k_1KX + k_2K^2X^2}{(1 + KX)^2} + \frac{k_{-0} + 2k_{-1}K'X + k_{-2}K'^2X^2}{(1 + K'X)^2} \quad (12)$$

where k_0, k_1, and k_2 are the rate constants for the isomerization of unliganded and mono- and diliganded E species to their respective E′ species and k_{-0}, k_{-1}, and k_{-2} are the corresponding rate constants in the reverse direction. Correspondingly, the k_{slow} at zero and saturating concentrations of NAD$^+$ are given by eq 13. The data of Figure 1 (insert) show that relaxation

$$k_{slow}(\text{at 0 [NAD}^+\text{]}) = k_0 + k_{-0}$$
$$k_{slow}(\text{at saturating [NAD}^+\text{]}) = k_2 + k_{-2} \quad (13)$$

is faster at low [NAD$^+$] and approaches a smaller constant value at high (saturating) [NAD$^+$]. Therefore, $(k_0 + k_{-0}) > (k_2 + k_{-2})$. Both k_2 and k_{-2} can be readily calculated because their sum is known (0.013 s^{-1}), and their ratio is determined by the values of L, K, andd K'

$$k_2/k_{-2} = L(K'/K)^2 \quad (14)$$

Only lower limits on the magnitudes of k_0 and k_{-0} can be calculated by a similar procedure, because we cannot determine the relaxation rate at 0 NAD$^+$ concentration. Moreover, the shape of the curve in the insert of Figure 1 (increasing slope at lower [NAD$^+$]) does not permit a precise extrapolation to 0 NAD$^+$ concentration. From these minimum estimates of k_0 and k_{-0} and if $k_0 \geq k_1 \geq k_2$, approximate values can be obtained for the various isomerization rate constants. The curve in Figure 1 (insert) has been calculated according to eq 12 with the values given in Table V. It is interesting to note that the rate constants for the forward isomerization (E → E′) are not much affected by NAD$^+$ ligation but the reverse rate constants (E′ → E) decrease sharply with increasing ligation with NAD$^+$.

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Properties of P^3 Esters of Nucleoside Triphosphates as Substrates for RNA Polymerase from *Escherichia coli*†

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ABSTRACT: P^3-[(2,4-Dinitrophenyl)amino]ethyl (DNPNHEt) and P^3-methyl phosphate esters of nucleoside 5′-triphosphates have been synthesized. Their properties as substrates in the initiation and elongation steps of transcription have been examined by using RNA polymerase from *Escherichia coli* and poly[d(A-T)] or T7 DNA as templates. It is shown that transcription can be initiated by ATP-EtNHDNP and that 2,4-dinitrophenyl residues are incorporated at the 5′ end of the RNA molecules. Steady-state kinetic experiments of abortive initation on promoters A1 and A3 of T7 DNA revealed that ATP-EtNHDNP, ADP-EtNHDNP, and ATP-OCH_3 have lower K_m values and markedly reduced V_{max} values compared to those of ATP. The two classes of esters, NTP-EtNHDNP and NTP-OCH_3, were found to differ regarding their utilization as substrates for elongation. Both ATP-OCH_3 and UTP-OCH_3 are substrates for transcription. However, only the pyrimidine derivatives of NTP-EtNHDNP are elongation substrates which release DNPNHEt-PP upon utilization. This dramatic difference between the purine and pyrimidine derivatives of NTP-EtNHDNP reflects a selective process in the transcriptional complex for purines and pyrimidines.

Several methods for introducing labels into RNA have been developed in the past in order to provide the newly synthesized RNA with a spectroscopic or functional marker. Nucleotide analogues which can be incorporated into RNA polymers by RNA polymerase are useful probes of the structure and function of the RNA itself and also provide information about the requirements and mechanism of transcription (Chamberlin, 1974). Most of the analogues have been selected to study the substrate requirements of the enzymatic process for the chemical structure of the nucleotide base. Base analogues which have useful spectral characteristics include those that are fluorecent, such as formycin 5′-triphosphate (Darlix et al., 1971), n^2ATP and n^2h^6ATP (Ward et al., 1969; Rackwitz & Scheit, 1977), and 7-deazanebularine (Ward & Reich, 1972). s^4UTP (Cramer et al., 1971) and s^6GTP (Darlix et al., 1973) both have absorption maxima above 300 nm which can be easily recognized. s^2UTP, 5′-mecuripyridine (Livingstone et al., 1976), and s^4UTP (Rackwitz & Scheit, 1977) can interact with solid supports via sulfur–mecury bonds and are therefore easily recognizable. Although the usefulness of the latter analogues for separating RNA transcripts has been well demonstrated in the above studies, other functional groups on nucleotides which can be incorporated into RNA are needed, especially since the above sulfur analogues are unstable due to the functional group (van Broeckhoven & de Wachter, 1978).

Another method of labeling RNA takes advantage of the initiation step of RNA synthesis (Chamberlin, 1976). This initiation reaction involves the phosphorylation of the 3′-ribofuranosyl position of a purine nucleoside 5′-triphosphate, ATP or GTP, by the 5′-α-phosphate of the next substrate, and therefore the purines act as primers of RNA synthesis. The 5′-triphosphate residues of the primers constitute the 5′ end of the newly synthesized RNA chain, and it should be feasible to link substituents to the γ-phosphate of these primers without significantly impairing their primer function.

Such derivatives of ATP have been known for some time. Grachev & Zaychikov (1974) showed that ATP-P^3-anilidate functions efficiently as a substitute for ATP in elongation, and although not explicitly shown, it is anticipated that this analogue participates in initiation. Armstrong & Eckstein (1976) investigated the properties ATP P_3 esters and fluoro esters as substrates for RNA polymerase from *Escherichia coli* and observed that those analogues functioned either as substrates with unfavorable kinetic parameters or as inhibitors. They did not investigate if such analogues function as primers in

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