Noncovalent Modulation by ATP of the Acyl Transfer from Acyl-glyceraldehyde-3-phosphate Dehydrogenase to Phosphate†

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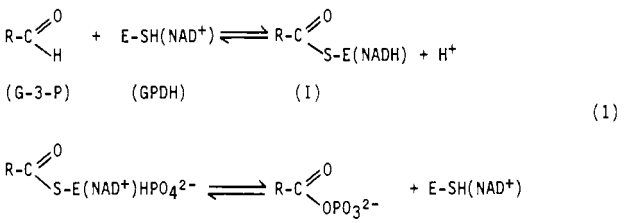
ABSTRACT: The effect of ATP on the formation, spectral properties, and reactions of [β-(2-furyl)acryloyl]glyceraldehyde-3-phosphate dehydrogenase (FA-GPDH) has been investigated. The chromophoric FA-GPDH has the advantage of providing spectrophotometric signals of the interaction of acyl enzyme with nucleotides and dinucleotides. The results are consistent with the exclusive existence of two acyl-enzyme conformations previously inferred from the interaction of the acyl enzyme with NAD^+ and NADH. ATP interaction stabilizes a conformation different from that stabilized by NAD^+. The inhibitory effects of ATP on these reactions are consistent with the reported inhibitory effect of ATP on the steady-state reaction with the true substrate. The physiological significance of these results to the regulation of glycolysis, via the ligand-dependent fate of 3-phosphoglycerol-GPDH, is discussed.

Glyceraldehyde-3-phosphate dehydrogenase [GPDH (EC 1.2.1.12)] catalyzes the two-step oxidative phosphorylation of glyceraldehyde 3-phosphate (eq 1). GPDH is present in very



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large concentrations in mammalian and fish muscle. In muscle, a substantial fraction of this enzyme exists in the form of the covalent enzyme–substrate intermediate, namely, 3-phosphoglyceroyl-GPDH (I) (Bloch et al., 1971). This energy-rich intermediate is known to interact differently with NAD^+ and NADH; these ligands activate acyl-GPDH for the acyl-group (I) (Bloch and reduction reaction, respectively. A potential role for the 3-phosphoglycerol enzyme is in modulation of the NAD^+/NADH ratio (Schwendimann et al., 1976). It is interesting to note that the free apoenzyme binds NAD^+ exceedingly tightly (Seydoux et al., 1973; Conway & Koshland, 1968), thus precluding competition by any other nucleotide. Formation of the acyl-enzyme intermediate drastically reduces the affinity for NAD^+, while the affinity for NADH remains unaltered (Malhotra & Bernhard, 1973; Keleman et al., 1975; Seydoux et al., 1976; Kellershohn et al., 1979). Under the physiological conditions the two coenzymes can effectively compete with each other for binding to 3-phosphoglyceroyl-GPDH. Thus, GPDH is an example par excellence for metabolic regulation via covalent E–S formation (Malhotra & Bernhard, 1973).

It has been reported that ATP inhibits the GPDH-catalyzed oxidative phosphorylation of G-3-P. This has been attributed to competition for the adenine subsite of the NAD^+ binding domain of the enzyme (Yang & Deal, 1969; Oguchi et al., 1973). It has also been suggested that ATP binds to a specific allosteric site (Ovadi et al., 1972), thus affecting transfer stability of subunit interactions (Constantinides & Deal, 1969). The molecular details of interaction among enzyme, substrate, and ATP are not clear from the steady-state kinetic reaction studies.

To facilitate studies on the role of ATP in the acyl-transfer reaction, we have utilized the synthetic pseudosubstrate, β-(2-furyl)acryloyl phosphate (FAP). FAP reacts with GPDH to form the corresponding FA-cysteinyl thiolester of the enzyme (FA-GPDH). FA-GPDH is analogous to the naturally occurring 3-phosphoglyceroyl-GPDH in its various physical and chemical properties (Malhotra & Bernhard, 1968, 1973); FA-GPDH reacts with NADH and with inorganic phosphate or arsenate, analogous to the reactions with the true acyl enzyme (eq 1). However, the pseudosubstrate reactions are considerably slower, allowing more detailed analysis of the transient reaction processes than is possible with the true 3-phosphoglyceroyl enzyme. The chromophoric acyl group provides an excellent tool to monitor not only the chemical reactions but also the protein conformational changes that accompany the interaction of acyl enzyme with NAD^+ and NADH. The affinities of FA-GPDH for NAD^+ and NADH are comparable to those of the naturally occurring acyl enzyme (Malhotra & Bernhard, 1973; Keleman et al., 1975; Seydoux et al., 1976; Kellershohn et al., 1979). The binding of NAD^+ and NADH to FA-GPDH brings about a red and a blue shift, respectively, in its acyl spectrum (Malhotra & Bernhard, 1973; Bernhard & Malhotra, 1974; Schwendimann et al., 1976). These spectral changes reflect protein conformational changes that precede and are prerequisites for the two chemical reaction steps of eq 1 (Malhotra & Bernhard, 1981; Malhotra et al., 1981).

In this paper the role of ATP in the modulation of acyl-enzyme formation and reactions is probed by its effects on the characteristic absorption properties of FA-GPDH in its two conformational states.

Experimental Procedures

Materials. GPDH was isolated from sturgeon muscle by the method of Seydoux et al. (1973). β-(2-Furyl)acryloyl phosphate barium salt was prepared and solubilized as de-

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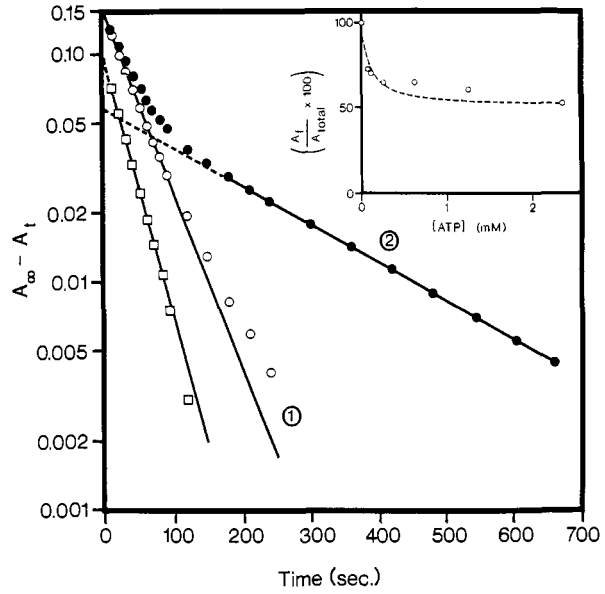


FIGURE 1: Kinetics of the reaction of holo-GPDH with FAP in the absence and presence of ATP. The concentration of holoenzyme is 0.33 mg/mL (2.37 μM containing 4 mol of NAD^+/mol of enzyme). FAP concentration is 1.53 mM. ATP concentration is 0 and 0.62 mM for curves 1 and 2, respectively. The reaction was started with an aliquot of the holoenzyme and monitored at 360 nm. The initial fast phase of curve 2 (difference between the experimental value and extrapolated slow phase) is plotted separately (□). (Inset) Relative amplitude of the fast phase in the reaction of holo-GPDH (2.37 μM containing 4 mol of NAD^+/mol of enzyme) with FAP (1.53 mM) in the presence of various concentrations of ATP. The data were simulated according to Scheme I with K_{ATP} equal to 0.1 mM.

scribed earlier (Malhotra & Bernhard, 1968). Its concentration was assayed spectrophotometrically ($\epsilon_{307nm} = 2.62 \times 10^4$ OD M^{-1} cm^{-1}). NAD^+, ATP, and sodium arsenate were purchased, in the highest quality available, from Sigma Chemical Co.

All experiments were performed at 25 °C in 10 mM ethylenediamine buffer containing 0.1 M KCl and 1 mM EDTA, pH 7.0.

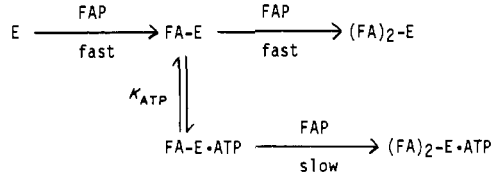
NAD^+-free GPDH and unliganded FA-GPDH were prepared as described earlier (Malhotra & Bernhard, 1981). NAD^+ content of GPDH and FA-GPDH was assayed on the basis of absorption at 260 and 280 nm, as described previously (Seydoux et al., 1973). The extent of reaction of enzyme with FAP was assayed at the isosbestic point between unliganded (or ATP liganded) and NAD^+-bound acyl enzyme as described previously (Malhotra & Bernhard, 1981).

Kinetics of Deacylation (Arsenolysis). FA-GPDH and NAD^+ were preincubated to UV spectral equilibration at 390 nm (ca. 5 min). When utilized, ATP was added to FA-GPDH before the addition of NAD^+. The kinetics of arsenolysis were initiated by the addition of microliter quantities of aqueous arsenate to the nucleotide-equilibrated acyl enzyme. The loss of acyl groups from FA-GPDH was monitored at 360 nm by using a Cary Model 14 spectrophotometer. The reaction progress was usually kinetically biphasic. The amplitudes, A_{fast} and A_{slow}, and the first-order rate constants, k_{fast} and k_{slow} (see eq 2), were derived by iterative curve fitting procedures using a computer program.

RESULTS

Previously we have reported on the reaction of GPDH with FAP and on the spectral and reactive properties of the resultant FA enzyme. Such equilibrium studies dealt with the effects of bound dinucleotides (NAD^+ and NADH) on the spectral and kinetic properties of the FA enzyme. All of these properties are affected by the presence of ATP, as is detailed below.

Scheme I



Effect of ATP on the Reaction of Holo-GPDH with FAP. First-order kinetics (linear semilog plots) are observed at 360 nm in the reaction of holo-GPDH with FAP (Malhotra & Bernhard, 1968, 1981). In the presence of ATP this reaction is kinetically biphasic. Each of the individual phases is expressible by a single-exponential decay rate law (Figure 1). The complete time course of the reaction is described by eq 2, where A_∞ and A_t are absorbance values at infinity and at

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

time t, respectively. A_{fast} and A_{slow} are the total individual amplitudes of absorbance change in the fast and slow phases, respectively; k_{fast} and k_{slow} are the corresponding first-order rate constants. The rates and amplitudes of reaction in the fast and slow phases are differently affected by variation in ATP concentration as follows.

(1) k_{fast} is independent of ATP concentration and is equal to the pseudo-first-order rate constant observed in the absence of ATP.

(2) k_{slow} decreases as ATP concentration increases.

(3) Although the total amplitude is nearly unaffected by variations of ATP concentration, the individual amplitudes (A_{fast} and A_{slow}) are variable in a complementary manner. The relative amplitude of the fast phase ($\%A_{fast}$) decreases from 100% in the absence of ATP to 50% at saturating concentrations of ATP (see inset in Figure 1). The total amplitude change at 360 nm at equilibrium corresponds to the formation of two acyl thiolesters per GPDH tetramer.

At the concentrations of ATP employed here, there is no effect on either the *initial* rate of reaction of holo-GPDH with excess FAP or the stoichiometry of acylation. After one acyl group per GPDH tetramer has been introduced, the residual acylation reaction is markedly slower.

The ATP concentration-dependent rate data at intermediate concentrations are consistent with the minimal model of Scheme I. In this scheme, E stands for the tetrameric enzyme. K_{ATP} is the dissociation constant of FA-E·ATP into FA-E and ATP. The calculated curve shown in the inset to Figure 1 is based on the model of Scheme I and its derived quantitative expression. The strong correspondence of the data with the model of Scheme I argues that there is a substantial increase in ATP affinity at acylated sites and that such ATP-liganded acyl sites affect the properties of adjacent nonacylated sites.

Effect of ATP on the Dinucleotide-Perturbed and the Unperturbed Spectra of FA-GPDH. The reaction of holo-GPDH with FAP gives rise to FA-GPDH(NAD^+). The acyl enzyme has a far lower affinity for NAD^+ than does the nonacylated enzyme (Malhotra & Bernhard, 1981). At these holoenzyme concentrations some of the bound NAD^+ dissociates upon acylation. The two species (FA-GPDH·NAD^+ and FA-GPDH) have distinct spectra with an isosbestic point at 360 nm. λ_{max} shifts from 346–347 nm in FA-GPDH to 360–363 nm in FA-GPDH(NAD^+). The FA-GPDH·NADH spectrum is severely blue-shifted relative to apo-FA-GPDH.

Unlike NAD^+ and NADH, ATP has little effect on the acyl (furylacryloyl) spectrum; only a very small blue shift of 2–3

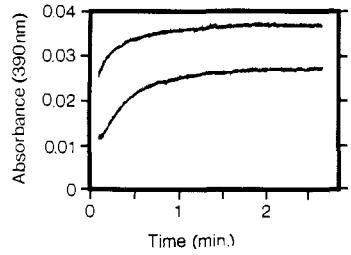


FIGURE 2: Time dependence of the spectral perturbation of *FA*-GPDH by NAD^+ in the absence (upper curve) and the presence (lower curve) of ATP. The protein concentration was 0.24 mg/mL containing 1.4 FA groups/mol of enzyme and 0.5 mol of NAD^+/mol of enzyme. In each case, NAD^+ was added at time zero. For the lower curve, addition of ATP (1.1 mM) preceded that of NAD^+. The absorbance *increase* was monitored at 390 nm.

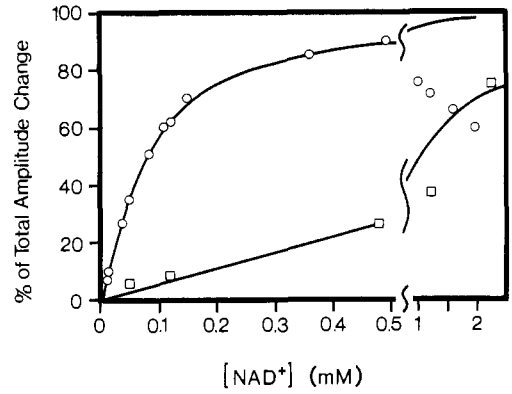


FIGURE 3: Equilibrium spectral perturbation of *FA*-GPDH by NAD^+ in the presence and absence of ATP. The protein concentration was 0.24 mg/mL containing 2.1 FA groups/mol of enzyme and 0.5 mol of NAD^+/mol of enzyme. An excess of NAD^+ was added to a solution of FA-GPDH in the absence (○) and in the presence of 1.14 mm ATP (□). Absorbance at 390 nm was monitored until it became constant (as in Figure 2). The final absorbance change expressed as percent of the value at NAD^+ saturation has been plotted against NAD^+ concentration. The solid lines are calculated as described in the text.

nm in λ_{max} and a 5–7% decrease in ϵ_{max} are observed (data not shown). ATP influences the NAD^+ perturbation of the FA-GPDH spectrum. In the presence of ATP, a larger proportion of the absorbance change takes place in the slow phase (Figure 2). Moreover, at equilibrium, the spectral shift induced by NAD^+ binding is smaller in the presence of ATP (Figure 3).

ATP also effects the equilibrium spectral perturbation of FA-GPDH by NADH. Increasing ATP concentrations tend to restore the apo-FA-GPDH spectrum. The effect of ATP concentration on the NADH-induced spectral perturbation is illustrated in Figure 4.

Effect of ATP on the Arsenolysis of FA-GPDH. FA-GPDH reacts with phosphate and arsenate only in the presence of bound NAD^+; FA-GPDH·NADH is not a competent species for the phosphorolysis and arsenolysis reactions. The two reactions are apparently analogous, but, unlike the acyl phosphate, the acyl arsenate is highly unstable, undergoing rapid hydrolysis (Malhotra & Bernhard, 1968).

Arsenolysis was initiated by the addition of arsenate to NAD^+-equilibrated FA-GPDH. The reaction was monitored spectrophotometrically at 360 nm. The reaction progress in the absence of ATP and after preincubation with a near saturating concentration of ATP is shown in Figure 5. The arsenolysis reaction is kinetically biphasic both in the absence and in the presence of ATP. The two first-order rate constants, k_{fast} and k_{slow}, are lower in the presence of ATP. However, the relative amplitudes of the two phases are strongly influenced by ATP concentration. A_{fast} decreases with increasing ATP concentration. In the experiments of Figure 5, A_{fast} accounts for 75% of the total amplitude in the absence of ATP. In the presence of 1.1 mM ATP, A_{fast} accounts for only 35% of the total amplitude. As the concentration of preincubated NAD^+ increases, the effect of ATP on the biphasicity of the

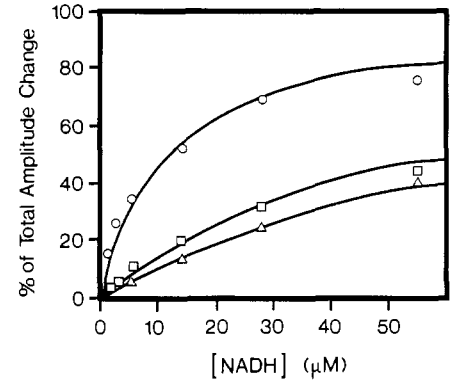


FIGURE 4: Equilibrium spectral perturbation of *FA*-GPDH by NADH in the presence and absence of ATP. The protein concentration was 0.156 mg/mL (1.12 μM) containing 2.05 FA groups/mol of enzyme and 0.8 mol of NAD^+/mol of enzyme. NADH was added to a solution of FA-GPDH in the presence of 0.39 mM ATP (□) and 0.77 mM ATP (△) and in the absence of ATP (○). The absorbance at 360 nm was monitored until it became constant. The final absorbance change expressed as percent of the value at NADH saturation has been plotted against NADH concentration. The solid lines are calculated as described in the text.

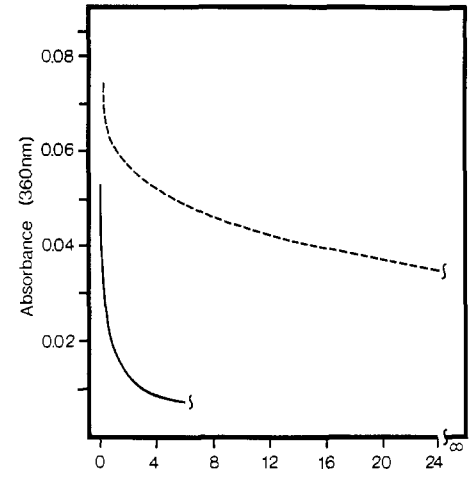


FIGURE 5: Kinetics of arsenolysis of FA-GPDH, as monitored at 360 nm, in the presence and absence of ATP. The protein concentration was 0.24 mg/mL (1.7 μM) containing 2.1 FA groups/mol of enzyme. The concentrations of NAD^+ and arsenate are 0.49 and 0.5 mM, respectively. The solid and dashed curves show the data in the absence and presence of 1.1 mM ATP, respectively. The final absorbance value (A_∞) in each case was close to zero.

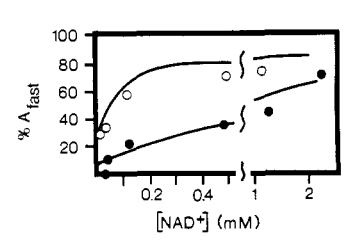


FIGURE 6: Fractional amplitude of the arsenolysis reactions occurring in the fast phase (A_{fast}) as a function of the preincubated NAD^+ concentration: A_{fast} in the absence (○) and in the presence (●) of 1.1 mM ATP. The solid lines are calculated by utilizing previously derived dissociation parameters as discussed in the text (see Results and Table I).

Table I: Binding Affinities for NAD^+, NADH, and ATP to Different Conformations of Furylacryloyl-GPDH

ligand	nature of experiment	dissociation constant (μM) E'-Ac	E-Ac
NAD^+	(i) spectral perturbation (ii) arsenolysis amplitudes	30 ± 3	140 ± 15
NADH	spectral perturbation	33 ± 6	9.5 ± 1.0
ATP	(i) competition with NAD^+ in spectral perturbation and arsenolysis amplitudes	167	83.3
	(ii) competition with NADH	184 ± 17	91 ± 9

reaction progress becomes less competitive, as is illustrated in Figure 6.

Previously, we have demonstrated the existence of two conformational states for FA-GPDH. The partition between the two states is affected by the presence of bound NAD^+. No additional conformational state need be invoked for the quantitative kinetic effects of NADH, although the two dinucleotides affect the partition differently (Malhotra & Bernhard, 1981). The kinetics of arsenolysis of FA-GPDH·NAD^+ in the presence of ATP are likewise quantitatively consistent with this two-state model and the assumption that ATP, like NADH, binds preferentially to the arsenolysis-incompetent acyl-enzyme state. The solid lines in Figure 6 are calculated on the basis of dissociation constants for dinucleotides and ATP previously determined, and determined herein, as listed in Table I.

Discussion

We have already demonstrated the existence of two distinct acyl-enzyme conformations. One of these is stabilized by interaction with NAD^+ and is competent and obligatory for acyl-transfer reactivity. The other conformation is stabilized by interaction with NADH and is obligatory for reduction of the acyl enzyme (Schwendiman et al., 1976; Malhotra & Bernhard, 1981). The dependence of the transient deacylation of the 3-phosphoglyceroyl-GPDH reaction on [NAD^+] is the same or very similar to the dependence of the color of the FA enzyme on [NAD^+] (Malhotra & Bernhard, 1973; Malhotra et al., 1981; Seydoux et al., 1974). Thus, the binding of NAD^+ to the acyl enzyme appears to be little affected by the structure of the acyl group, although acylation profoundly reduces the NAD^+ binding affinity. Likewise, the transient reduction of the acyl enzyme shows an NADH concentration dependence that is independent of the particular acyl group (Keleman et al., 1975; Kellershohn & Seydoux, 1979). Unlike the effect of acylation on NAD^+ affinity, the NADH binding affinity is not significantly affected by acylation. These facts argue strongly that acylation results in two new conformational states, rather than in two different acyl–coenzyme interactions, whose relative populations are determined by the structure of the coenzyme and the acyl derivative exclusively. In the presence of ATP, the kinetics of both arsenolysis and NAD^+-induced spectral perturbation of the FA enzyme are quantitatively accountable without recourse to further conformational states, on the assumption that ATP inhibition is a consequence of its preferential interaction with the NADH-stabilized acyl-enzyme conformation. We have previously shown that the acyl-enzyme conformational transitions are exceedingly slow with *FA*-GPDH. Even in the absence of ATP, a considerable fraction of the kinetically biphasic arsenolysis reaction occurs at the slow rate. This slow phase reflects the proportion of acyl sites in the NADH-affine conformation; this proportion is NAD^+ concentration dependent (Malhotra & Bernhard, 1981). The amplitude of this slow-phase contribution is increased in the presence of ATP. Quantitatively similar biphasic rates and amplitudes are obtained from the kinetic analysis of NAD^+-induced spectral perturbation. Thus, all of the data we present herein on arsenolysis kinetics, NAD^+-induced spectral perturbation, and acylation kinetics are explained on the assumption that ATP functions exclusively as a positive effector of the NADH-stabilized acyl-enzyme state. The solid curves in Figures 3, 4, and 6 are based on this assumption, using the dissociation constants of Table I.

ATP does not affect the initial rate of acylation of GPDH with FAP; its inhibitory effect becomes noticeable only after the statistical incorporation of one FA group per four GPDH monomeric subunits. ATP appears to interact much more strongly with FA enzyme than it does with nonacylated GPDH. This might be a consequence either of the increased affinity of the FA enzyme for ATP or of a decreased affinity of the FA enzyme for the competitive ligand, NAD^+. The affinity of nonacylated enzyme for NAD^+ is very high; under physiological conditions, no other ligand could effectively compete with NAD^+ for binding to free unacylated GPDH. The affinity for NAD^+ is known to decrease sharply on acylation (Malhotra & Bernhard, 1973). The relative affinities for NAD^+, NADH, and ATP change drastically on the formation of FA-GPDH (Table I). The physiologically relevant 3-phosphoglyceroyl-GPDH has apparent affinities for NAD^+ and NADH similar to those of the FA enzyme; the similarity might extend to ATP ligation as well. If so, given the in situ concentrations of these effectors (Srivastava & Bernhard, 1986), the three ligands (NAD^+, NADH, and ATP) can effectively compete with each other for binding to 3-phosphoglyceroyl-GPDH.

Both the reaction kinetics and the equilibrium spectral perturbations are affected by the oligomeric nature of the GPDH structure. Although composed of identical subunits, the tetrameric enzyme interacts with specific ligands in a manner unanticipated for a high symmetry (D_2) tetrameric structure. Most characteristically, the tetrameric enzyme interacts with four NAD^+ as if there were two distinct classes of NAD^+ binding sites of differing affinity (Seydoux et al., 1973; Scheek & Slater, 1978). The enzyme reacts with an excess of the pseudosubstrate, FAP, to yield a stoichiometric diacyl-enzyme tetramer (Malhotra & Bernhard, 1968). Consequently, the simplest structure for the enzyme molecule must be that of a dimer of dimers [cf. Malhotra et al. (1983) and Malhotra and Srinivasan (1985)]. Within the tetramer, the acyl-enzyme dimers can exist in two alternate conformations, dependent on specific nucleotides ligation (Malhotra & Bernhard, 1981). The question arises as to whether dimers of opposing conformation can exist within a single tetramer. Previously, we have shown that the state of ligation at one acyl site of the molecule affects the ligand affinity and/or the reactivity at the other acyl site in the diacyl-enzyme tetramer. Positive cooperativity is evident in the binding of NAD^+ to the diacyl enzyme (Malhotra & Bernhard, 1981).

A substantial fraction of the data we present herein, as well as data presented previously for NAD^+ interaction with the acyl enzyme, can be quantitatively explained on the basis of the concerted model of Monod et al. (1965) on the assumption that the diacyl enzyme is a dimer of dimers. These data include all of the spectral equilibrium of measurements and all of the kinetic amplitude data as presented in Table I. However, the minimal concerted model is inadequate to explain

the NAD^+ concentration dependence of the (biphasic) kinetic rate constants. Introduction of ATP adds further complexity to the interpretation of these rate constants, although analyses according to the concerted model are still entirely consistent with the kinetic amplitudes and equilibrium data. Whatever the detailed kinetic mechanisms, the variations in the magnitudes of these rate constants indicate a greater diversity of states in the kinetic pathway than is evident or required to account for the equilibrium populations of acyl-enzyme states.

The results we have presented here indicate that the formation of acyl phosphate from acyl enzyme in the GPDH pathway may be an important control point in glycolysis. ATP, by its substantial interaction with the phosphorolysis-incompetent form of acyl enzyme, provides a mechanism for switching off glycolysis. We note in this regard the relatively high concentration of ATP as compared with other metabolites in the glycolytic pathway (including NAD^+, NADH, and all of the three carbon intermediates of glycolysis). If glycolysis is turned off due to a nearly stoichiometric formation of E-acyl·ATP, the system will be sensitive to small fluxes in ATP concentration.

It has been shown that GPDH is capable of direct enzyme–enzyme metabolite transfer of 1,3-diphosphoglycerate to phosphoglycerate kinase (Weber & Bernhard, 1982) and of NADH from E-acyl·NADH to lactate dehydrogenase (Srivastava & Bernhard, 1985). Direct transfer to NADH to lactate dehydrogenase and of 1,3-diphosphoglycerate to phosphoglycerate kinase requires alternate acyl-GPDH conformations. It is tempting to speculate that the alternative recognitions of acyl-enzyme NADH by lactate dehydrogenase and acyl-enzyme NAD^+ by phosphoglycerate kinase are a consequence of their alternative conformations. Hence, in the direction of glycolysis, the recognition of acyl-GPDH·NADH by lactate dehydrogenase and its conversion to acyl-GPDH·NAD^+ by pyruvate must precede the transfer of 1,3-diphosphoglycerate to phosphoglycerate kinase. Excessive ATP synthesis, beyond the tight binding capacity of the kinase, will autoregulate subsequent ATP synthesis by inactivation of acyl-GPDH toward high-energy acyl-phosphate formation.

Registry No. ATP, 56-65-5; NAD, 53-84-9; NADH, 58-68-4.

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