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Nicotinamide Adenine Dinucleotide-specific Glyceraldehyde 3-Phosphate Dehydrogenase from *Pisum sativum*

EFFECT OF NICOTINAMIDE ADENINE DINUCLEOTIDE AND RELATED COMPOUNDS ON THE ENZYME-CATALYZED ARSENOLYSIS OF 1,3-DIPHOSPHOGLYCERIC ACID*

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SUMMARY

NAD$^+$-specific glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) from pea seeds is shown to catalyze the arsenolysis of 1,3-diphosphoglyceric acid. The reaction shows an absolute requirement for NAD$^+$ or analogs which will replace NAD$^+$ in the enzyme-catalyzed oxidative phosphorylation of glyceraldehyde 3-phosphate. NADH, glyceraldehyde 3-phosphate, or NAD$^+$ analogs which are inhibitory or inactive in the oxidative phosphorylation reaction, are unable to stimulate arsenolysis. NAD$^+$ is also required for the reductive dephosphorylation of 1,3-diphosphoglyceric acid by NADH.

The specificity and kinetics of the effect of NAD$^+$ in the arsenolysis reaction are consistent with the kinetic mechanism previously proposed (DUGGLEBY, R. G., AND DENNIS, D. T. (1974) *J. Biol Chem.* 249, 167–174). In view of the similarity between the enzyme from pea seeds and that from other sources, it is suggested that this mechanism may operate in the enzyme from other sources. The mechanism is capable of providing a simple explanation for a number of the catalytic properties which have been reported for the enzyme from other sources.

Glyceraldehyde 3-phosphate dehydrogenase is capable of catalyzing a number of reactions. Colowick *et al.* (1) list seven, to which must be added the oxidative activity (2, 3) and the formation of "alkali-stable NAD$^+$" (4). Of these nine activities, NAD$^+$ appears to be required for enzyme activity in the reductive dephosphorylation of 1,3-diphosphoglyceric acid by NADH (5, 6), the transacylase activity (6–8), the acyl phosphatase activity (9), NADH-X[1] formation (10), and the diaphorase activity (1, 11). Of the four remaining activities, NAD$^+$ is a stoichiometric participant in three. Only the esterase activity shows no NAD$^+$ requirement (12), although NAD$^+$ may inhibit this reaction (13). NAD$^+$ is required for the acylation of the enzyme by aldehydes (14) or by acyl phosphates (15, 16) and for the alkylation of the enzyme by iodoacetic acid (17). NAD$^+$ also protects the enzyme against an intramolecular acyl transfer (18) and against inactivation by proteolytic enzymes (19, 20). It has generally been assumed that NAD$^+$ induces a conformational change from an inactive to a catalytically active form of the enzyme. While it is clear that NAD$^+$ does induce a conformational change (21–24), there is no satisfactory explanation of why it should be the enzyme-NAD$^+$ complex which is catalytically active.

In the preceding paper (25), kinetic evidence was presented which suggests that glyceraldehyde 3-phosphate dehydrogenase has a ping-pong mechanism. In this mechanism, the free enzyme does not participate in the catalytic cycle, whereas the enzyme-NAD$^+$ complex is an obligatory intermediate. Amongst other things, this mechanism requires that the enzyme should be capable of catalyzing the arsenolysis of acyl phosphates and, further, that this reaction should show an absolute requirement for NAD$^+$. As noted above, previous work would appear to confirm this aspect of the mechanism. However, in view of the limited amount of published data on the arsenolysis of 1,3-diphosphoglyceric acid and the poorly understood role of NAD$^+$, we undertook a detailed study of this reaction. The requirements of a related reaction, the reductive dephosphorylation of 1,3-diphosphoglyceric acid by NADH were also investigated. The results agree with the mechanism deduced on kinetic grounds (25), which provides a simple explanation for a variety of properties of glyceraldehyde 3-phosphate dehydrogenase.

MATERIALS AND METHODS

Materials—Glyceraldehyde 3-phosphate dehydrogenase was purified from pea seeds as previously described (26). Other enzymes, biochemicals, and buffers were purchased from Sigma Chemical Co.; other chemicals were of the highest grade commercially available, except for ammonium sulfate, which met ACS specifications.

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[1] The following abbreviations are used: NADH-X, monohydroxytetrahydronicotinamide adenine dinucleotide; TNAD, thionicotinamide adenine dinucleotide; APAD, acetylpyridine adenine dinucleotide; NHD, nicotinamide hypoxanthine dinucleotide; PAHD, pyridinealdehyde hypoxanthine dinucleotide; PAAD, pyridinealdehyde adenine dinucleotide; ADPR, ADP-ribose; APHD, acetylpyridine hypoxanthine dinucleotide; NA, nicotinamide.

Preparation of NAD$^+$-free Glyceraldehyde 3-Phosphate Dehydrogenase—The widely used charcoal method (27) only removes 50 to 70% of the NAD$^+$ from the pea seed enzyme, and the following procedure was therefore adopted. Glyceraldehyde 3-phosphate dehydrogenase was dissolved in 10 mM potassium phosphate buffer, pH 7.2, containing 1.5 mM EDTA (EDTA-phosphate buffer), to give a protein concentration of 1 to 2 mg per ml. Glyceraldehyde 3-phosphate was added to give a final concentration of 0.1 mM, then 6 mg of activated charcoal per mg protein were added. The charcoal was added in the form of a suspension containing 200 mg per ml of activated charcoal in EDTA-phosphate buffer, adjusted to pH 7.2 with ammonium hydroxide. The enzyme solution was incubated for 5 min at 0° and the charcoal was removed by centrifugation. At this stage, analysis of the enzyme showed that it is acylated (approximately 1.5 moles of 3-phosphoglyceric acid per mole of enzyme, using the methods of Bloch *et al.* (28)). Solid ammonium sulfate was added until saturation was reached, the pH being maintained between 6.5 and 7.0 by the addition of 3.5 N ammonium hydroxide. The precipitated protein was collected by centrifugation and redissolved in sufficient EDTA-phosphate buffer, pH 7.2, to give a protein concentration of 5 to 10 mg per ml. This solution was left for 24 hours at 4° during which time the enzyme was found to spontaneously deacylate. The enzyme was then precipitated by the addition of 9 volumes of saturated ammonium sulfate in EDTA-phosphate buffer, pH 7.2, and stored as a suspension. This preparation contains about 0.05 moles of NAD$^+$ per subunit, and is stable for a period of several weeks.

Assay Procedures—Glyceraldehyde 3-phosphate dehydrogenase was assayed in the forward direction using the previously described kinetic assay (25), with 1 mM glyceraldehyde 3-phosphate, 10 mM potassium phosphate, and nucleotide substrates and inhibitors as described in the text and figure legends. The reaction was followed at the absorption maximum of the reduced form of the nucleotide substrate (29).

The routinely used arsenolysis assay mixture contained 5 mM ATP, 5 mM 3-phosphoglyceric acid, 10 mM magnesium chloride, 1 mM EDTA, 3 mM β-mercaptoethanol, and 80 mM sodium arsenate in 150 mM Tris-chloride buffer, pH 6.9; other additions are noted in the text and figure legends. Yeast 3-phosphoglyceric acid kinase (3.2 units of Sigma type IV per ml of reaction mixture) was added, which was found to be sufficient to allow the 3-phosphoglyceric acid kinase system to come to equilibrium in less than 2 min. This mixture was incubated for 10 to 15 min in order to establish a basal rate for the nonenzymic hydrolysis of 1,3-diphosphoglyceric acid. The arsenolysis reaction was then initiated by the addition of NAD$^+$-free glyceraldehyde 3-phosphate dehydrogenase.

ADP was estimated at intervals throughout this procedure by withdrawing 1-ml samples and treating them as follows. The reaction was stopped by the addition of 1 ml of 54 mM hydrochloric acid containing 1 mg per ml of pepsin (Sigma type "1: 10,000 powder"). After approximately 20 min at pH 2.0, 1 ml of 110 mM potassium carbonate was added to raise the pH to 6.8. One-milliliter samples were then assayed for ADP, using the method of Adams (30), making duplicate determinations of ADP.

Departures from this procedure are noted at the appropriate places in the text. When the sodium arsenate concentration was varied, the concentrations of hydrochloric acid and potassium carbonate were also varied, so that the pH values after acidification and neutralisation were 2.0 and 6.8, respectively. The method of calculating the arsenolysis of 1,3-diphosphoglyceric acid from ADP concentrations is given in the "Appendix."

RESULTS

The arsenolysis of 1,3-diphosphoglyceric acid was followed by coupling the reaction to the hydrolysis of ATP using 3-phosphoglyceric acid and 3-phosphoglyceric acid kinase and by measuring the increase in ADP concentration with time. It should be noted that the reaction occurred over a period of several minutes, so that the hydrolysis of 1-arseno-3-phosphoglyceric acid was essentially complete. As was discussed in the previous paper (25), the hydrolysis of this compound may not be complete over the short periods required for steady state kinetic measurements. The reaction sequence is:

3-Phosphoglyceric acid + ATP

$$\xleftarrow{\text{3-phosphoglyceric acid kinase, } Mg^{2+}}$$

ADP + 1,3-diphosphoglyceric acid

1,3-Diphosphoglyceric + arsenate

$$\xrightarrow{\text{glyceraldehyde 3-phosphate dehydrogenase}}$$

1-arseno-3-phosphoglyceric acid + P_i

$$\text{1-Arseno-3-phosphoglyceric acid} + H_2O \xrightarrow{\text{spontaneous}}$$

3-phosphoglyceric acid + arsenate

$$\text{Sum: ATP} + H_2O \rightarrow \text{ADP} + P_i$$

The system has an absolute requirement for NAD$^+$ (Fig. 1*A*). The slow rate observed before NAD$^+$ addition is probably due to the nonenzymic hydrolysis of 1,3-diphosphoglyceric acid. The system also has an absolute requirement for 3-phosphoglyceric acid and 3-phosphoglyceric acid kinase (Fig. 1*B*), glyceraldehyde 3-phosphate dehydrogenase (Fig. 1*C*), and arsenate (Fig. 1*D*). These results show that the activity observed is not due to an ATPase, some activity in the 3-phosphoglyceric acid kinase, or an acyl phosphatase. Thus, the system measures the arsenolysis of 1,3-diphosphoglyceric acid. The rate of arsenolysis is proportional to the glyceraldehyde 3-phosphate dehydrogenase concentration (Fig. 2*A*) and shows a hyperbolic dependence on arsenate concentration below 40 mM (Fig. 2*B*). At arsenate concentrations above 80 mM, there is an marked inhibition (Fig. 2*C*). The apparent K_m for NAD$^+$ is lowered by increasing the arsenate concentration (Table I).

The specificity of the NAD$^+$ effect was examined by using a number of NAD$^+$ analogs. Each analog was also tested for its ability to replace NAD$^+$ as a substrate in the oxidative phosphorylation of glyceraldehyde 3-phosphate. Those analogs which failed to act as substrates were tested for their ability to inhibit this reaction when NAD$^+$ is used as the substrate, in order to determine if the analogs which failed to act as substrates will bind to the enzyme. NAD$^+$, TNAD, APAD, and NHD were found to act as substrates (Fig. 3*A*), and PADH, PAAD, and ADPR were found to act as inhibitors (Fig. 3*B*). APHD, NADP$^+$, NMN, and NA do not appear to bind to the enzyme. Only those compounds which act as substrates were found to be capable of stimulating the glyceraldehyde 3-phosphate dehydrogenase-catalyzed arsenolysis of 1,3-diphosphoglyceric acid (Table II).

NADH was tested for its ability to stimulate the arsenolysis of 1,3-diphosphoglyceric acid. Interpretation is made difficult by the fact that, in the presence of NADH, the reverse reaction can proceed, which will appear to indicate that 1,3-diphospho-

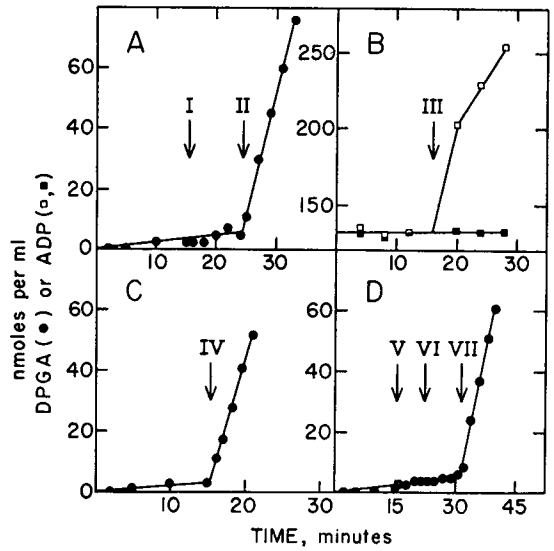

FIG. 1. Requirements for the arsenolysis reaction catalyzed by pea seed glyceraldehyde 3-phosphate dehydrogenase. Experiments were performed as described under "Materials and Methods," except as noted below. In *A*, the reaction mixture contained 5 mM sodium arsenate and had a pH of 6.87. At *I* (15.5 min), NAD^+-free glyceraldehyde 3-phosphate dehydrogenase was added at a concentration of 2.30 μg per ml and, at *II* (24.5 min), NAD^+ was added at a concentration of 100 μM. In *B*, the reaction mixtures contained 100 μM NAD^+ and had a pH of 6.93. 3-Phosphoglyceric acid kinase was omitted from one mixture (□), and both 3-phosphoglyceric acid and 3-phosphoglyceric acid kinase were omitted from the other (■). The reactions were initiated by the addition of NAD^+-free glyceraldehyde 3-phosphate dehydrogenase at a concentration of 2.52 μg per ml (0.0 min), and at *III* (16.0 min), 3-phosphoglyceric acid kinase was added at a concentration of 3.2 units per ml. In the reaction mixture containing 3-phosphoglyceric acid (□), the ADP concentration rose rapidly as the 3-phosphoglyceric acid kinase equilibrium was established, and this was followed by a slower increase due to the arsenolysis of 1,3-diphosphoglyceric acid. In *C*, the reaction mixture contained 10 μM NAD^+ and had a pH of 6.96. At *IV* (15.4 min), NAD^+-free glyceraldehyde 3-phosphate dehydrogenase was added at a concentration of 2.10 μg per ml. In *D*, sodium arsenate was omitted from the reaction mixture which had a pH of 6.90. At *V* (15.5 min), NAD^+-free glyceraldehyde 3-phosphate dehydrogenase was added at a concentration of 2.30 μg per ml; at *VI* (22.5 min) NAD^+ was added at a concentration of 100 μM; and at *VII* (31.5 min), sodium arsenate was added at a concentration of 5 mM. In *B*, ADP concentration is plotted against time, and in *A*, *C*, and *D*, ADP concentrations are converted to 1,3-diphosphoglyceric acid hydrolyzed, as described under the "Appendix," and are plotted against time.

glyceric acid is being hydrolyzed. One may correct for this reaction by simultaneously following the oxidation of NADH at 340 nm. Furthermore, as the reverse reaction proceeds, NAD^+ is formed which, as has already been shown, will stimulate the arsenolysis of 1,3-diphosphoglyceric acid. Nevertheless, a lag in arsenolysis should be observed if NADH does not stimulate arsenolysis. In such an experiment (Fig. 4), a lag of 0.7 min was observed.

In addition to its effect in the arsenolysis reaction, NAD^+ has been reported to be required in catalytic amounts for the reductive dephosphorylation of 1,3-diphosphoglyceric acid catalyzed by glyceraldehyde 3-phosphate dehydrogenase from rabbit muscle (6). This experiment was repeated with the pea seed enzyme, following NADH oxidation at 340 nm in a stopped flow apparatus (Fig. 5). A lag of about 80 ms is observed (*Curve A*), but preincubation of the enzyme with 0.1 mM NAD^+ reduces this lag to about 5 ms (*Curve B*) which probably represents the time re-

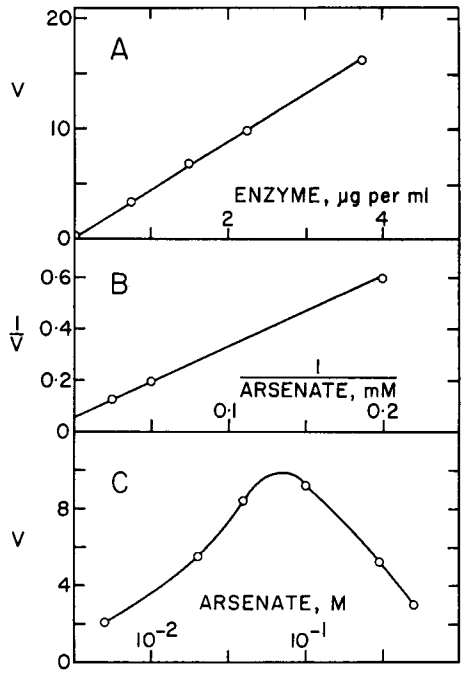

FIG. 2. Effect of pea seed glyceraldehyde 3-phosphate dehydrogenase and arsenate concentrations on the rate of arsenolysis of 1,3-diphosphoglyceric acid. Experiments were performed as described under "Materials and Methods," except as noted below. In *A*, the reaction mixtures contained 5 mM sodium arsenate, 100 μM NAD^+, and NAD^+-free glyceraldehyde 3-phosphate dehydrogenase as indicated and each had a pH of 6.88. Rates of arsenolysis were calculated, corrected for the nonenzymic hydrolysis of 1,3-diphosphoglyceric acid (0.14 nmoles per min per ml), and are shown plotted against enzyme concentration. In *B*, reaction mixtures contained 10 μM NAD^+, 2.30 μg per ml of NAD^+-free glyceraldehyde 3-phosphate dehydrogenase, and sodium arsenate as indicated, and each had a pH of 6.90. Rates of arsenolysis were calculated, corrected for the nonenzymic hydrolysis of 1,3-diphosphoglyceric acid (0.21 nmole per min per ml), and are shown plotted against arsenate concentration in double reciprocal form. In *C*, experiments were performed as in *B*, and the results were plotted as rate against log [arsenate]. In all cases, rates are expressed in nanomoles per min per ml.

TABLE I

Effect of NAD^+ concentration on rate of arsenolysis of 1,3-diphosphoglyceric acid

Experiments were performed as described under "Materials and Methods," using 2.15 μg (5 mM arsenate) or 2.65 μg (80 mM arsenate) per ml of NAD^+-free glyceraldehyde 3-phosphate dehydrogenase. From the rates at 10^{-4} M NAD^+ and 10^{-5} M NAD^+, the K_m for NAD^+ was calculated to be 0.156 mM (5 mM arsenate) or 0.0244 mM (80 mM arsenate). Rates have been corrected for the nonenzymic rate of hydrolysis of 1,3-diphosphoglyceric acid (0.21 nmole per min per ml).

NAD^+	Rate of arsenolysis	
	5 mM arsenate	80 mM arsenate
M	*nmoles/min/ml*	
0	<0.10	<0.10
10^{-7}	<0.10	0.16
10^{-6}	<0.10	1.48
10^{-5}	1.04	8.12
10^{-4}	6.74	22.44

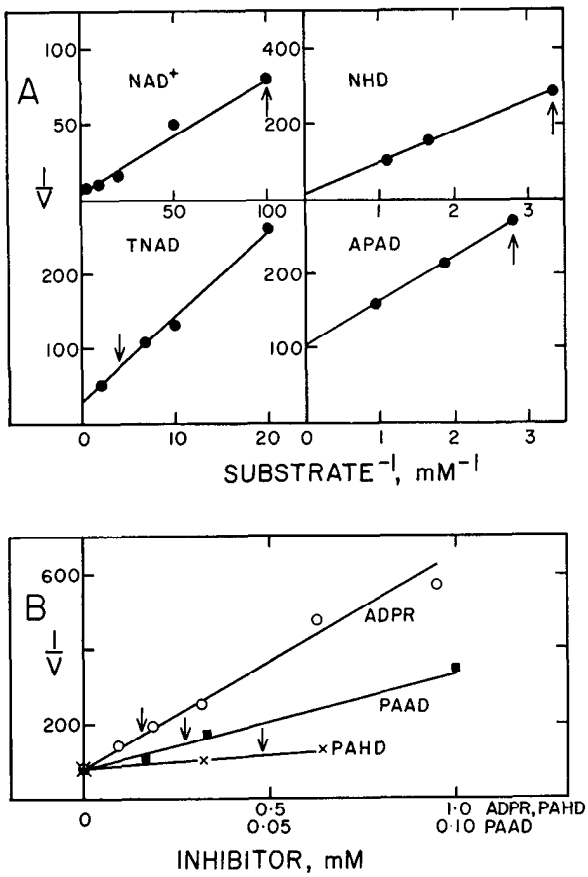

FIG. 3. Effect of NAD+ analogs on the oxidation of glyceraldehyde 3-phosphate by pea seed glyceraldehyde 3-phosphate dehydrogenase. Assays were performed as described under "Materials and Methods," using 1 μg of NAD+-free glyceraldehyde 3-phosphate dehydrogenase and NAD+ analogs as indicated. In *A*, the concentration of the nucleotide substrate was varied as indicated and is plotted against rate in double reciprocal form. In *B*, assays contained 10 μM NAD+, and ADPR (○), PAAD (□), or PAHD (×) as indicated. The inhibitor concentration is shown plotted against 1/rate. In both *A* and *B*, rate are expressed as micromoles of product formed per min. The *arrows* indicate the concentrations used in the arsenolysis experiment (Table II).

quired to attain steady state conditions. Thus, NAD+ appears to be required in the reverse reaction.

DISCUSSION

In the preceding paper (25), a ping-pong mechanism for glyceraldehyde 3-phosphate dehydrogenase was suggested on the basis of steady state kinetic studies. This mechanism predicts various partial reactions. The simplest of these to measure is the arsenolysis of 1,3-diphosphoglyceric acid (Fig. 6).

This scheme predicts that the free enzyme should be catalytically inactive, the reaction having an absolute requirement for NAD+. This prediction was confirmed (Fig. 1*A*). The effects of NAD+ in several of the catalytic activities of glyceraldehyde 3-phosphate dehydrogenase have been noted by other workers (1, 5–11), and these have previously been ascribed to an effect of NAD+ in inducing the correct conformation of the enzyme. These NAD+ effects are usually considered to be distinct from the substrate role of NAD+ (31). In the mechanism we propose, these NAD+ effects are a necessary consequence of the substrate role of NAD+, which is consistent with the finding that only NAD+ analogs with substrate activity are able to stimulate arsenolysis (Table II). Kaplan *et al.* (32) have noted

TABLE II

Effect of NAD+ analogs on arsenolysis of 1,3-diphosphoglyceric acid

Experiments were performed as described under "Materials and Methods," using NAD+-free glyceraldehyde 3-phosphate dehydrogenase at a concentration of 2.7 μg per ml. Rates have been corrected for slight variations in the amount of enzyme used in different experiments, and for the nonenzymic hydrolysis of 1,3-diphosphoglyceric acid.

Analog	Concentration	Rate of arsenolysis
	μM	*nmoles/min/ml*
NAD+ (substrate)	10	8.23
TNAD (substrate)	237	5.46
NHD (substrate)	300	2.77
APAD (substrate)	356	0.60
PAAD (inhibitor)	27	<0.10
PAHD (inhibitor)	482	<0.10
ADPR (inhibitor)	157	<0.10
APHD (no effect)	640	<0.10
NADP+ (no effect)	100	<0.10
NMN (no effect)	648	<0.10
NA (no effect)	100	<0.10
Glyceraldehyde 3-phosphate	100	<0.10
None		<0.10

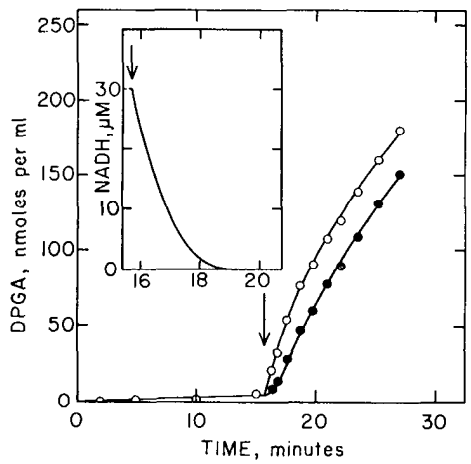

FIG. 4. Effect of NADH on the arsenolysis of 1,3-diphosphoglyceric acid catalyzed by pea seed glyceraldehyde 3-phosphate dehydrogenase. Conditions were as described under "Materials and Methods," except that the reaction mixture contained 30 μM NADH and each had a pH of 6.95. At the point indicated (15.7 min), NAD+-free glyceraldehyde 3-phosphate dehydrogenase was added at a concentration of 2.00 μg per ml, and the disappearance of 1,3-diphosphoglyceric acid was followed (○). The reduction of 1,3-diphosphoglyceric acid by NADH was simultaneously followed at 340 nm (*inset*), and the arsenolysis of 1,3-diphosphoglyceric acid was found by subtraction of this reaction (●).

that, for the rabbit muscle enzyme, the ability of NAD+ analogs to stimulate the arsenolysis of acetyl phosphate is related to their ability to act as substrates in the oxidative phosphorylation of glyceraldehyde 3-phosphate.

The results presented in this report are also consistent with an alternative model in which NAD+ serves a dual role, both as a substrate and as an activator of the enzyme. To some extent, this model is similar to the one we propose, in that in both cases it is the $E \cdot NAD^+$ complex rather than the free enzyme which is the active species in the arsenolysis of acyl phosphates. The value of the model we propose is that it provides an explanation for this phenomenon in terms of a simple mechanism.

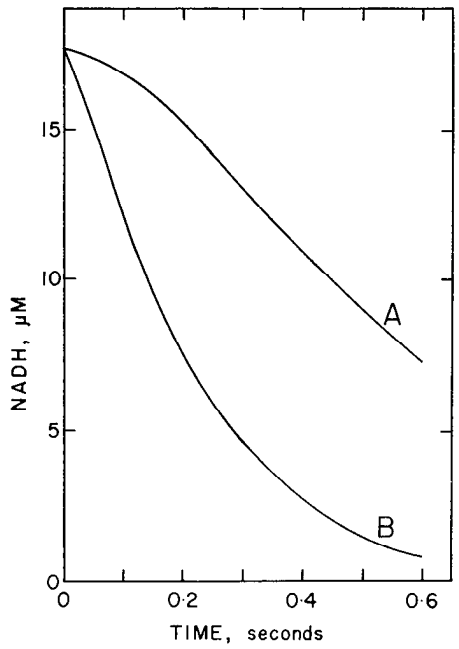

FIG. 5. NAD^+ requirement for the oxidation of NADH by 1,3-diphosphoglyceric acid catalyzed by pea seed glyceraldehyde 3-phosphate dehydrogenase. The oxidation of NADH was followed at 340 nm using a Durrum-Gibson stopped flow spectrophotometer at 25°. The curves shown are redrawn from photographs of oscilloscope tracings obtained with this instrument. One syringe contained 10 mM ATP, 10 mM 3-phosphoglyceric acid, 20 mM magnesium chloride, 2 mM EDTA, 3 mM β-mercaptoethanol, 35.4 μM NADH, and 3.2 units per ml of 3-phosphoglyceric acid kinase (Sigma type IV), in 150 mM Tris-chloride buffer, pH 6.95. This mixture was incubated at 25° for 10 to 20 min before use. The second syringe contained 2 mM EDTA, 3 mM β-mercaptoethanol, and approximately 1 mg per ml of NAD^+-free glyceraldehyde 3-phosphate dehydrogenase, in 150 mM Tris-chloride buffer, pH 6.95 (*Curve A*), or the same mixture plus 0.1 mM NAD^+ (*Curve B*).

Several workers (33–35) have studied the binding of NAD^+ to glyceraldehyde 3-phosphate dehydrogenase and have observed binding constants as high as 10^{11} M^{-1} for the first mole of NAD^+ bound to the tetramer. The mechanism we propose suggests that NAD^+ is required in the arsenolysis reaction to convert the catalytically inactive free enzyme into the active enzyme-NAD^+ complex. Thus, it might be expected that the concentrations of NAD^+ required would be lower than Table I indicates. However, it must be remembered that the acyl·*E*·NAD^+ complex may also release NAD^+, with a dissociation constant of the same order of magnitude as the K_m determined by kinetic methods (25, 36). Thus, it was predicted that at low arsenate concentrations (when a large fraction of the enzyme will exist as the acyl·*E*·NAD^+ complex) the apparent K_m in arsenolysis will be similar to the kinetic K_m of 0.10 to 0.15 mM. At 5 mM arsenate, the apparent K_m was found to be 0.156 mM (Table I). Furthermore, it was predicted that increasing the arsenate concentration would decrease the apparent K_m for NAD^+, and this effect is observed (Table I). At a saturating concentration of arsenate, the concentration of the acyl·*E*·NAD^+ complex should be zero, and the apparent K_m for NAD^+ should approach the dissociation constant of the enzyme-NAD^+ complex. Since high arsenate concentrations are inhibitory (Fig. 2*C*), this hypothesis could not be tested.

Glyceraldehyde 3-phosphate dehydrogenase catalyses quite a wide range of reactions, and it might be argued that the arsenolysis of 1,3-diphosphoglyceric acid is such an activity and, hence, that kinetic studies of the forward reaction have no relevance to the arsenolysis reaction. Indeed, Colowick *et al.* (1) classified it as a separate activity, and grouped it with other transacylase activities, such as the acylation of thiolalcohols. However, limited proteolysis of the enzyme with chymotrypsin does not destroy the transacylase activity (2), whereas this treatment leads to a simulataneous loss of the dehydrogenase and arsenolysis activities (2, 3). Furthermore, the relative activities of

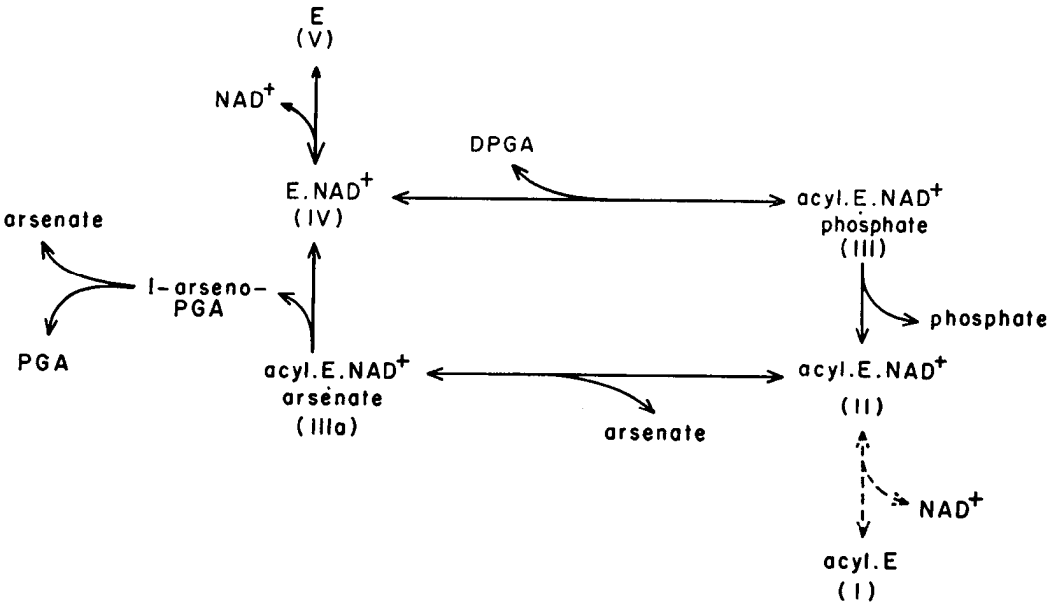

FIG. 6. Proposed cycle of reactions in the glyceraldehyde 3-phosphate dehydrogenase-catalyzed arsenolysis of DPGA. The catalytic cycle is composed of the reactions *IV* → *III* → *II* → *IIIa* → *IV*. If glyceraldehyde 3-phosphate dehydrogenase is added as the free enzyme (*V*), arsenolysis will be absolutely dependent on NAD^+. When arsenate is not saturating, a finite concentration of *II* will exist, from which NAD^+ may dissociate to give the catalytically inactive complex, *I*. The numbering of the enzyme forms is as given by Duggleby and Dennis (25), except for *IIIa*, which is the arsenate-containing equivalent of *III*. *PGA*, 3-phosphoglyceric acid; *DPGA*, 1,3-diphosphoglyceric acid.

the pea seed enzyme in the arsenolysis and reverse reactions are quite similar under identical conditions (from Fig. 4, the relative activities are 1.00 and 1.07, respectively). Since the rate of the arsenolysis reaction is similar to that of the reverse reaction, we believe that we are justified in assuming that the arsenolysis reaction is a partial reaction which is directly related to the mechanism derived from steady state kinetic studies.

Previously proposed kinetic mechanisms (36–40) appear to be deficient in various respects. All predict that NADH would be necessary in order to observe arsenolysis, but this does not appear to be the case (Fig. 4). Racker and Crimsky (41) have reported that NADH will stimulate the arsenolysis of 1,3-diphosphoglyceric acid but, under the conditions they employ, the reverse reaction may occur, and they may not have been measuring NADH-stimulated arsenolysis.

From the evidence presented in Fig. 5, and the data of De Vijlder *et al.* (6), NAD^+ appears to be required in the reverse reaction, which agrees with the proposed mechanism (25), in which the free enzyme is not catalytically active. The free enzyme may be formed by removal of NAD^+ from the enzyme-NAD^+ complex, or by the spontaneous deacylation of the acyl enzyme. This latter method was used to prepare the free enzyme. The enzyme was acylated using glyceraldehyde 3-phosphate, when the nucleotide is released presumably as NADH. The acyl enzyme then spontaneously hydrolyzes over a period of several hours. The rate of deacylation appears to be similar to that reported by Bloch *et al.* (28) for the rabbit muscle enzyme.[2]

We have shown in an accompanying paper (26) that glyceraldehyde 3-phosphate dehydrogenase from pea seeds resembles that from other sources on the basis of its molecular weight, subunit structure, amino acid composition, and specific activity. To this must be added its ability to catalyze the arsenolysis of 1,3-diphosphoglyceric acid, the requirement for NAD^+ in both the arsenolysis and reverse reactions, and its ability to form acyl enzyme and enzyme-NAD^+ complexes. The pea seed enzyme also resembles that from other sources in its specificity towards NAD^+ analogs (Table III). We therefore feel justified in extending the mechanism proposed in the preceding paper to the enzyme from other sources. Virtually all of the data on glyceraldehyde 3-phosphate dehydrogenase in the literature is consistent with this mechanism.

TABLE III

Effect of NAD^+ analogs on oxidative phosphorylation of glyceraldehyde 3-phosphate by glyceraldehyde 3-phosphate dehydrogenase

Analog	Relative V_{max}				Relative K_m		
	Muscle[a]	Yeast[b]	Fish[c]	Pea[d]	Muscle	Fish	Pea
NAD^+	100 (100)	100	100	100	1 (1)	1	1
APAD	10 (4.5)	8.7	2.8	5.3	10 (8.8)	8.1	3.4
NHD	80 (—)	8.3		30	46 (—)		24
TNAD	20 (9.3)		7.2	18	6.2 (2.4)	3.1	4.6
APHD	1 (3.2)	1.7	1.3	0	— (79.5)	107	

	K_I		
	Muscle	Yeast	Pea[e]
	mM		
PAAD	0.025	0.013	0.031
ADPR	0.18		0.14

[a] The data are for the rabbit muscle enzyme, and are taken from Eby and Kirtley (42) and from Greene and Feeney (43), the latter being in parentheses.

[b] Taken from Stockell (44).

[c] The data are for *Dissostichus mawsoni*, reported by Greene and Feeney (43).

[d] From Fig. 3*A*.

[e] These are the concentrations required to give 50% inhibition under the conditions employed (Fig. 3*B*). Assuming that the inhibitions are competitive with NAD^+, these values will be approximately equal to the inhibition constants.

APPENDIX

The arsenolysis of 1,3-diphosphoglyceric acid was followed by coupling it to the hydrolysis of ATP. However, there is not a stoichiometric relationship between the amount of ATP hydrolyzed and 1,3-diphosphoglyceric acid arsenolysis, since the accumulation of ADP during the course of the reaction significantly disturbs the 3-phosphoglyceric acid kinase equilibrium. Provided equilibrium conditions are maintained,

$$\frac{[ADP_t][\text{1,3-disphosphoglyceric acid}_t]}{[ATP_t][\text{3-phosphoglyceric acid}_t]} = \frac{[ADP_0][\text{1,3-disphosphoglyceric acid}_0]}{[ATP_0][\text{3-phosphoglyceric acid}_0]} \quad (1)$$

where the subscripts t and 0 refer to the concentrations at time t and zero, respectively. [3-Phosphoglyceric acid$_t$] and [3-phosphoglyceric acid$_0$] will be approximately equal, since the 3-phosphoglyceric acid kinase equilibrium lies heavily in favour of ATP and 3-phosphoglyceric acid (45). Provided the amount of ATP hydrolyzed is small, relative to [ATP_0], this simplifies to:

$$[ADP_t][\text{1,3-diphosphoglyceric acid}_t] = [ADP_0][\text{1,3-diphosphoglyceric acid}_0] \quad (2)$$

If the amount of 1,3-diphosphoglyceric acid hydrolyzed is x,

$$[\text{1,3-diphosphoglyceric acid}_t] = [\text{1,3-diphosphoglyceric acid}_0] + ([ADP_t] - [ADP_0]) - x \quad (3)$$

Substituting Equation 3 into Equation 2 and solving for x, we get:

$$x = \frac{([ADP_t] - [ADP_0])([\text{1,3-disphosphoglyceric acid}_0] + [ADP_t])}{[ADP_t]} \quad (4)$$

[ADP_0] was estimated by extrapolation to zero time (*i.e.* the time of addition of 3-phosphoglyceric acid kinase) of the ADP concentrations of samples taken before the addition of glyceraldehyde 3-phosphate dehydrogenase (or which ever component was used to initiate the arsenolysis reaction). [1,3-Diphosphoglyceric acid$_0$] was estimated from the difference between [ADP_0] and the ADP concentration before the addition of 3-phosphoglyceric acid kinase.[3] With this information, x can be calculated using Equation 4.

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[2] R. G. Duggleby and D. T. Dennis, unpublished results.

[3] Commercial ATP preparations are contaminated with ADP.

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