Interaction of Phosphate Analogues with Glyceraldehyde-3-phosphate Dehydrogenase†

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ABSTRACT: The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase catalyzes the oxidative phosphorylation of D-glyceraldehyde 3-phosphate. A variety of phosphonates have been shown to substitute for phosphate in this reaction [Gardner, J. H., & Byers, L. D., (1977) *J. Biol. Chem. 252*, 5925–5927]. The dependence of the logarithm of the equilibrium constant for the reaction on the pK_{a2} value of the phosphonate is characterized by a Brønsted coefficient, β_{eq}, of ~ 1. This represents the sensitivity of the transfer of the phosphoglyceroyl group between the active-site sulfhydryl residue (in the acyl-enzyme intermediate) and the acyl acceptor on the basicity of the acyl acceptor. Molybdate (MoO_4^{2-}) can also serve as an acyl acceptor in the glyceraldehyde-3-phosphate dehydrogenase catalyzed reaction. The second-order rate constant for the reaction with molybdate is only ~ 12 times lower than the reaction with phosphate even though the pK_{a2} of molybdate is 3.1 units lower than the pK_{a2} of phosphate. The immediate product of the molybdate reaction is the acyl molybdate, 1-molybdo-3-phosphoglycerate. The acyl molybdate, like the acyl arsenate (the immediate product of the reaction when arsenate is the acyl acceptor), is kinetically unstable. At pH 7.3 (25 °C), the half-life for hydrolysis of the acyl molybdate, or the acyl arsenate, is less than 2.5 s. Thus, hydrolysis of 1-molybdo- and 1-arseno-3-phosphoglycerate is at least 2000 times faster than hydrolysis of 1,3-diphosphoglycerate under the same conditions. Glyceraldehyde-3-phosphate dehydrogenase has a fairly broad specificity for acyl acceptors. Most tetrahedral oxy anions tested are substrates for the enzyme (except SO_4^{2-} and SeO_4^{2-}). Tetrahedral monoanions such as ReO_4^- and $GeO(OH)_3^-$ are not substrates but do bind to the enzyme. These results suggest the requirement of at least one anionic site on the acyl acceptor required for binding and another anionic group on the acyl receptor required for nucleophilic attack on the acyl enzyme.

Inorganic phosphate is a substrate for at least 58 different enzymic reactions. The products of these reactions (phosphoric anhydrides and phosphate esters), while thermodynamically labile, are generally kinetically stable under physiological conditions. Since the early investigations of Harden & Young (1906), it has become apparent that arsenate can substitute for inorganic phosphate in many of these reactions. The resulting arsenic analogues of the physiological phosphorus compounds are unstable. This proposal was orignally made by Braunstein (1931) and verified by Needham & Pillai (1937) and by Warburg & Christian (1939) in the reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase. Because of the relative instability of the arsenic anhydrides and esters resulting from substitution of arsenate for phosphate in these enzymic reactions, the net reaction is an arsenate-catalyzed hydrolysis, or "arsenolysis" (Doudoroff et al., 1947). Arsenolysis has been demonstrated for many enzymic reactions in addition to the reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase. These include glycogen phosphorylase (Katz & Hassid, 1951), sucrose phosphorylase (Doudoroff et al., 1947), purine nucleoside phosphorylase (Klein, 1935), and ornithine carbamoyltransferase (Knivett, 1954). Slocum & Varner (1960) have shown, by using ^{18}O-labeling techniques, that these arsenolysis reactions involve a transient acyl arsenate or arsenate ester intermediate. The elegant studies of Long & Ray (1973) have demonstrated that the instability of the arsenic esters is a result of a *kinetic*, rather than a *thermodynamic*, lability. Indeed, Long & Ray (1973) have shown that the equilibrium constant for hydrolysis of glucose 6-arsenate is the same as that of glucose 6-phosphate but the former undergoes hydrolysis $\sim 10^5$ times faster than the latter.

Most of the enzymic reactions which are subject to arsenolysis are specific with respect to the nucleophile. Thus, the thiol ester intermediate in the reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase can readily undergo an arsenate-catalyzed hydrolysis but the rate of reaction of water directly with the acyl-enzyme is over 50 000 times slower than the rate of reaction of the acyl-enzyme with arsenate or phosphate (Byers & Koshland, 1975). Recently, however, it has been demonstrated that phosphonates can readily substitute for phosphate in the reactions catalyzed by glyceraldehyde-3-phosphate dehydrogenase and purine nucleoside phosphorylase (Gardner & Byers, 1977). To gain further insight into the specificity of the enzymes which are subject to arsenolysis as well as the factors responsible for the kinetic lability of the immediate arsenic products, we investigated the effects of a variety of potential analogues of phosphate on the reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase. We also reinvestigated the stability of 1-arseno-3-phosphoglycerate.

Experimental Section

Yeast glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) was prepared as described previously (Byers, 1978). Glyceraldehyde 3-phosphate, phosphoglycerate kinase (yeast), and NAD[1] were obtained from Sigma Chemical Co. Disodium germanate was obtained from K & K Laboratories, Inc., and molybdate and perrhenate (sodium salts) were obtained from Alfa Division of Ventron Corp. Aminomethylphosphonic acid was obtained from Sigma Chemical Co., chloromethylphosphonic acid was obtained from Chemical Procurement Laboratories, Inc., and chloroethylphosphonic

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[1] Abbreviations used: Bicine, *N,N'*-bis(2-hydroxyethyl)glycine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; GPD, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); PGK, phosphoglycerate kinase (EC 2.7.2.3); NAD, β-nicotinamide adenine dinucleotide; 1,3-DPG, 1,3-diphosphoglyceric acid.

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acid was obtained from Polysciences, Inc. Methyl- and ethylphosphonic acids were prepared as described by Crofts & Kosolapoff (1953). Monomethylphosphoric acid was prepared by the general procedure of Khwaja et al. (1970). This phosphate ester, and all the phosphonates, were found to contain less than 0.5% inorganic phosphate contamination by ^{31}P NMR (JEOL FX 60).

Glyceraldehyde-3-phosphate dehydrogenase was assayed by following the appearance of NADH either spectrophotometrically (on a Beckman DU spectrophotometer modified with an Update Model 122 digital display log converter amplifier connected to a Sargent recorder) or fluorometrically (λ_{ex} 340 nm, λ_{em} 450–470 nm, on a Turner Model 430 spectrophotometer). The cuvettes were thermostated at 25 (±0.1) °C. The kinetic parameters for the enzymic reaction under initial velocity conditions were evaluated by the graphical technique of Eisenthal & Cornish-Bowden (1974).

The reaction in the "reverse direction" (i.e., conversion of 1,3-diphophoglycerate + NADH to glyceraldehyde 3-phosphate + NAD) was carried out essentially as described by Kirschner & Voigt (1968). The 1,3-diphosphoglycerate is generated in situ by coupling the dehydrogenase reaction to the phosphoglycerate kinase reaction in the presence of MgATP (1 mM) and D-phosphoglyceric acid (3 mM). The phosphoglycerate kinase (~240 units) was present in large excess over the glyceraldehyde-3-phosphate dehydrogenase (~0.6 unit) to ensure the rate of decrease of NADH is a measure of the dehydrogenase activity (Storer & Cornish-Bowden, 1974). In addition to NADH, NAD (0.1 mM) was also present to facilitate acylation of the enzyme by 1,3-diphosphoglyceric acid (see Trentham, 1971a). The reaction was followed by the decrease in absorbance at 340 nm ([NADH] < 0.2 mM) or at 366 nm ([NADH] < 0.3 mM).

The thermodynamic dissociation constants of the phosphonates were determined by the method of Kresge and Yang (1977), by using a Brinkmann pH meter (Model E512 with a Metrohm AH combination glass electrode). The equilibrium constants for the glyceraldehyde-3-phosphate dehydrogenase reaction with phosphate and phosphonates were determined at 25.0 (± 0.1) °C in buffered solutions (0.05 M buffer, 1 mM EDTA, 0–1.5 M sodium acetate) containing known amounts of NAD (~1 mM), D-glyceraldehyde 3-phosphate (~1 mM), and acyl acceptors (0.03–0.17 M). The enzyme concentration was $\sim 5 \times 10^{-7}$ M. The equilibrium concentration of NADH was determined by extrapolation of the slow linear increase in absorbance (340 nm) to $t = 0$. The absorbances were converted to concentrations by a calibration curve based on a standard NADH solution (prepared from a preweighed sample obtained from Sigma Chemical Co.).

Results

Equilibria Measurements of Acyl Transfer Reactions. Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) catalyzes the oxidative phosphorylation of D-glyceraldehyde 3-phosphate (RCHO):

$$\mathrm{RCHO + NAD^+ + HPO_4^{2-} \rightleftharpoons R\overset{\overset{\mathrm{O}}{\|}}{\mathrm{C}}OPO_3^{2-} + NADH + H^+} \quad (1)$$

The reaction catalyzed by the yeast enzyme (or by the enzyme from other sources) is known to involve formation of an acyl-enzyme (thiolester) intermediate (Segal & Boyer, 1953; Velick & Hayes, 1953; Byers & Koshland, 1975). Phosphonates (XPO_3^{2-}) can be substituted for phosphate in this reaction (Gardner & Byers, 1977). The use of phosphonates provides a convenient means of determining the sensitivity of the acyl transfer equilibria (reaction 2b) to the pK_a of the acyl acceptor:

$$\mathrm{RCHO + NAD^+ + E^{SH} \overset{K_1}{\rightleftharpoons} E^{S\overset{\overset{\mathrm{O}}{\|}}{\mathrm{C}}R} + NADH + H^+} \quad (2a)$$

$$\mathrm{E^{S\overset{\overset{\mathrm{O}}{\|}}{\mathrm{C}}R} + XPO_3^{2-} + H^+ \overset{K_2}{\rightleftharpoons} E^{SH} + RC(O)OP(O)(O^-)X} \quad (2b)$$

$$\mathrm{RCHO + NAD^+ + XPO_3^{2-} \overset{K_{eq}}{\rightleftharpoons} RC(O)OP(O)(O^-)X + NADH} \quad (2c)$$

Preliminary studies indicate that reaction 2b is kinetically competent. Thus, the isolated acyl-enzyme rapidly reacts with chloromethyl- and methylphosphonate. Measurement of the equilibrium constant, K_{eq}, for the overall reaction

$$K_{eq} = \frac{(RCO_2PO_2X^-)(NADH)}{(RCHO)(NAD^+)(XPO_3^{2-})} \quad (3)$$

with a variety of phosphonates provides a direct measure of the sensitivity of K_2 to the basicity of the acyl acceptor. $K_{eq} = K_1K_2$ and K_1 is independent of the nature of the acyl acceptor. Thus, the slope of a plot of log K_{eq} vs. pK_{a2} (i.e., β_{eq}) is the Brønsted coefficient for reaction 2b.

The equilibrium constants for the overall reaction (eq 2c) were evaluated by measuring the production of NADH (which is stoichiometric with the production of the phosphonic anhydride) spectrophotometrically.

Upon addition of the phosphonate to a solution containing glyceraldehyde 3-phosphate, NAD, and enzyme, there is a rapid "burst" of NADH production. The amplitude of this burst is independent of the enzyme concentration (0.1–1 μM). This burst phase is followed by a slow, enzyme-independent, linear production of NADH. This linear phase represents the spontaneous hydrolysis of the acylphosphonate product. The phosphonic anhydride products are kinetically stable with respect to hydrolysis ($t_{1/2} \approx 10$–100 min). Thus, extrapolation of the linear phase of NADH production to zero time provides a reliable means of evaluating the equilibrium concentrations of NADH and of the phosphonic anhydride product.

The equilibrium constants were found to have a small dependence on the ionic strength of the medium. For the reactions with most of the phosphonates, the equilibrium constants were found to decrease by ~20% as the ionic strength (maintained with sodium acetate) was reduced from 1.5 to 0.1 M at pH 8.2–8.6. The equilibrium constants were evaluated at 4–5 ionic strengths (0.1 M ≤ μ ≤ 1.5 M) and extrapolated to $\mu = 0$ by the linear technique described by Kresge & Yang (1977). The results are summarized in Figure 1. The β_{eq} value is 0.94. This represents the sensitivity of the equilibrium constant for transfer of the phosphoglyceroyl group (from the active-site sulfhydryl group to the acyl acceptor) to the pK_a of the acyl acceptor.

Formation and Stability of 1-Molybdo-3-phosphoglycerate. To further explore the acyl acceptor specificity of the enzyme as well as to examine the properties of the organometallic anhydride products, a variety of metal oxy anions were tested as potential substrates for glyceraldehyde-3-phosphate dehydrogenase.

Molybdate is structurally similar to phosphate, being tetrahedral with an average Mo–O bond distance (1.83 Å) about 19% longer than the P–O bond distance (1.54 Å) in phosphate (Sutton, 1958). Molybdate is known to undergo polymeri-

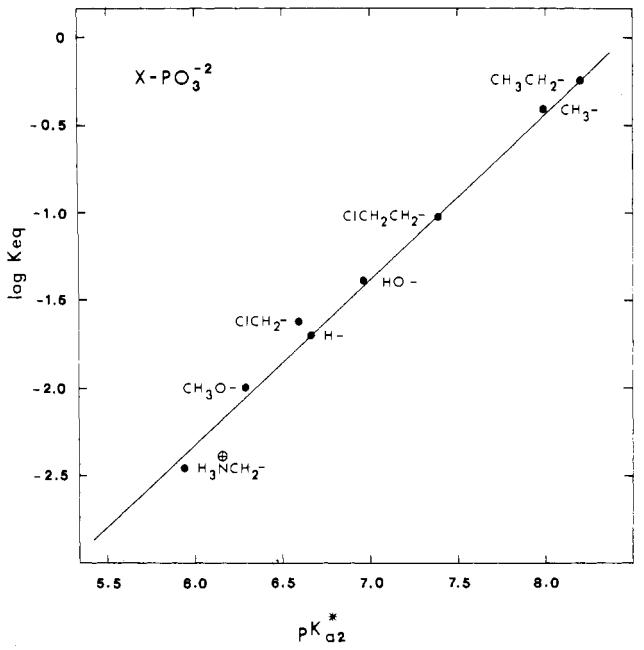


FIGURE 1: Brønsted plot for the formation of D-3-phosphoglyceroylphosphoric anhydrides. The K_{eq} values are the equilibrium constants for the reaction $NAD^+ + RCHO + XPO_3^{2-} \rightleftharpoons NADH + RC(=O)OP(=O)(O^-)X$ and were determined as described in the text. These values were determined at several ionic strengths (maintained with sodium acetate) and extrapolated to $\mu = 0$. The abscissa is the thermodynamic pK_{a2} value and, in the case of phosphate, is statistically corrected. The reactions were carried out at pH 8.2–8.6 and 25 °C. The K_{eq} for the reaction with phosphate was corrected for the ionization constant for this acyl phosphate which was found to be $\sim 2 \times 10^{-6}$ M from the pH dependence of K_{eq} ($5.8 \leq pH \leq 8.5$). This corresponds to a pK_a of ~ 5.7 which can be compared with the corresponding pK_as of acetyl phosphate (4.95; Lipmann & Tuttle, 1947; Koshland, 1952) and butyryl phosphate (5.1; Lipmann & Tuttle, 1947) determined at 37 °C. The concentration of the dianionic species of the phosphonate, XPO_3^{2-}, was determined from the pK_{a2} of the phosphonate at the ionic strength of the experiment. The solid line is the best least-squares fit of the data: $\log K_{eq} = 0.94\ pK_{a2}^* - 7.97$. The interval estimator of the slope ($= \beta_{eq}$) at 90% confidence is 0.06.

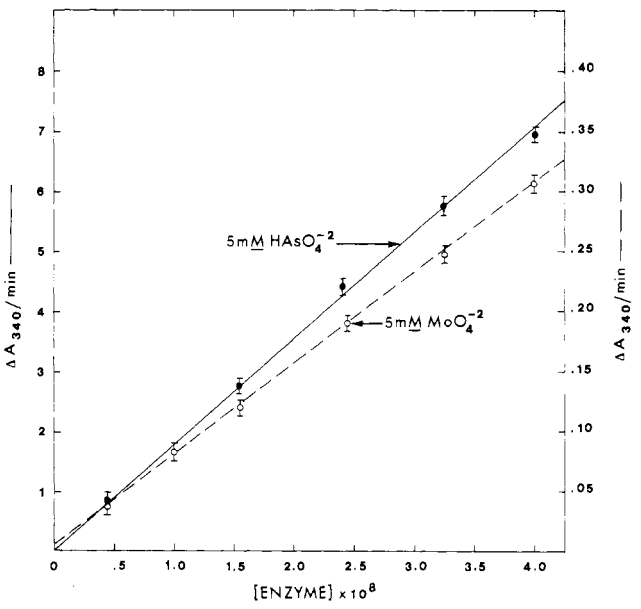


FIGURE 2: Dependence of the arsenolysis and molybdenolysis reaction on enzyme concentration. Reaction conditions are: 1 mM D-glyceraldehyde 3-phosphate, 0.05 M Hepes, 1 mM EDTA, 0.5 M NaCl, pH 7.3, 25 °C. After the reaction with the contaminating inorganic phosphate (~0.2 mM) was allowed to reach equilibrium, the reaction was initiated by the addition (to a final concentration of 5 mM) of either sodium arsenate (solid line) or sodium molybdate (broken line). The lines are based on a linear least-squares fit of the data. The relative slope of the arsenolysis and molybdenolysis lines is 22.5.

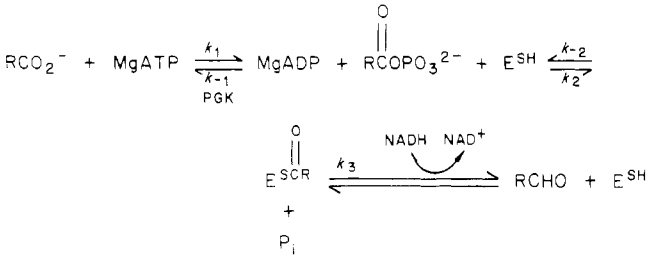
zation (Travers & Malaprade, 1926), and this makes the measurement of the pK_a values particularly difficult (Travers & Malaprade, 1926; Schwarzenback, 1958; Aveston et al., 1964). However, from the data of Sasaki et al. (1959), a pK_{a2} value (for $HMoO_4^- \rightleftharpoons H^+ + MoO_4^{2-}$) of 4.08 (±0.15) has been estimated and at pH >6.5 it is clear that the predominate species is MoO_4^{2-} (i.e., there is negligible paramolybdate, $H_nMo_7O_{24}^{n-6}$, present in neutral aqueous solutions). Thus, the basicity of molybdate dianion is greater than that of sulfate but comparable to the basicity of trichloromethylphosphonate ($pK_{a2} = 4.93$) which is a substrate for glyceraldehyde-3-phosphate dehydrogenase (Gardner & Byers, 1977). Therefore, molybdate was tested as a potential substrate for the enzyme.

Molybdate was found to indeed be a substrate for glyceraldehyde-3-phosphate dehydrogenase at pH 7.3 and 8.5. While not as good a substrate as phosphate or arsenate, the rate of NADH production in the presence of MoO_4^{2-} is significant. Furthermore, molybdate behaves as arsenate (vide infra) in that (1) the production of NADH is linear in time indicating either a very large equilibrium constant for the reaction or a kinetic instability of the acyl molybdate product and (2) the coupling system (phosphoglycerate kinase + MgADP) has no effect on the rate of NADH production.

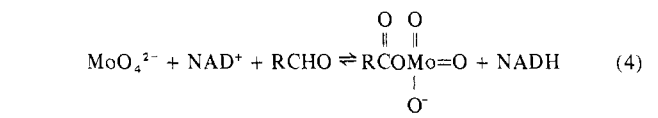
No change in absorbance at 340 nm was observed when molybdate (0.1 M) was incubated with the substrates (glyceraldehyde 3-phosphate and NAD) either separately or in combination. Also, molybdate plus enzyme in the presence of NAD or NADH had no effect on the absorbance at 340 nm during the time course of the usual kinetic runs (< 10 min). The rate of production of NADH was linearly dependent on the enzyme concentration at all concentrations tested (up to 8×10^{-8} M enzyme). The dependence of the molybdate reaction on enzyme concentration is compared with that of the arsenate reaction in Figure 2.

The observation that production of NADH in the presence of molybdate is a linear function of time (i.e., the progress curve does not level off to a equilibrium value as in the case of phosphate or the phosphonates) suggests that, if an acyl molybdate is indeed formed, it is kinetically labile. By analogy with the physiological reaction (with phosphate), or with the arsenolysis reaction which is known to involve the transient presence of an acyl arsenate (Slocum & Varner, 1960), it is likely that the immediate product of the molybdate reaction is the unstable acyl molybdate. It is possible, however, that molybdate may catalyze production of 3-phosphoglyceric acid by an indirect mechanism (e.g., general base catalysis or induction of a conformation change in the protein upon binding to the acyl-enzyme facilitating hydrolysis of the acyl-enzyme) rather than by a direct nucleophilic attack on the acyl-enzyme. ^{18}O-Labeling studies would be useful in distinguishing between these possibilities. However, these experiments are technically difficult because of the short half-time for nonenzymic exchange of molybdate oxygen with water (~2 s at neutral pH; Felten et al., 1978). This exchange process is about 100 times slower with arsenate (Slocum & Varner, 1960). Nevertheless, it is likely that an acyl molybdate is indeed produced for the following reasons. (1) Oxydianions such as sulfate and selenate can bind to the enzyme at the acyl-acceptor site but do not serve as substrates (vide infra). (2) As in the case of the reactions with phosphate and arsenate (Byers & Koshland, 1975), deacylation of the isolated acyl-enzyme with molybdate (or with the phosphonates) requires the presence of NAD (H.

Scheme I



S. She and L. D. Byers, unpublished observations). (3) The V_{max} for the reaction with molybdate is independent of pH ($6.5 \leq pH \leq 9.0$) (A. Alayoff and L. D. Byers, manuscript in preparation), indicating no significant direct attack by hydroxide ion on the acyl-enzyme in the presence of molybdate. Furthermore, molybdate is isosteric and isoelectronic with the phosphonates. Indeed, by analogy with the reaction with phosphonates (reaction 2), the equilibrium constant for formation of the acyl molybdate (reaction 4) can be estimated:



(4)

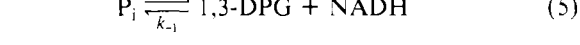
From the β_{eq} value for the glyceraldehyde-3-phosphate dehydrogenase reaction (Figure 1) and the pK_{a2} value of molybdate (4.1), the K_{eq} value for the reaction 4 can be estimated (see Discussion). The equilibrium constant for production of 1-molybdo-3-phosphoglycerate is, thus, expected to be $\sim 7 \times 10^{-5}$ M^{-1}. Therefore, the equilibrium concentration of the acyl molybdate is expected to be $\sim 6 \times 10^{-7}$ when 1 mM NAD, 1 mM D-glyceraldehyde 3-phosphate and 5 mM Na_2MoO_4 are incubated at pH 7.3 in the presence of glyceraldehyde-3-phosphate dehydrogenase. This corresponds to an absorbance change of $\sim 4 \times 10^{-3}$ OD unit due to the equilibrium concentration of NADH produced. However, when this experiment is carried out (i.e., the phosphate contamination was allowed to reach equilibrium and the system was then made 5 mM in MoO_4^{2-}), the production of NADH does not level off but rather increases linearly with time. The linear production of NADH is not due to a rate-limiting spontaneous hydrolysis of the acyl molybdate since the rate of NADH production is directly proportional to the enzyme concentration (Figure 2). Thus, the hydrolysis of the acyl molybdate must be more rapid than the enzyme-catalyzed production of the acyl molybdate.

To further test the kinetic stability of the 1-molybdo-3-phosphoglycerate, molybdate was tested as an inhibitor of the "reverse" reaction (see Experimental Section). This reaction, illustrated in Scheme I, utilizes phosphoglycerate kinase (PGK), D-3-phosphoglyceric acid (RCO_2^-), and ATP as a system to generate the substrate 1,3-diphosphoglycerate ($RC(=O)OPO_3^{2-}$) for the glyceraldehyde-3-phosphate dehydrogenase reaction.

Here E^{SH} represents glyceraldehyde-3-phosphate dehydrogenase and the reaction is monitored by the decrease in absorbance due to NADH. The rate of decrease of NADH will depend on the partitioning of the acyl-enzyme between the reduction step and the acyl transfer step. Thus, if molybdate reacts with the acyl-enzyme to produce an acyl-molybdate which hydrolyzes faster than reacylation of the enzyme, molybdate should inhibit the "reverse" reaction (see Discussion). Molybdate is an effective inhibitor of this reaction. At pH 8.0, 50% inhibition is observed at a MoO_4^{2-} concentration of 15 mM. The K_m for MoO_4^{2-} in the forward direction is 6 mM. Phosphate, unlike molybdate, is not as effective an inhibitor of the "reverse" reaction even though phosphate is a better substrate for the dehydrogenase (in the direction of aldehyde oxidation) than is molybdate (k_{cat}/K_m is about 12 times larger for phosphate than for molybdate). No inhibition of the "reverse" reaction can be detected at low phosphate concentrations and 50% inhibition is observed only when phosphate is present at 15 mM (which is larger than its K_m value under these conditions by a factor of 10). This is expected since the phosphoglycerate kinase maintains a constant (equilibrium) concentration of 1,3-diphosphoglycerate.

Stability of 1-Arseno-3-phosphoglycerate. There have been reports that the anhydride, 1-arseno-3-phosphoglycerate, may have a finite stability with a half-life for spontaneous hydrolysis on the order of 1 min at pH 7 (Teipel & Koshland, 1970; Trentham, 1971b; Duggleby & Dennis, 1974). This conclusion, originally made by Teipel & Koshland (1970), was based on the nonlinearity of the initial production of NADH in the glyceraldehyde-3-phosphate dehydrogenase catalyzed oxidative arsenolysis of D-glyceraldehyde 3-phosphate. However, it is known that glyceraldehyde 3-phosphate contains varying amounts of inorganic phosphate contamination (see, e.g., Gardner & Byers, 1977; Seydoux et al., 1976; Armstrong & Trentham, 1976). Because of the different reactivities of arsenate and phosphate with the enzyme (see, e.g., Byers & Gardner, 1977; Furfine & Velick, 1965; Orsi & Cleland, 1972; Meunier & Dalziel, 1978), it is possible that contaminating phosphate may be responsible for this nonlinearity. It is noteworthy that Teipel & Koshland (1970) did not observe this nonlinearity when nonphosphorylated aldehydes (glyceraldehyde, propionaldehyde, or acetaldehyde) were substituted for glyceraldehyde 3-phosphate. Because arsenate esters are known to be kinetically labile, we reexamined the stability of the acyl arsenate.

In the absence of any additional acyl acceptor, other than the small contamination of inorganic phosphate in the glyceraldehyde 3-phosphate, the production of NADH can be represented by the expression



(5)

where 1,3-DPG is the phosphoric anhydride, 1,3-diphosphoglyceric acid. k_1 is the "rate constant" for the forward reaction and is a function of the enzyme concentration and the concentrations of NAD^+ and glyceraldehyde 3-phosphate.[2] Since these concentrations are greater than the concentration of the contaminating phosphate, $[P_i]$, the dependence of the NADH concentration on time (see Appendix) is given by

$$[NADH] = [NADH]_{eq'} \tanh\left(\frac{[P_i]_0 k_1 t}{[NADH]_{eq'}}\right) + k_h[NADH]_{eq'}t \quad (6)$$

where $NADH_{eq'}$ is the concentration of NADH when equilibrium is initially established (i.e., $[NADH]_{eq'} = [1,3\text{-DPG}]_{eq}$) and k_h is the rate constant for spontaneous hydrolysis of

[2] For example, at fixed concentrations of NAD and glyceraldehyde 3-phosphate, the initial rate of disappearance of inorganic phosphate, P_i, is given by: $-d[P_i]/dt = k_{cat}^{app}[E_T][P_i]/(K_m^{app} + [P_i])$, where $[E_T]$ is the total enzyme concentration. The apparent kinetic parameters, k_{cat}^{app} and K_m^{app}, are dependent on the kinetic mechanism and the concentrations of NAD and the aldehyde. If $[P_i] \ll K_m^{app}$, then this expression reduces to the pseudo-first-order expression: $-d[P_i]/dt = k_1[P_i]$, where $k_1 = k_{cat}^{app}[E_T]/K_m^{app}$.

1,3-DPG. k_h, unlike k_1, is enzyme independent and thus the two terms in eq 6 can be readily distinguished. The hydrolysis of 1,3-DPG is a slow process. The k_h value was found to be $\sim 10^{-2}$ min^{-1} between pH 5.5 and 8.4 at 25 °C.

In the presence of arsenate, the immediate product of the reaction is the acyl arsenate. If the acyl arsenate is kinetically labile in the time course of the assay ($t_{1/2} < 0.2$ min), the progress curve for NADH production is given by

$$[\text{NADH}] = [\text{NADH}]_{eq'} \tanh\left(\frac{[P_i]_0 k_1' t}{[\text{NADH}]_{eq'}}\right) + fV_m t \quad (7)$$

where $[As_i]$, the inorganic arsenate concentration, is constant (because of the rapid hydrolysis of the low steady-state level of the acyl arsenate), NAD and glyceraldehyde 3-phosphate concentrations are constant (initial velocity conditions), f is the fractional degree of saturation by the arsenate ($f = [As_i]/(K_m' + [As_i])$, K_m' is the apparent K_m of arsenate), and V_m is the maximal velocity obtained with saturating arsenate concentrations. V_m is directly proportional to the enzyme concentration. k_1' may be different from k_1 in eq 6 due to inhibition of the phosphorolysis reaction by arsenate (see, e.g., Segel, 1975, pp 59–64).

Thus, in the presence of arsenate the progress curve for NADH production should be biphasic if there is any phosphate contamination present (eq 7). In the absence of arsenate the progress curve (at sufficiently high enzyme concentrations) will consist of a "burst" of NADH, corresponding to production of an equilibrium concentration of products, followed by a linear, enzyme-independent, production of [NADH]. This linear production of NADH is a result of the hydrolysis of 1,3-diphosphoglycerate and will be zero-order process since the phosphoric anhydride concentration will remain at its equilibrium concentration due to the presence of the enzyme and excess concentration of NAD and glyceraldehyde 3-phosphate. Thus, the equilibrium constant for the dehydrogenase reaction as well as the rate constant for hydrolysis of 1,3-diphosphoglyceric acid can be readily determined.

The phosphate contamination in glyceraldehyde 3-phosphate can be readily detected by incubation of the aldehyde with NAD in the absence of alternate acyl acceptors (e.g., arsenate). In a typical run, 1 mM D-glyceraldehyde 3-phosphate (2 mM racemate) and 1 mM NAD were incubated in buffer (0.1 M Hepes, 1 mM EDTA, 0.5 M NaCl) at pH 7.3, 25 °C. At time = 0 a sample of glyceraldehyde-3-phosphate dehydrogenase ($\sim 2 \times 10^{-8}$ M final concentration) was added and the NADH produced was monitored by following the increase in absorbance at 340 nm. A rapid burst ($\Delta A_{340} = 0.12$ OD unit) was observed followed by a slow linear increase in absorbance. This linear increase in absorbance is independent of enzyme concentration (2–20 $\times 10^{-8}$ M) consistent with this phase being due to spontaneous hydrolysis of 1,3-diphosphoglycerate. Since the linear phase is independent of enzyme concentration, it does not represent hydrolysis of the thiol ester (acyl-enzyme) intermediate. The amplitude (but not the rate) of the burst phase is also independent of the enzyme concentration. This burst corresponds to an equilibrium concentration of NADH = 19.3 μM. The equilibrium constant for formation of the phosphoric anhydride was measured independently under these reaction conditions (pH 7.3, $\mu = 0.6$ M, 25 °C) and found to be 1.11 M^{-1}. From the equilibrium constant at pH 7.3, an original inorganic phosphate concentration of ~ 0.40 mM is estimated which represents a 20% contamination of the total DL-glyceraldehyde 3-phosphate initially present.

The arsenolysis reaction was followed by adding enzyme to an assay mixture containing the substrates NAD, glyceraldehyde 3-phosphate, and arsenate. The progress curves appear linear and from this data the "burst" cannot be easily resolved from the rapid linear phase. (Similar progress curves are obtained in the molybdenolysis reaction, but because of the lower reactivity of MoO_4^{2-} relative to that of arsenate, the initial nonlinear phase is well resolved from the linear phase.) The nonlinearity in the arsenolysis reaction becomes more apparent at higher phosphate contamination or lower arsenate concentrations. The phosphate contamination in glyceraldehyde 3-phosphate can be removed either by treatment with purine nucleotide phosphorylase and inosine (Byers & Gardner, 1977) or by ion-exchange chromatography (Belasco et al., 1978). When the experiment is repeated with the purified aldehyde, the arsenolysis progress curve is linear. However, the arsenolysis reaction can be monitored directly, even in the presence of contaminating inorganic phosphate, by initiating the reaction by the addition of arsenate after the phosphate reaction has reached equilibrium. This is illustrated in Figure 3.

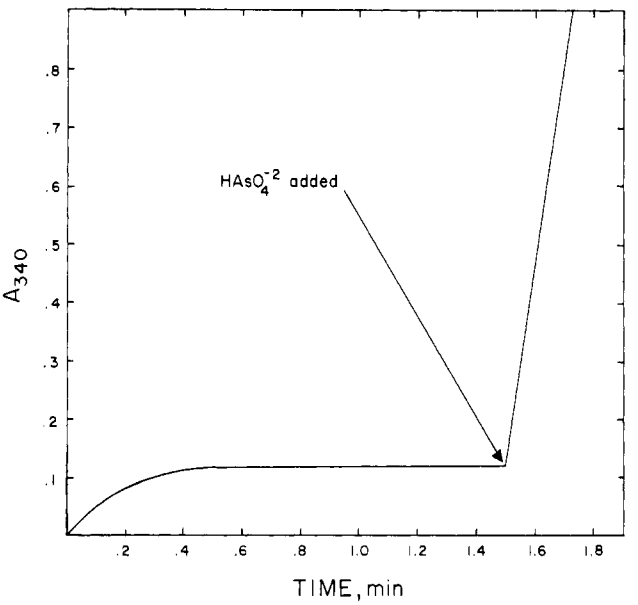


FIGURE 3: Arsenolysis in the presence of contaminating phosphate. The reaction is initiated by the addition of glyceraldehyde-3-phosphate dehydrogenase (final concentration = 2×10^{-8} M) to a mixture of NAD (1 mM) and D-glyceraldehyde 3-phosphate (1 mM). The initial "burst", due to phosphate contamination, has a hyperbolic tangent dependence on time consistent with eq 6. A plot of $\ln\{([\text{NADH}]_{eq} + [\text{NADH}])/([\text{NADH}]_{eq} - [\text{NADH}]\}$ vs. time is linear ($0 < t < 1.5$ min), consistent with eq 8 (Appendix). After 1.5 min, arsenate was added (to a final concentration of 5 mM), and the production of NADH is a linear function of time (correlation coefficient > 0.999).

If the rate constant for hydrolysis of the acyl-arsenate, k_h', is less than fV_m (eq 7), then the progress curve for NADH production will be similar to the progress curve for the phosphorolysis reaction in the absence of arsenate (i.e., eq 6). The increase in [NADH] will be a nonlinear function of time which is dependent on the enzyme concentration (see Segal, 1975). This, however, is not observed. When 5 mM arsenate is added to a solution containing NAD (1 mM), D-glyceraldehyde 3-phosphate (1 mM) and enzyme ($0.2–4.0 \times 10^{-8}$ M in 0.1 M Hepes, 1 mM EDTA, 0.5 M NaCl, pH 7.3, 25 °C) after the phosphate contamination was allowed to reach equilibrium, a linear production of NADH (from $A_{340} = 0.12$ to 0.80) was observed (Figure 3). Since the pK_{a2} of arsenate (6.98) is similar to that of phosphate (7.21), the equilibrium constant for formation of the acyl arsenate is expected to be similar to the equilibrium constant for formation of the acyl phosphate (i.e., reaction 1). The expected equilibrium con-

centration of the arsenic anhydride is ~68 μM (corresponding to a change in A_{340} of 0.43 OD unit). Since the NADH production is a linear function of time, this indicates that $k_h' > fV_m$. Furthermore, the rate of NADH production is linearly dependent on the enzyme concentration which is also consistent with $k_h' > fV_m$. Arsenate also inhibits the "reverse" reaction (Scheme I). At pH 8.0, 50% inhibition is observed in the presence of 6 mM arsenate.

Phosphate Analogue Inhibitors. Deacylation of the phosphoglyceroylated glyceraldehyde-3-phosphate dehydrogenase (i.e., the thiolester intermediate) is highly specific in that phosphorolysis is at least 5×10^4 times faster than hydrolysis (Byers & Koshland, 1975). However, tetrahedral oxy anions such as phosphonates, arsenate, and molybdate are all good acyl acceptors in this reaction. To further examine the specificity in the deacylation reaction, we examined the effect of several inorganic analogues of phosphate on the reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase.

The nitrate anion was the first competitive inhibitor (with respect to the acyl acceptor) reported for glyceraldehyde-3-phosphate dehydrogenase. Orsi & Cleland (1972) found that nitrate is a linear competitive inhibitor (with respect to arsenate) of the rabbit muscle enzyme (K_i = 8.1 mM). We have obtained similar results with the yeast enzyme. It is interesting that nitrate is not a substrate for the enzyme but does bind to the "acyl acceptor binding site" as evidenced by the competitive component of inhibition with respect to the acyl acceptor (phosphate or arsenate). This lack of reactivity of nitrate may be due to several factors: (1) its poor basicity (pK_a = −1.3); (2) its planar structure; and/or (3) its monoanionic character. While it is not clear whether it is the trianionic form of phosphate or arsenate which is the reactive species or the dianionic form which exclusively reacts, it is clear that tetrahedral dianions are effective acyl acceptors (Gardner & Byers, 1977). This is not unique to the yeast enzyme since we find similar results with the rabbit muscle and lobster muscle enzymes. The pH dependence of the reaction of phosphate, carried out by Keleti & Batke (1966) with pig muscle glyceraldehyde-3-phosphate dehydrogenase, indicates that the dianionic form of phosphate may be the most reactive species. However, it is not clear whether the monoanionic species of phosphate or phosphate analogues can serve as substrates for the enzyme. To further investigate this possibility, we examined the effects of germanate and perrhenate on the enzyme.

In dilute (<0.1 M) aqueous solutions, near neutrality "germanate" exists as a monomer (Gayer & Zajicek, 1969; Glockling, 1969), the most prevalent species being the tetrahedral hydrate (Ingri & Lundgren, 1963), $Ge(OH)_4$ and its monoanion, $GeO(OH)_3^-$ (Gayer & Zajicek, 1969; Ingri, 1963). The pK_a (at 20 °C) which characterizes the interconversion of these two species has been estimated by several authors to be 8.8 (±0.2), although Gayer & Zajicek (1969) report a pK_{a1} of 11.6. Pugh (1929) determined the pK_{a1} of germanate to be 8.5 and the second dissociation constant, pK_{a2}, to be 12.7. Thus, under the reaction conditions employed in these studies (~0.08 M, pH 7–8.5), germanate exists predominately as the tetrahedral species, $Ge(OH)_4$, with varying amounts of the monoanion, $GeO(OH)_3^-$, depending on the pH and pK_{a1} value.

Perrhenate is a tetrahedral monoanion in aqueous solution (pK_a = −1.25). It is a reasonably good oxidizing agent (Cotton & Wilkinson, 1972). Perrhenate, however, was not found to oxidize the active-site sulfhydryl group of glyceraldehyde-3-phosphate dehydrogenase at an appreciable rate. Thus, when

Scheme II

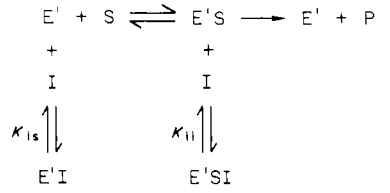


Table I: Effects of Inorganic Analogues of Phosphate on the Reaction Catalyzed by Glyceraldehyde-3-phosphate Dehydrogenase[a]

A. Substrates

ion	pK_a[b]	vol, rel[c]	K_m[d] (mM)	k_{cat}/K_m (M^{-1} s^{-1})
HPO_4^{2-}	7.21[e]	1.00	1.5 (± 0.1)	6.7 (± 0.4) × 10^5
$HAsO_4^{2-}$	6.98[e]	1.48	*f*	1.0 (± 0.2) × 10^5
MoO_4^{2-}	4.1[g]	1.68	6.0 (± 0.5)	5.7 (± 0.3) × 10^4

B. Inhibitors

ion	pK_a[b]	vol, rel[c]	K_{is}[d,h] (mM)	K_{ii}[d,h] (mM)	varied substrate
$Ge(OH)_4$	*i*	1.44	4.44 (± 0.4)	5.6 (± 0.9)	HPO_4^{2-}
			1.9 (± 0.6)	8 (± 2)	MoO_4^{2-}
ReO_4^-	−1.25[e]	1.60	3.6 (± 0.8)	7 (± 3)	MoO_4^{2-}
NO_3^-	−1.30[e]	0.51	84 (± 12)	146 (± 10)	MoO_4^{2-}
			200 (± 22)	103 (± 13)	HPO_4^{2-}
SO_4^{2-}	1.99[e]	0.85	>100	>100	MoO_4^{2-}
SeO_4^{2-}	2.00[e]	1.14	~60	>100	MoO_4^{2-}
$HP_2O_7^{3-}$	6.60[e]	3.96	~500	>500	MoO_4^{2-}

[a] Reactions were carried out at 25 °C at pH 8.5 (0.05 M Bicine buffer, 1 mM EDTA, and 0.5 M sodium acetate with saturating amounts of NAD (1.5 mM) and D-glyceraldehyde 3-phosphate (1.5 mM). [b] pK_a value for production of the indicated ionic species. [c] Ionic volume relative to that of phosphate. Volumes are based on the central atom–oxygen bond distance (obtained from Sutton, 1958). [d] Values obtained from the direct linear plot of Eisenthal & Cornish-Bowden (1974). The 90% confidence region of these values (indicated in parentheses) were evaluated from the direct linear plot as described by Porter & Trager (1977). [e] Values from Jencks & Regenstein (1968). [f] Saturation kinetics not observed with arsenate (Gardner & Byers, 1977). [g] Sasaki et al. (1959). [h] Dissociation constants defined in text (Scheme II). [i] See text.

$NaReO_4$ (45 mM) is incubated with the enzyme (pH 8.5, 25 °C, 10 min) no loss of enzymic activity could be detected. Neither perrhenate (45 mM) nor hydroxygermanate[3] (50 mM) has any effect on the stability of NAD (1 mM), NADH (1 mM), or glyceraldehyde 3-phosphate (1 mM), at pH 7 or 8.5, within a period of 25 min. Furthermore, neither perrhenate nor hydroxygermanate, at concentrations up to 50 mM, was found to be a substrate for glyceraldehyde-3-phosphate dehydrogenase. Both of these compounds, however, were found to be reversible inhibitors of the enzymic reaction with phosphate, arsenate, or molybdate as acyl acceptors.

The effect of hydroxygermanate on the initial velocity of the enzymic reaction with molybdate is illustrated in Figure 4. This noncompetitive inhibition pattern, which is also observed with perrhenate, is consistent with that shown in Scheme II. Here E′ represents the form of the enzyme which binds and reacts with the acyl acceptor, S. The form of this complex depends on the kinetic mechanism but most likely is the holoacylenzyme[4] (e.g., see Byers & Koshland, 1975;

[3] The term "hydroxygermanate" is used here to refer to the hydrated species of germanate which is present in aqueous solutions. This hydroxygermanate is predominately $Ge(OH)_4$ with varying amounts of $GeO(OH)_3^-$ depending on pH.

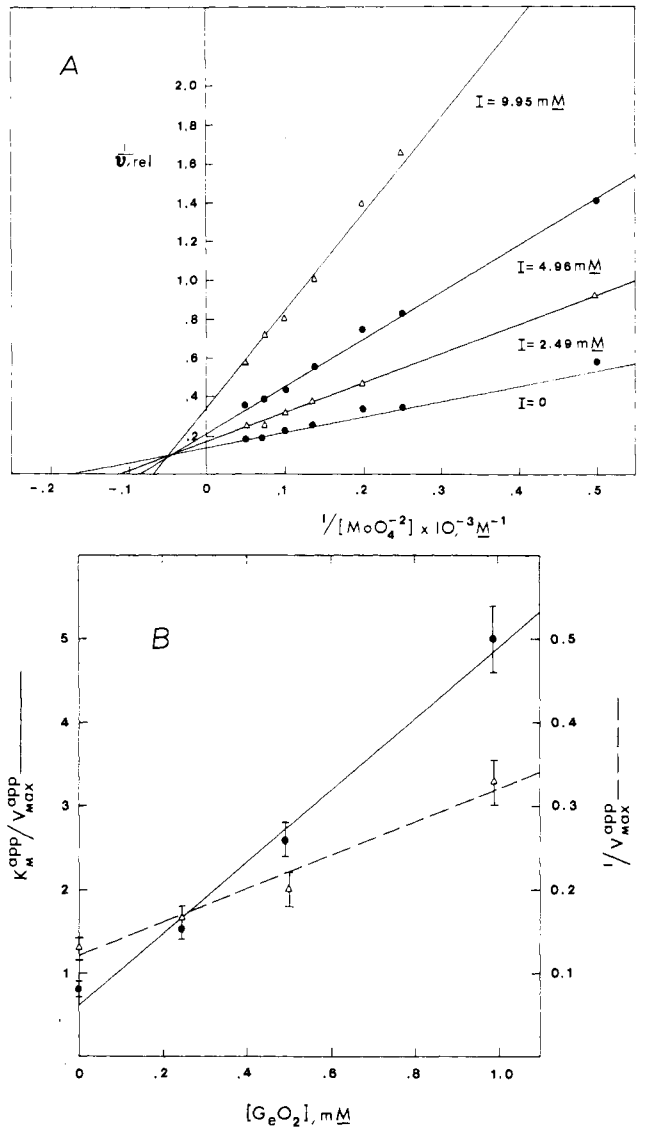


FIGURE 4: Inhibition of glyceraldehyde-3-phosphate dehydrogenase by germanate. The reaction was carried out at pH 8.5 (0.05 M Bicine buffer, 1 mM EDTA, 0.5 M sodium acetate, 25 °C) in the presence of saturating concentrations of NAD (1.5 mM) and D-glyceraldehyde 3-phosphate (1.5 mM). The enzyme concentration was $\sim 10^{-9}$ M and the appearance of NADH was monitored fluorometrically. The variable substrate is sodium molybdate. (A) Double-reciprocal representation of the experimental data. The lines are based on $1/V_{max}^{app}$ and $1/K_m^{app}$ values obtained from the direct linear plot of Eisenthal & Cornish-Bowden (1974). The velocities were determined as a function of $[MoO_4^{2-}]$ in the presence of various hydroxygermanate (I) concentrations. (B) Replot illustrating the dependence of the kinetic parameters on the inhibitor concentration. The K_m^{app} and V_{max}^{app} were obtained from Eisenthal–Cornish-Bowden plots and the error bars indicate the 90% confidence region obtained by the method of Porter & Trager (1977). The lines for the dependence of K_m^{app}/V_{max}^{app} (O) on the germanate concentration are based on a least-squares fit.

Kirschner & Voight, 1968). Under the experimental conditions, the acyl acceptor is the varied substrate and NAD and glyceraldehyde 3-phosphate are present at saturating concentrations ($>10K_M^{app}$).

Results similar to those represented in Figure 4 are obtained when phosphate is substituted for molybdate. The K_{is} and K_{ii} values for hydroxygermanate and perrhenate, as well as the inhibition constants for some other inhibitors, are summarized in Table I.

Sulfate and selenate are not substrates for the yeast, rabbit muscle, or lobster muscle enzymes. Both of these dianions were found to be very poor inhibitors of the yeast enzyme (Table I).

Discussion

The β_{eq} value for phosphoglyceroyl transfer between the active site sulfhydryl group (Cys-149) of glyceraldehyde-3-phosphate dehydrogenase and various phosphate analogues (Figure 1) is 0.94 (± 0.06). This β_{eq} value is lower than the Brønsted coefficient for the acetyl transfer equilibria between thiols and oxygen or nitrogen acceptors. Thus, Hupe & Jencks (1977) report a β_{eq} value of 1.6 (± 0.1) for acetyl transfer between *p*-nitrothiophenol and a series of alcohol monoanions. The reason for the smaller sensitivity of the transfer of the phosphoglyceroyl group (from the enzyme to phosphonates) than for the acetyl group (from *p*-nitrothiophenol to monooxy anions) is not yet clear. This may be a result of the different acyl groups involved in these two reactions or a result of the nature of the acyl acceptors (phosphonate dianions vs. alkoxides and phenoxides). In this respect, it is interesting to note that Hupe & Jencks (1977) find a lower β_{eq} value (=1.38) for acetyl transfer (from thiol esters) to thiol anions than to oxy anions or nitrogen.

Molybdate can substitute for phosphate in the reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase. Unlike the reactions where phosphate or phosphonates serve as acyl acceptors, the immediate product of the reaction (1-molybdo-3-phosphoglycerate) is kinetically unstable. In this respect, molybdate behaves like arsenate. The conclusion of Teipel & Koshland (1970) that 1-arseno-3-phosphoglycerate is kinetically stable ($t_{1/2} \sim 1$ min) was based on the nonlinear increase of NADH with time. However, this nonlinearity can be accounted for by the contamination of glyceraldehyde 3-phosphate with inorganic phosphate.

The kinetic instability of 1-arseno- and 1-molybdo-3-phosphoglycerate is suggested by the ability of arsenate and molybdate (but not phosphate) to effectively inhibit the "reverse" reaction (Scheme I). Since phosphate reacts with the acyl-enzyme to produce the kinetically stable 1,3-diphosphoglycerate ($t_{1/2} \sim 1$ h), and phosphoglycerate kinase (+ MgADP and MgATP) is present, a constant concentration of the phosphoric anhydride will be maintained and no significant inhibition of the dehydrogenase reaction is expected. The inhibition at high phosphate concentration probably reflects a shift in the extent of the equilibrium between the acyl-enzyme and 1,3-diphosphoglycerate. This shift in equilibrium will not be as severe with molybdate replacing phosphate since the equilibrium constant ($=k_{-2}/k_2$, Scheme I) will be smaller. Nevertheless, molybdate as well as arsenate is an effective inhibitor for the reverse reaction. Fifty percent inhibition of the reverse reaction is observed in the presence of 15 mM MoO_4^{2-} or 6 mM $HAsO_4^{2-}$. The simplest interpretation of this observation is that in the presence of either molybdate, arsenate, or phosphate the acyl-enzyme (Scheme I) can partition between reduction (k_3) and acyl transfer (k_{-2}). If 1-molybdo- and 1-arseno-3-phosphoglycerate are kinetically unstable (unlike 1,3-diphosphoglycerate) inhibition of the

[4] Further evidence for this is obtained from the following experiment. When the "reverse" reaction (1,3-DPG + NADH → D-G3P + P_i + NAD^+) is followed in the absence of any added NAD, a lag in the disappearance of NADH is observed (~1 min). In the presence of 0.2 mM NAD, the lag in NADH disappearance is reduced to <0.1 min. In the presence of NAD nucleosidase (which hydrolyzes NAD to nicotinamide and ADP-ribose but does not hydrolyze NADH), the lag phase is substantially increased (e.g., 2.8 min in the presence of 0.2 unit of NADase). This suggests that NAD is required for acylation of the enzyme by the phosphoric anhydride and, therefore, NAD must also be required for the phosphorolysis of the acyl-enzyme.

reverse reaction will be expected in the presence of molybdate and arsenate.

An estimate of the kinetic stability of 1-arseno-3-phosphoglycerate and of 1-molybdo-3-phosphoglycerate can be made from the kinetics and thermodynamics of the overall reaction. In the absence of free inorganic phosphate, the appearance of NADH is a linear function of time when molybdate or arsenate is added to a mixture of NAD, glyceraldehyde 3-phosphate, and the dehydrogenase. The rate of NADH production is a linear function of the enzyme concentration (Figure 2). These results further indicate that the acyl arsenate and the acyl molybdate are kinetically labile species. An upper limit for the kinetic stability of these anhydrides can be obtained by assuming that substitution of the central atom (As or Mo) for phosphorus does not result in a deviation of these oxy anions from the Brønsted relationship obtained with the phosphonates (Figure 1). This is a reasonable assumption since the major difference between MoO_4^{2-} and $HOAsO_3^{2-}$ and the phosphonates is only in the length of the central atom–oxygen bond. Like the phosphonates, molybdate and arsenate are tetrahedral (Sutton, 1958), and acyl transfer to these species involves acylation of an oxygen atom. We are not aware of any previous studies of acyl transfer reactions involving oxy dianions. However, acyl transfer equilibria to oxy monoanions do follow the Brønsted relationship (i.e., a linear dependence of log K_{eq} on the pK_a of the conjugate acid of the oxy anion) for a variety of substituted oxyanions (see, e.g., Hupe & Jencks, 1977). Acyl transfer to atoms other than oxygen, however, do result in deviations from the Brønsted relationship based on acyl transfer to oxygen (Hupe & Jencks, 1977). Arsenate and molybdate, which are structurally and electronically homologous with respect to the phosphonates (i.e., tetrahedral dianions), are expected to follow the Brønsted relationship for acyl transfer equilibria based on the reaction with the phosphonates. Thus, Long & Ray (1973) find that the K_{eq} for hydrolysis of arsenate esters and phosphate esters are nearly identical. The pK_{a2} of arsenate (6.98) is similar to that of phosphate (7.21) but the central atoms differ. Thus, with a knowledge of the pK_{a2} values of arsenate (6.98) and molybdate (4.1) and the relationship between pK_{a2} and the equilibrium constant (log K_{eq} = 0.94 pK_{a2} − 7.97) obtained with the phosphonates, the equilibrium constants for the reaction with molybdate and arsenate can be estimated.

The estimated equilibrium constant for formation of the acyl arsenate monoanion is $\sim 3.9 \times 10^{-2}$ M^{-1}. At pH 7.3 this monoanion will ionize to produce the acyl arsenate dianion. Since the pK_as of phosphoric acid and arsonic acid are similar, the overall equilibrium constant for the reaction with arsenate at pH 7.3 will be similar to that for the reaction of phosphate at this pH (viz., 1.1 M^{-1}). The equilibrium constant for formation of 1-molybdo-3-phosphoglycerate (and NADH) from NAD, MoO_4^{2-}, and glyceraldehyde 3-phosphate, based on a pK_{a2} of 4.1, is estimated to be $\sim 7 \times 10^{-5}$ M^{-1}. Thus, when 1 mM NAD and 1 mM D-glyceraldehyde 3-phosphate are incubated at pH 7.3 with either 5 mM arsenate or 5 mM molybdate, the equilibrium concentrations of the anhydride products are expected to be 68 μM (acyl arsenate) and ~ 0.6 μM (acyl molybdate). With either arsenate or molybdate as acyl acceptors, there is no evidence for accumulation of the anhydride products (i.e., the progress curves are linear). This indicates that hydrolysis of these anhydrides is faster than their formation and, thus, the steady-state level of the anhydrides must be less than their equilibrium concentration. A *minimum* value for the rate constant for spontaneous hydrolysis is $k_h' \leq v/[\text{anhydride}]_{eq}$, where v is the initial velocity for the production of NADH (= production of anhydride). At the highest enzyme concentration tested (Figure 2), the rate of NADH production is 1.14×10^{-3} M min^{-1} with 5 mM arsenate and 5.3×10^{-5} M min^{-1} with 5 mM molybdate. Thus, the rate constant for hydrolysis of 1-arseno-3-phosphoglycerate must be >17 min^{-1} ($t_{1/2}$ < 2.5 s) and the rate constant for spontaneous hydrolysis of 1-molybdo-3-phosphoglycerate must be >88 min^{-1} ($t_{1/2}$ < 0.5 s) at pH 7.3. Under these same conditions, the rate constant for spontaneous hydrolysis of 1,3-diphosphoglycerate is 1.2×10^{-2} min^{-1} ($t_{1/2}$ = 58 min).

The greater stability of the acyl phosphate than of the acyl arsenate or the acyl molybdate is expected. For example, pyrophosphate is a kinetically stable molecule, while pyroarsenate ($t_{1/2} \sim 12$ s, pH <9) hydrolyzes rapidly (Richmond et al., 1977). In aqueous solution, MoO_4^{2-} undergoes rapid substitution reactions (Dienann & Muller, 1972). Indeed, Mo(VI) rapidly undergoes expansion of coordination number from 4 to 6 [e.g., the rapid polymerization of MoO_4^{2-} (Schwarzenbach, 1958)], and it has been suggested that the species "$HMoO_4^-$" may in fact be the octahedral species $OMo(OH)_5^-$ (Schwarzenbach, 1958). Indeed, the longer Mo–O (1.83 Å) and As–O (1.75 Å) bond in molybdate and arsenate than in phosphate (P–O bond = 1.54 Å) may indicate less p_π–d_π bonding corresponding to a weaker central atom–oxygen bond energy and, thus, a more rapid hydrolysis of the anhydrides. ^{18}O-Labeling studies have shown that hydrolysis of $RC(=O)OAsO_3^{2-}$ (Slocum & Varner, 1960) as well as acid-catalyzed hydrolysis of $RC(=O)OPO_3^{2-}$ (Bentley, 1949) involve cleavage of O–As and O–P bonds. Dahms et al. (1973) have shown that, while O–P bond cleavage occurs in hydrolysis of acetyl phosphate (75 °C) under acidic conditions (pH 3.8), C–O bond cleavage predominates at alkaline conditions (pH 13.4). The greater stability of the P–O bond relative to that of As–O and Mo–O bonds is further indicated by the rapid exchange of $H_2{}^{18}O$ with arsenate and molybdate anions (Hall & Alexander, 1940; Felten et al., 1978) compared with the slow exchange with HPO_4^{2-} [none detected after 2 days at 100 °C (Hall & Alexander, 1940; Winter et al., 1940)] or with $H_2PO_4^-$ [$t_{1/2} \approx 48$ h at 100 °C (Bunton et al., 1961)].

Even though molybdate and arsenate are larger in volume than phosphate (by 65% and 48%, respectively), they are good substrates for glyceraldehyde-3-phosphate dehydrogenase. The relative k_{cat}/K_M values for molybdate, arsenate, and phosphate (at pH 8.5, μ = 0.5 M, 25 °C) are: 1:1.85:11.8. The high reactivity of molybdate, as well as the reactivity of the phosphonates (Gardner & Byers, 1977), indicates the kinetic competency of the phosphate dianion in the reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase.

Neither sulfate nor selenate is a substrate for the enzyme. Both these tetrahedral oxy anions are poor bases (p$K_{a2} \sim 2$). Nevertheless, they are only $\sim$100 times less basic than molybdate and, since the overall reaction rate is not very sensitive to the pK_a of the acyl acceptor (Gardner & Byers, 1977), sulfate and selenate are expected to also be acyl acceptors in this reaction. Indeed, the presence of a sulfate ion bound to the lobster muscle enzyme (Buehner et al., 1974) and the *B. stearothermophilus* enzyme (Biesecker et al., 1977) (between Ser-148 and Thr-208) observed in the crystal structures have led to the suggestion that this is the phosphate binding site in the acyl-enzyme (Garavito et al., 1977). It is not clear why sulfate or selenate is not a substrate for glyceraldehyde-3-phosphate dehydrogenase. It is even more surprising that sulfate or selenate, at concentrations as high as 50 mM, have little effect on the arsenolysis, molybdenolysis,

or phosphorolysis of the enzyme. Since MoO_4^{2-} binds to the enzyme, it will be interesting to further examine the factors responsible for the poor binding of SO_4^{2-} and SeO_4^{2-}.

$Ge(OH)_4$ and ReO_4^- are reversible inhibitors of glyceraldehyde-3-phosphate dehydrogenase, but are not substrates. Water (or OH^-) is a poor substrate for the enzyme, reacting $\sim 5 \times 10^4$ times less rapidly than phosphate at pH 8.5 (Byers & Koshland, 1975). These observations suggest the requirement of a dianionic species for the proper binding and orientation of the acyl acceptor in the deacylation of the thiol ester–enzyme intermediate.

Acknowledgments

We would like to thank Drs. John Perdew, Joel Mague, Gary McPherson, and Bill Alworth for helpful discussions.

Appendix: Relationship between NADH Production and Phosphoric Anhydride Production

The reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase is

$$NAD^+ + \text{D-G3P} + P_i \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{1,3-DPG} + NADH + H^+$$

In the absence of any additional acyl acceptor, other than a small amount of contaminating inorganic phosphate, P_i, the rate of approach of NADH to equilibrium (where $[NAD^+]_0$, $[\text{D-G3P}]_0 \gg [P_i]_0$) is given by (Benson, 1960)

$$\frac{[NADH]_{eq}}{2[P_i]_0} \ln\left(\frac{[NADH]_{eq} + [NADH]}{[NADH]_{eq} - [NADH]}\right) = k_1 t \quad (8)$$

where $[NADH]_{eq}$ is the equilibrium concentration of NADH (= $[\text{1,3-DPG}]_{eq}$) and k_1 is a function of enzyme, NAD^+, and G3P concentrations. This equation holds when $[P_i]_0 \gg [NADH]_{eq}$, a condition which is readily met since the initial concentration of phosphate is less than the initial concentrations of NAD^+ and G3P and the pH-dependent equilibrium constant for the reaction is less than 13 M^{-1} (pH <8.4). Rearrangement of eq 8 yields

$$[NADH] = [NADH]_{eq} \tanh\left(\frac{[P_i]_0 k_1 t}{[NADH]_{eq}}\right) \quad (9)$$

which predicts a hyperbolic tangent dependence on time for production of NADH. NADH, however, can also be produced by hydrolysis of the acyl-enzyme and by spontaneous hydrolysis of 1,3-DPG (which shifts the equilibrium to the right). The first situation is not significant since it is known that in the presence of NAD phosphorolysis of the acyl-enzyme is at least 5×10^4 times more rapid than hydrolysis (Byers & Koshland, 1975). The nonenzymic hydrolysis of 1,3-DPG is a slow process. The rate constant for this reaction was found to be $\sim 10^{-2}$ min^{-1} between pH 5.5 and 8.4 at 25 °C. Negelein & Brömel (1939, 1969) reported a rate constant for hydrolysis of 1,3-DPG of 2.6×10^{-2} min^{-2} (pH 7, 38 °C). Thus, when the enzyme is present, a constant equilibrium concentration of 1,3-diphosphoglyceric acid will be maintained since the rate constant for hydrolysis of the anhydride, k_h, is much less than k_1 and k_{-1}. When 1,3-diphosphoglyceric acid is hydrolyzed, phosphate is regenerated and with it a stoichiometric amount of NADH is produced to maintain the equilibrium of the dehydrogenase reaction (as long as NAD and glyceraldehyde 3-phosphate are present in excess). Thus, the production of NADH is given by eq 6, where $[NADH]_{eq'}$ is the concentration of NADH when equilibrium is initially established (i.e., $[NADH]_{eq'} = [\text{1,3-diphosphoglycerate}]_{eq}$).

$$[NADH] = [NADH]_{eq'} \tanh\left(\frac{[P_i]_0 k_1 t}{[NADH]_{eq'}}\right) + k_h[NADH]_{eq'} t \quad (6)$$

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Relaxation Kinetics of Glutamate Dehydrogenase Self-Association by Pressure Perturbation†

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ABSTRACT: The kinetics of self-association for beef liver glutamate dehydrogenase (EC 1.4.1.3) have been measured by using pressure perturbation in both the time domain and the frequency domain by monitoring scattered light intensity. The kinetic behavior is entirely consistent with the random self-association model proposed by Thusius et al. [Thusius, D., Dessen, P., & Jallon, J. M. (1975) *J. Mol. Biol. 92*, 413–432]. The activation volume $\Delta V^{\ddagger}$ for association is estimated to be positive, and it is shown that this provides further corroboration of the molecular mechanism advanced by these same authors. A rapid shift in scattered light intensity is attributed to preferential interaction between the phosphate anion and the protein, proceeding with a positive volume change (2–5 mL/mol of phosphate). A description of the instrument developed for this study is also included.

Beef liver glutamate dehydrogenase (EC 1.4.1.3) (GDH)[1] exists in solution as a molecule of 336 000 molecular weight, each molecule consisting of six apparently identical polypeptide chains. These molecules of GDH self-associate to form a concentration-dependent statistical distribution of rodlike aggregates, with a single equilibrium constant describing the addition of a unit to a growing chain. This phenomenon has been studied extensively and is covered in recent reviews devoted to GDH (Sund et al., 1975; Eisenberg et al., 1976). Although the mode of the interactions has been well characterized as a linear indefinite (or isodesmic) self-association by a variety of equilibrium techniques, equilibrium measurements, of course, leave the question of mechanism unresolved. For GDH self-association, it would be interesting to know if any particular oligomeric species plays a special role in the self-association process. The initial stopped-flow kinetic measurements (Fisher & Bard, 1969) showed uniphasic kinetics, suggesting the existence of but a single kinetic interaction. A more detailed temperature-jump study (Thusius et al., 1975) corroborates Fisher's observation and points out

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[1] Abbreviations used: GDH, bovine liver glutamate dehydrogenase (EC 1.4.1.3).

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