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Interaction of the Substrate Phosphate Substituent with Glyceraldehyde-3-phosphate Dehydrogenase†

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ABSTRACT: Linear free energy relationship studies were carried out to examine the nature of the interaction of aldehydes with glyceraldehyde-3-phosphate dehydrogenase. The association constant of most aldehydes with the enzyme is dependent on the Taft polar substituent constant, characterized by a ρ^* value of 1.7. This is identical with the ρ^* value for thiohemiacetal formation between aldehydes and glutathione and suggests that the binding of aldehydes to the enzyme results in the formation of a thiohemiacetal adduct. D-Glyceraldehyde 3-phosphate, however, shows a positive deviation from the linear correlation, indicating a specific interaction of the phosphate substituent with the enzyme. Thiohemiacetal formation contributes 3.9 kcal/mol to the formation of the enzyme–aldehyde complex with D-glyceraldehyde 3-phosphate, and the interaction of the phosphate moiety on the aldehyde with the phosphate binding site on the enzyme contributes 3.7 kcal/mol. The interaction of inorganic phosphate with the aldehyde phosphate binding site on the enzyme is characterized by a $\Delta G^{\circ\prime} > -1.4$ kcal/mol, indicating that most of the apparent free energy for binding the phosphate substituent on the aldehyde is entropic in origin. Phosphorylation of irreversible inhibitors of glyceraldehyde-3-phosphate dehydrogenase has been shown to enhance their reactivity with the enzyme by 3.8 (±1) kcal/mol [Byers, L. D. (1977) *J. Am. Chem. Soc. 99*, 4146–4149]. Phosphorylation of the aldehyde substrates also enhances the stereoselectivity of the overall oxidative reaction [Byers, L. D. (1978) *Arch. Biochem. Biophys. 186*, 335–342]. In addition to these effects, and the enhanced binding of aldehydes, the phosphate substituent was found to alter the conformational mobility of the protein, the specificity of deacylation of the acyl enzyme (with respect to acyl acceptors), and the reactivity of the aldehydes as substrates in the overall oxidative arsenolysis reaction. On the basis of electronic effects of the acyl substituent, D-glyceraldehyde 3-phosphate is more reactive than are nonphosphorylated aldehydes by a factor of 10^4.

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) catalyzes the oxidative phosphorylation of D-glyceraldehyde 3-phosphate. The early studies of Racker & Krimsky (1952) and of Harting & Velick (1954) have demonstrated that glyceraldehyde-3-phosphate dehydrogenase (from yeast and rabbit muscle) can also catalyze the oxidation of a variety of aldehydes but at substantially reduced rates relative to that for oxidation of D-glyceraldehyde 3-phosphate. In addition to enhancing the reactivity of the aldehyde, the phosphate substituent enhances the stereoselectivity of the enzyme. D-Glyceraldehyde 3-phosphate is at least 30 000 times more reactive than L-glyceraldehyde 3-phosphate, but D-glyceraldehyde is only 180 times more reactive than its enantiomer (Byers, 1978). The reaction of irreversible inhibitors with the enzyme is also enhanced by the presence of a phosphate moiety. After correction for electronic effects (Byers, 1977), glycidol phosphate is found to be 630 times more reactive than other epoxides with the enzyme, and chloracetol phosphate is found to be 680 times more reactive than other modifying reagents with the enzyme (Byers, 1977). This enhanced reactivity of the phosphorylated irreversible inhibitors with the enzyme (which corresponds to a lowering of the free energy of activation by 3.8 ± 0.1 kcal/mol) is a measure of the specific interaction of the phosphate moiety with the enzyme. This study was undertaken to examine the nature of the interaction of the phosphate substituent with the enzyme and evaluate the role of this interaction in the binding and catalytic steps of the enzyme reaction.

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Experimental Section

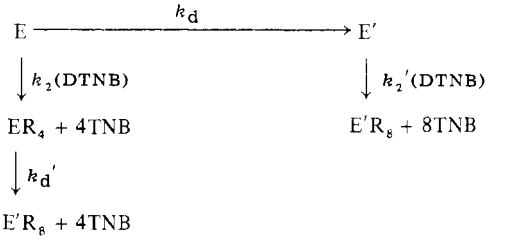
Materials

The preparation and purification of the reagents were carried out as described previously (Kanchuger & Byers, 1979). DL-Lactaldehyde was prepared by the method of Hough & Jones (1952). Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) from *Saccharomyces cerevisiae* was prepared as described previously (Byers, 1978). Covalently modified enzymes were prepared by treating the enzyme with propylene oxide, glycidol, or glycidol phosphate until over 98% of the active-site sulfhydryl groups were modified as detected by titration with DTNB[1] [see McCaul & Byers (1976)]. The enzyme was then separated from the unreacted epoxides by gel filtration on a small Sephadex G-50 column. Guanidine hydrochloride (Sequanal Grade) was obtained from Pierce Chemical Co.

Methods

"Denaturation" Kinetics. The unfolding of glyceraldehyde-3-phosphate dehydrogenase, and alkylated derivatives, was monitored by three probes: (1) loss of catalytic activity, (2) reduction of fluorescence intensity (λ_{ex} = 283 nm; λ_{em} = 340 nm), and (3) exposure of the buried sulfhydryl group. The yeast glyceraldehyde-3-phosphate dehydrogenase is a tetramer consisting of four identical polypeptide chains. Each subunit contains two cysteine residues (Cys-149 and Cys-153). One of these residues (Cys-149) contains the active-site SH group which is reactive toward a variety of modifying reagents while the other residue (Cys-153) is buried in an environment which reduces its reactivity toward modifying reagents (Jones & Harris, 1972). Vas & Boross (1974) have described an approach for the characterization of the fluctuational motion around Cys-153 in the pig muscle enzyme. By using the *p*-mercuribenzoate mercaptide of 2-nitro-5-mercaptobenzoate as a chromophoric probe, Vas and Boross were able to evaluate the accessibility of Cys-153 to this reagent under a variety of conditions. Our approach was based on this method. A sample of the enzyme was added to a buffered solution (0.1 M Hepes and 0.1 M NaCl, pH 7.5, and 25 °C) containing various concentrations of guanidine hydrochloride and 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). The reaction of DTNB with free SH groups releases the chromophore 2-nitro-5-mercaptobenzoate (Ellman, 1959) which has an extinction coefficient at 412 nm of 1.36×10^4 M^{-1} cm^{-1} in guanidine hydrochloride concentrations ranging from 0 to 6 M. Addition of the enzyme to a solution of DTNB in the absence of guanidine hydrochloride results in a rapid release of 3.5–4.0 thionitrobenzoates/tetramer, followed by a very slow increase in absorbance at 412 nm. The rate of this second phase is increased in guanidine hydrochloride. When the enzyme is mixed with 6 M guanidine hydrochloride and allowed to stand 5 min and then DTNB is added, thionitrobenzoate (corresponding to seven to eight molecules per tetramer) is rapidly released in a single-exponential process ($k_2 \sim 7 \times 10^4$ M^{-1} min^{-1}). Guanidine hydrochloride has little effect on the rate of reaction of DTNB with glutathione. The use of DTNB to monitor the structural motility (in guanidine hydrochloride) of Cys-153 is summarized in Scheme I. Here E′ represents the "denatured" enzyme and TNB is the thionitrobenzoate chromophore. Under these conditions the

Scheme I



rate of reaction of DTNB with the four active-site SH groups [k_2(DTNB) = 2.5×10^3 min^{-1} (Byers, 1977)] or with the eight equivalent SH groups in the denatured enzyme [k_2′(DTNB) $\approx$ 70 min^{-1}] is faster than the denaturation rates (k_d and k_d′). Thus, the rate equation for production of TNB is

$$[TNB] = 4[E]_0(1 - e^{-k_2(DTNB)t}) + 4[E]_0(1 - e^{-k_d't}) \quad (1)$$

Since the experiments were carried out in a conventional spectrophotometer, the kinetics of only those processes occurring after ~0.3 min could be followed. Thus, eq 1 reduces to

$$[TNB] \approx 4[E]_0(2 - e^{-k_d't}) \quad (2)$$

For the reactions with the modified enzymes, the active-site SH is no longer available for reaction with DTNB (McCaul & Byers, 1976), and, therefore, the production of TNB will be characterized by a single-exponential process without a burst phase:

$$[TNB] = 4[E]_0(1 - e^{-k_d't}) \quad (3)$$

The data obtained with the various species of enzyme fit eq 2 (native enzyme) or eq 3 (modified enzyme). The rate constants were evaluated by a linear least-squares regression to the logarithmic forms of eq 2 and 3.

The rate of loss of enzymatic activity was followed by removing aliquots of the enzyme–guanidine hydrochloride solution at various times, diluting ~20-fold into a standard assay mixture containing 1 mM NAD, 1 mM D-glyceraldehyde 3-phosphate, and 25 mM arsenate (0.05 M Bicine and 1 mM EDTA, pH 8.5) and measuring the rate of appearance of NADH (λ = 340 nm). The loss of activity is a first-order process. Incubation of the enzyme in 1 M NaCl for 15 min had no effect on the activity of the enzyme.

Aldehdye Binding. The association constants of the various aldehydes with the enzyme were determined by measuring the alkylation rate of the enzyme in the presence and absence of the aldehydes at various aldehyde concentrations. The alkylation rate was measured by loss of enzymic activity. At an ionic strength of 0.4 M (sodium acetate and 1 mM EDTA, 25 °C), the rates of alkylation of the enzyme with a variety of modifying reagents (iodoacetamide, chloroacetamide, epifluorohydrin, *N*-ethylmaleimide, glycidol, and propylene oxide) all depend on an ionizing group with pK_a = 9.14 (±0.15) (Jurovich and Byers, unpublished experiments). The aldehyde binding experiments were carried out in the presence of 0.4 M sodium acetate (0.05 M Bicine and 1 mM EDTA, pH 8.2) with either iodoacetamide or chloroacetamide as the alkylating reagent. Under these conditions the second-order rate constants for alkylation of the enzyme (in the absence of aldehyde) are 2.6×10^3 M^{-1} min^{-1} with iodoacetamide and 16 M^{-1} min^{-1} with chloroacetamide. The binding constants of the aldehydes were determined by the method described earlier (Kanchuger & Byers, 1979). The corrected association constants, K_{GPD}, were calculated from the observed association constants by correcting for aldehyde hydration and thiol ionization by the relationship (Kanchuger & Byers, 1979)

[1] Abbreviations used: Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; Gdn·HCl, guanidine hydrochloride; GPD, glyceraldehyde-3-phosphate dehydrogenase [D-glyceraldehyde-3-phosphate:NAD oxidoreductase (phosphorylating)]; GSH, glutathione; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

$$K_{GPD} = \frac{[EA]}{[E][A]} = K_{GPD}^{obsd}\left(1 + \frac{K_a}{[H^+]}\right)(1 + K_h)$$
$$= K_{GPD}^{obsd}(1.126)(1 + K_h) \quad (4)$$

at pH 8.2, where K_a is the dissociation constant of the active-site SH group ($=7.9 \times 10^{-10}$ M) and K_h is the equilibrium constant for hydration of the free aldehyde, A. The K_h values were determined as described previously (Kanchuger & Byers, 1979) from the Taft relationship, $\log K_h = 1.68\sigma^* - 0.033$, for those aldehydes for which a directly measured value is not available. The ^1H NMR spectrum of glyoxalic acid (pD 8) in D_2O showed no evidence of an aldehydic proton at 9–10 ppm, indicating that $K_h > 16$. Since the σ^* value for the $-CO_2^-$ is variable, the effective σ^* value was determined by a comparison of the K_h value of pyruvate (0.054) and acetone (0.002) (Greenzaid et al., 1967), which is consistent with a σ^* value of 0.87 based on a ρ^* value of 1.68 [see Greenzaid et al. (1967)]. This implies a K_h value of 27 for the glyoxalic acid anion. A pH dependence of K_{GPD}^{obsd} for propanal was carried out ($7.0 \leq$ pH ≤ 9.0) and K_{GPD}^{obsd} varied from 6.5 (pH 9) to 11.6 M^{-1} (pH 7), consistent with eq 4 which is based on the assumption that aldehyde binding (and subsequent protection against alkylation) involves formation of a thiohemiacetal.

Aldehyde Reactivity. The aldehydes examined in this study are much poorer substrates for glyceraldehyde-3-phosphate dehydrogenase than is D-glyceraldehyde 3-phosphate (Fife & Rikihisa, 1970; Byers, 1978). In order to minimize inactivation of the enzyme by nonspecific reaction of the aldehydes with amino groups on the protein, and to minimize the nonenzymatic reaction of the aldehydes with NAD (which results in formation of an adduct which absorbs at 340 nm) (Byers, 1978), we carried out the reactions at low aldehyde concentrations ($\leq$0.01 M). Under these conditions the initial velocity, $v = d[NADH]/dt$, is directly proportional to the aldehyde concentration:

$$v = k_{obsd}[\text{aldehyde}] \quad (5)$$

$$k_{obsd} = V_{max}/K_M^{app} \quad (6)$$

where K_M^{app}, the apparent K_M for the aldehyde, is a function of [NAD] and $[HOAsO_3^{2-}]$. In these studies [NAD] = 1 mM, which is saturating when either D-glyceraldehyde 3-phosphate or propionaldehyde is the aldehyde substrate. At very low concentration of the aldehydes ($\sim$0.5 mM), the increase in NADH is a first-order process characterized by a rate constant given in eq 6. Equation 5 was verified by plotting the initial velocity as a function of aldehyde concentration. The slope of this line, k_{obsd}, agrees well ($\pm$10%) with k_{obsd} obtained under first-order conditions ([aldehyde] $\leq$ 0.5 mM). It has been established that the free aldehyde form of D-glyceraldehyde 3-phosphate (and not the hydrate) is the actual substrate for glyceraldehyde-3-phosphate dehydrogenase (Trentham et al., 1979). Therefore, the second-order rate constants, k_2, were corrected for aldehyde hydration by using the equation

$$k_2 = \frac{k_{obsd}}{(1 + K_h)[E]_T} = \frac{k_{cat}}{K_M^{cor}} \quad (7)$$

where K_M^{cor} is the K_M value corrected for aldehyde hydration and $[E]_T$ is the total enzyme concentration ($\sim$0.8–5 μM).

Results

Kinetics of Denaturation. Figure 1 shows the dependence of the pseudo-first-order rate constant for denaturation on the guanidine hydrochloride concentration as a function of three different probes. At guanidine hydrochloride concentrations between 0.5 and 4.0 M, the dependence of the denaturation rate on the guanidine hydrochloride concentration ([Gdn·HCl]) was found to obey the empirical relationship

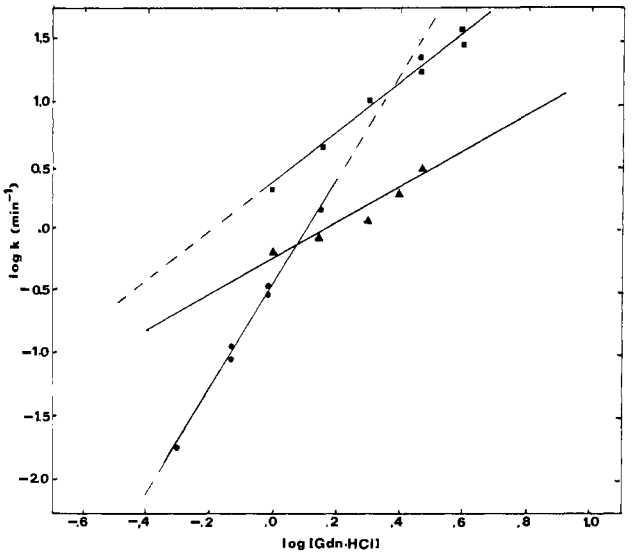


FIGURE 1: Denaturation of glyceraldehyde-3-phosphate dehydrogenase as monitored by various probes. The enzyme was added to a buffered solution (0.1 M Hepes, pH 7.5, at 25 °C) containing various concentrations of guanidine hydrochloride, and the pseudo-first-order denaturation was monitored by loss of enzymic activity [(●); protein concentration = 4.6×10^{-7} M], decrease in fluorescence intensity [(▲); protein concentration = 7.7×10^{-8} M], and exposure of the buried sulfhydryl group [(■); protein concentration = 6×10^{-8} M)].

$$k = a[\text{Gdn·HCl}]^n \quad (8a)$$

or

$$\log k = \log a + n \log [\text{Gdn·HCl}] \quad (8b)$$

It is of interest that the three probes yield different rate constants for "denaturation". Thus, the observed rate constants for unfolding of the native enzyme in the presence of 1 M Gdn·HCl are 0.40, 0.60, and 2.7 min^{-1} as measured by activity loss, fluorescence decrease, and buried sulfhydryl group reactivity, respectively. Furthermore, activity loss is more sensitive to the denaturant concentration (n = 4.07) than is the fluorescence decrease (n = 2.00) or the buried sulfhydryl group reactivity (n = 1.89).

For examination of the effect of the phosphate substituent on the denaturation (unfolding) of glyceraldehyde-3-phosphate dehydrogenase, the enzyme was modified with propylene oxide, glycidol, or glycidol phosphate (McCaul & Byers, 1976). It has been shown that epoxides modify only one of the two cysteine residues per subunit of glyceraldehyde-3-phosphate dehydrogenase (McCaul & Byers, 1976). A sample of the propylene oxide modified enzyme was subjected to amino acid analysis, and no significant decrease of any amino acid (other than one Cys per subunit) was observed when compared to the native enzyme, suggesting that only the active-site cysteine residue (Cys-149) has been modified (Gardner and Byers, unpublished experiments). The stability of the propylene oxide, glycidol, and glycidol phosphate modified enzyme to guanidine hydrochloride denaturation was monitored by the rate of exposure of the buried sulfhydryl group (and subsequent reaction with DTNB). These results are summarized in Table I.

Aldehyde Binding. To further examine the interaction of the phosphate substituent with the enzyme, we measured the association constants of various aldehydes with the enzyme.

Table I: Conformational Motility of Modified GPD[a]

species	n^b	a^c (min^{-1})	$\Delta G^{\ddagger}_{0}{}^{d}$ (kcal/mol)
native enzyme	1.88	2.68	19.2
glycidol modified	1.39	1.18	19.7
propylene oxide modified	0.78	0.50	20.2
glycidol phosphate modified	2.16	0.31	20.5

[a] Conformational motility of glyceraldehyde-3-phosphate dehydrogenase (GPD) at pH 7.5, 25 °C, as monitored by the rate of exposure of the buried sulfhydryl groups. [b] Dependence of the observed rate constant on the Gdn·HCl concentration (1 M $\leq$ [Gdn·HCl] $\leq$ 4 M); see eq 8 (text). [c] Rate constant at [Gdn·HCl] = 1 M; see eq 8. [d] Standard free energy of activation in 1 M Gdn·HCl [see Chan & Shanks (1977)].

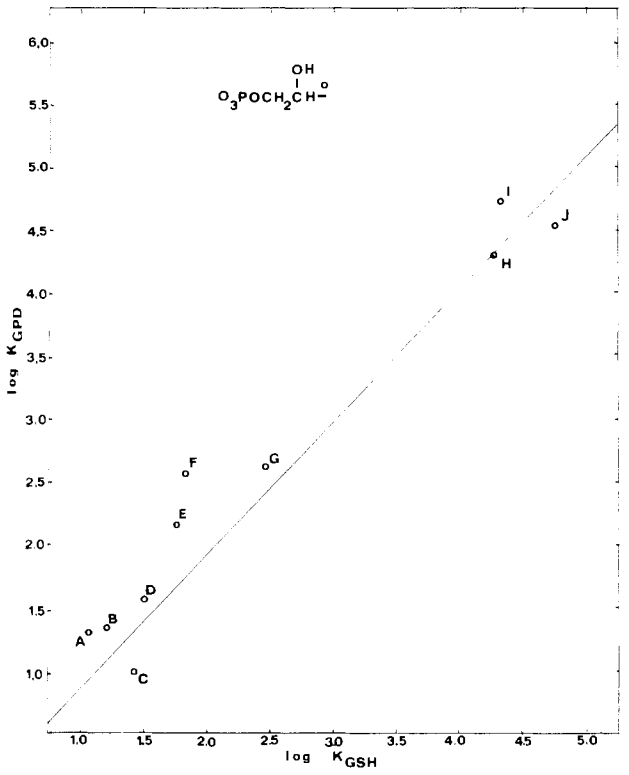


FIGURE 2: Correlation of association constants of aldehydes with glyceraldehyde-3-phosphate dehydrogenase (K_{GPD}) and with glutathione (K_{GSH}). The following are the aldehyde (XCHO) substituents: X = *i*-Pr- (A), X = Et- (B), X = CH_3- (C), X = $CH_3CH(OH)$- (D), X = $CH_3C(=O)NHCH_2$- (E), X = $HOCH_2$- (F), X = $HOCH_2CH(OH)$- (G), X = ^-O_2C- (H), X = $^+H_3NCH_2$- (I), and X = $CH_3C(=O)$- (J). The line is the least-squares fit of the data (A–J). The slope is 1.043 and the interval estimator of the slope (at 90% confidence) is ±0.038.

The Taft ρ^* value for thiohemiacetal formation with glutathione (K_{GSH}) is 1.65 (±0.18) (Kanchuger & Byers, 1979). Glutathione is a good model for the active-site sulfhydryl group of glyceraldehyde-3-phosphate dehydrogenase in that the pK_a values (~9.1) are identical (Byers, 1977). Furthermore, it has been established that thiohemiacetal equilibria are insensitive to the basicity of the thiol (Gilbert & Jencks, 1977; Lienhard & Jencks, 1966). Figure 2 shows the dependence of the association constant of the aldehydes with the enzyme (K_{GPD}) on the association constant of the aldehydes with the model thiol, glutathione (K_{GSH}). The data fit the relationship

$$\log K_{GPD} = 1.04\ (\pm 0.04)\ \log K_{GSH} - 0.231\ (\pm 0.098) \quad (9)$$
$$n = 10;\ r = 0.975$$

for all the aldehydes except D-glyceraldehyde 3-phosphate. The slope of the line is the ratio of the ρ^* values for the

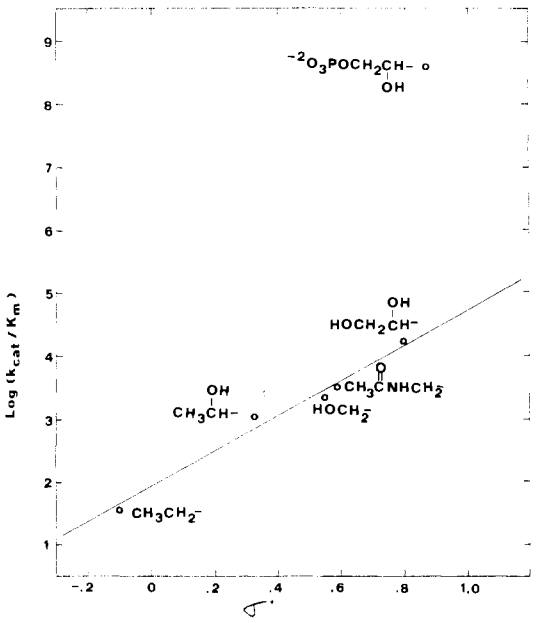


FIGURE 3: Dependence of the second-order rate constant for the glyceraldehyde-3-phosphate dehydrogenase catalyzed oxidative arsenolysis (k_{cat}/K_M) of aldehydes (XCHO) as a function of the Taft polar substituent constant. The following are the reaction conditions: pH 8.2 (0.05 M Bicine, 0.4 M sodium acetate, and 1 mM EDTA, at 25 °C), 1 mM NAD, and 25 mM arsenate. The K_M values are corrected for aldehyde hydration.

enzymic and nonenzymic ($\rho^* = 1.65$) equilibria. Thus, the ρ^* (=1.72) value for association of the aldehydes with the enzyme is essentially the same as the ρ^* value for the thiohemiacetal formation with the aldehydes and glutathione (Kanchuger & Byers, 1979).

Various aldehydes were tested as substrates for yeast glyceraldehyde-3-phosphate dehydrogenase (25 mM arsenate and saturating NAD). The second-order rate constants, k_{cat}/K_M^{cor} (eq 7), are dependent on the Taft polar substituent constant. The results are summarized in Figure 3. The ρ^* value is 2.81 with an interval estimator (at 90% confidence) of ±0.58:

$$\log (k_{cat}/K_M^{cor}) = 2.81\sigma^* + 1.98 \quad (10)$$
$$n = 5;\ r = 0.989$$

Phosphate Inhibition of Glyceraldehyde 3-Phosphate Binding. Equilibrium dialysis experiments suggest that there is no significant specific binding of inorganic phosphate to yeast glyceraldehyde-3-phosphate dehydrogenase (Velick & Hayes, 1953). At fixed concentrations of NAD and glyceraldehyde 3-phosphate (>10 K_M), there is no kinetic evidence for nonspecific binding of inorganic phosphate (Byers et al., 1979). However, when the aldehyde concentration is lowered (to ~K_M), and the varied substrate is inorganic phosphate, deviations from normal Henri–Michaelis–Menten kinetics are observed at $[P_i] \geq 15$ mM (Alayoff and Byers, unpublished experiments). This is similar to the situation observed by Meunier & Dalziel (1978) with the rabbit muscle enzyme. These authors found that the initial velocities for oxidative phosphorylation of glyceraldehyde 3-phosphate decreased with an increase in the phosphate concentration above 10 mM. No inhibition by phosphate (1–50 mM) was observed, however, when glyceraldehyde was used as a substrate. Meunier & Dalziel (1978) have shown that the phosphate inhibition is competitive with respect to glyceraldehyde 3-phosphate.

In order to estimate the dissociation constant of inorganic phosphate from the binding site for the phosphate moiety of

Scheme II

$$P_i + E{\cdot}A' \overset{K_S}{\rightleftharpoons} E + A' + P_i \xrightarrow{k} E' + A' + P_i$$

$$K_P \Big\Updownarrow$$

$$A' + E{\cdot}P_i \xrightarrow{k} E'{\cdot}P_i + A'$$

glyceraldehyde 3-phosphate on the yeast enzyme, we investigated the effect of inorganic phosphate on the binding of glyceraldehyde 3-phosphate to the enzyme. Because of the complexities of initial velocity studies [the kinetic mechanism of the yeast enzyme has not yet been fully established, and NAD binding is characterized by cooperativity (Mockrin et al., 1975)], it is difficult to extract a dissociation constant for phosphate from inhibition data. We, therefore, measured binding of glyceraldehyde 3-phosphate to the apoenzyme by protection against alkylation with chloroacetamide. High concentrations of phosphate induce dissociation of the tetrameric yeast enzyme (Jaenicke et al., 1968); therefore, these studies were carried out at low phosphate concentrations ($\leq$20 mM) in the presence of 0.4 M sodium acetate. The maximum increase in ionic strength was $\sim$15%. Phosphate (up to 20 mM) has no effect on the alkylation rate of the enzyme with chloroacetamide. At a fixed concentration of glyceraldehyde 3-phosphate, increasing concentrations of phosphate increase the alkylation rate (Table II). This is consistent with phosphate competing with glyceraldehyde 3-phosphate for binding to the enzyme according to Scheme II. A′ is the total (hydrated plus unhydrated) aldehyde, and k is the pseudo-first-order rate constant for alkylation of the enzyme in the absence of the aldehyde. The observed rate constant, k_{obsd}, for alkylation in the presence of both phosphate and aldehyde, according to Scheme II (i.e., mutually exclusive binding of P_i and A), is

$$k_{obsd} = \frac{k(1 + [P_i]/K_P)}{1 + [A']/K_S + [P_i]/K_P} \qquad (11)$$

Under these conditions $K_S = 8.9 \times 10^{-5}$ M. The data (Table II) are consistent with Scheme II with $K_P = 17$ ($\pm$3) mM. However, the data cannot rule out other mechanisms, and, therefore, the value of 17 mM must be considered a lower estimate of the dissociation constant.

Table II: Inorganic Phosphate Activation of Enzyme Alkylation in the Presence of Glyceraldehyde 3-Phosphate[a]

[G3P] (mM)	[P_i] (mM)	k_2^{calcd} (M^{-1} min^{-1})[b]	k_2^{obsd} (M^{-1} min^{-1})[c]
0	0	15.9	15.9 (±1.2)
0	20	15.9	15.4 (±0.7)
0.05	0	10.16	10.7 (±0.8)
0.05	10	11.73	11.5 (±0.5)
0.05	15	12.23	12.0 (±1.0)
0.05	20	12.62	12.5 (±1.0)
0.10	0	7.47	7.3 (±0.3)
0.10	10	9.29	7.9 (±0.9)
0.10	15	9.94	10.1 (±0.6)
0.10	20	10.47	12.0 (±1.0)
0.20	0	4.88	4.7 (±0.2)
0.20	10	6.56	6.3 (±0.3)
0.20	15	7.23	9.2 (±0.8)

[a] Alkylation of glyceraldehyde-3-phosphate dehydrogenase was carried out at pH 8.2 (0.05 M Bicine, 0.4 M sodium acetate, and 1 mM EDTA, at 25 °C) in the presence of 0.01 M chloroacetamide and various concentrations of D-glyceraldehyde 3-phosphate (G3P) and inorganic phosphate (P_i). [b] Calculated second-order alkylation rate constant based on eq 11 (see text) where $k = 15.9$ M^{-1} min^{-1}, $K_S = 8.9 \times 10^{-5}$ M, and $K_P = 17$ mM. The dissociation constant of inorganic phosphate, K_P, was determined by a nonlinear regression to eq 11 with K_S, the dissociation constant of the aldehyde, fixed at 8.9×10^{-5} M. [c] The observed second-order rate constant is the average value of two to three determinations.

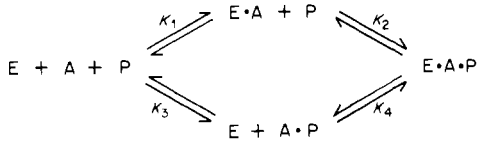
Discussion

The presence of a phosphate moiety on irreversible inhibitors of glyceraldehyde-3-phosphate dehydrogenase (glycidol phosphate and chloroacetol phosphate) enhances the reactivity of these reagents toward the enzyme by more than can be accounted for on electronic grounds alone (Byers, 1977). This has been attributed to an interaction of the phosphate moiety with a phosphate binding site on the enzyme. Another manifestation of the interaction of the phosphate substituent with the protein is seen in the effect of this substituent on the conformational mobility of the protein in guanidine hydrochloride (Table I).

Denaturation of glyceraldehyde-3-phosphate dehydrogenase in guanidine hydrochloride is a complex process. At high concentrations of guanidine hydrochloride, the tetrameric yeast enzyme is dissociated into unfolded monomers (Jaenicke & Rudolph, 1977). The fluorescence spectrum of the yeast enzyme is the same in 1 M guanidine hydrochloride (after 20 min = 16 half-lives) and in 6 M guanidine hydrochloride (after 2 min), suggesting that the final "denatured state" is the same at all guanidine hydrochloride concentrations tested. However, the denaturation most likely involves intermediate states. This is suggested by the observation that both the rate constants and the sensitivity to denaturant concentrations are dependent on the probe. Thus, in 1 M Gdn·HCl the rate of exposure of the buried sulfhydryl group (Cys-153) is more rapid than the decrease of the rate of fluorescence intensity or the irreversible loss of catalytic activity of the enzyme. It is not clear whether the exposure of the buried sulfhydryl group is simply a reflection of a local conformational fluctuation which is facilitated by the presence of guanidine hydrochloride or a property of an intermediate along the pathway to denaturation (as measured by activity loss). Nevertheless, alkylation of the active-site sulfhydryl group (Cys-149) reduces the rate of exposure of the buried sulfhydryl to the chromophoric modifying agent, DTNB. Glycidol phosphate is more effective than its nonphosphorylated analogue, glycidol, in restricting the accessibility of the buried sulfhydryl to DTNB.

Another manifestation of the interaction of the phosphate substituent with the enzyme is seen in the binding of aldehydes to the enzyme (Figure 2). Most aldehydes tested bind about equally well to the enzyme and to the model thiol, glutathione. Furthermore, the Taft ρ^* value is virtually the same for thiohemiacetal formation with glutathione (Kanchuger & Byers, 1979) and binding of the aldehyde to the enzyme. This suggests that thiohemiacetal formation is the dominant factor responsible for binding of aldehydes to the enzyme. Alcohol and carboxylic acid analogues of the aldehydes do not bind well to the enzyme. For example, the dissociation constant of glyceraldehyde 3-phosphate from the enzyme is $\sim 9 \times 10^{-5}$ M, and K_i for α-glycerol phosphate is 0.3 M (K. Gray and L. Byers, unpublished experiments). The K_i values (obtained from steady-state kinetics with glyceraldehyde 3-phosphate as the varied substrate) for acetate, propionate, lactate, *N*-acetylglycine, glycolate, glycerate, and glycine are all greater than 0.2 M under the same conditions at which the binding of the corresponding aldehydes was measured. Furthermore, there is no good correlation between aldehyde binding (log K_{GPD}) and the Hansch lipophilic substituent constant, π. Thus, $\log K_{GPD} = -0.54\pi + 2.0$ ($r = -0.478$) for the six aldehydes for which π values are available (Hansch et al., 1973). The

Scheme III



ρ^* value for formation of a thiohemiacetalate anion has been estimated to be $\sim$2.97 (Kanchuger & Byers, 1979), which is 75% larger than the observed ρ^* value (1.7). While the thiohemiacetalate anion is expected to be an intermediate in the oxidation of the aldehyde (i.e., hydride transfer to NAD), it does not appear likely that such an anion exists to any significant extent when aldehydes bind to the enzyme in the absence of NAD.

It is of interest that no significant deviation of any aldehyde, other than glyceraldehyde 3-phosphate, is observed in the correlation of K_{GPD} and K_{GSH} (Figure 2). Thus, lactaldehyde and glyceraldehyde fall on the same line as propionaldehyde. This indicates that there is no significant interaction of the C-2 (or C-3) hydroxyl group with the enzyme. The physiological substrate, D-glyceraldehyde 3-phosphate, however, shows a significant deviation from the correlation. No deviation of this aldehyde is observed in the Taft correlation (log K_{GSH} vs. σ^*) for thiohemiacetal formation with glutathione (Kanchuger & Byers, 1979). Thus, D-glyceraldehyde 3-phosphate binds 532 times more tightly to the enzyme than is expected in terms of the electronic effect of this acyl substituent (Figure 2). Therefore, as in the case of various irreversible inhibitors, a specific interaction of the phosphate moiety with the enzyme is indicated. This interaction provides an additional 3.7 kcal/mol ($=RT$ ln 532) of free energy of binding for glyceraldehyde 3-phosphate.

This 3.7 kcal/mol can be considered a *minimal* value for the intrinsic binding energy (Jencks, 1975) of the phosphate moiety since some of the intrinsic binding energy can be used to alter the conformation of the protein and/or the aldehyde when it binds to the enzyme. For example, the presence of the phosphate moiety reduces the "denaturation" rate of the enzyme, in 1 M guanidine hydrochloride, by a factor of 3.8 (relative to the glycidol-modified protein), which indicates stabilization of the protein structure by $\sim$0.8 kcal/mol. Thus, the intrinsic binding energy of the phosphate moiety must be *at least* 4.5 (=3.7 + 0.8) kcal/mol. Ionic or hydrogen-bonding interactions between the phosphate moiety and the phosphate binding site on the enzyme can readily account for this value of $\geq$4.5 kcal/mol. Unfortunately, direct evaluation of the intrinsic phosphate binding energy is not possible. Inorganic phosphate does not significantly inhibit the binding of glyceraldehyde 3-phosphate to the enzyme. If inorganic phosphate is bound to the phosphate binding site for the substrate, glyceraldehyde 3-phosphate would be expected to bind less tightly to the enzyme. Since the dissociation constant of inorganic phosphate is $\geq$0.017 M, inorganic phosphate must have a binding constant of $\leq$59 M^{-1}. This low binding constant most likely reflects an unfavorable entropic component since glyceraldehyde 3-phosphate binding is 532 times more favorable than predicted on electronic grounds alone. The entropic component can be estimated from the thermodynamic cycle shown in Scheme III. Here A represents the unhydrated aldehyde without the phosphate substituent and K_1 is the association constant of this aldehyde with the enzyme. This value is 726 M^{-1}, which is estimated from eq 9. [K_{GSH} is 940 M^{-1} with glyceraldehyde 3-phosphate (Kanchuger & Byers, 1979).] This is more reliable than using the association constant of an aldehyde such as glycolaldehyde (61 M^{-1}) since eq 9 provides a value of K_1 corrected for the electronic effect of the phosphate substituent (i.e., the association constant if no specific interaction of the phosphate moiety with the enzyme accompanies binding of glyceraldehyde 3-phosphate). K_2 is the binding constant of inorganic phosphate (P) to the aldehyde phosphate moiety binding site on the enzyme. K_3 is the association constant of phosphate with the aldehyde to form a noncovalent, bimolecular analogue of glyceraldehyde 3-phosphate (A•P). The A•P complex is one in which P has the same orientation relative to A as does the phosphate substituent relative to the rest of glyceraldehyde 3-phosphate. Since no covalent bonds are present in A•P, K_3 is predominantly an entropic term. Indeed, K_3 is expected to be $\sim$1/55.6 M (which corresponds to 8 eu) based on the *cratic entropy* which is the entropic correction of bringing a molecule of A near a molecule of P in a dilute solution (Gurney, 1953). K_4 is the binding constant of A•P to the enzyme which is 3.86×10^5 M^{-1} (i.e., K_{GPD} for glyceraldehyde 3-phosphate). Since $K_3 = K_1K_2/K_4$ and $K_2 \leq 59$ M^{-1}, K_3 must be $\leq$0.11 M^{-1}. This corresponds to a dissociation constant (of P from A) of $\geq$9 M. If K_3 is entirely entropic in nature, this corresponds to an entropy change of -4.4 eu in bringing A and P together in the same orientation as present in the monomolecular species, glyceraldehyde 3-phosphate. Thus, glyceraldehyde 3-phosphate binds more tightly to the enzyme than predicted on the basis of electronic effects because there is a phosphate binding site on the enzyme (which contributes 3.7 kcal/mol of energy to binding) and formation of the thiohemiacetal adduct (which contributes 3.9 kcal/mol = RT ln 726) fixes the phosphate substituent in a proper orientation for binding to the phosphate binding site (thus overcoming the unfavorable entropic term).[2]

In addition to enhancing binding of aldehydes to the apoenzyme, the phosphate substituent enhances the efficacy of the aldehyde substrates in the overall reaction (Figure 3). The dependence of the second-order rate constant (k_{cat}/K_M^{cor}) on σ^* is characterized by a ρ^* value of 2.81. Fife & Rikihisa (1970) found a dependence of k_{cat} on σ^* for the reaction of a series of aliphatic aldehydes with rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, characterized by a ρ^* value of 2.08. There is not as good a correlation between K_M and σ^* as there is between k_{cat} and σ^* for these aldehydes. When K_M is corrected for aldehyde hydration, a plot of log ($1/K_M^{cor}$) vs. σ^* yields a ρ^* value of 0.5 ($n = 6$; $r = 0.73$). Thus, a ρ^* value of $\sim$2.6 is estimated for k_{cat}/K_M^{cor} with the rabbit muscle enzyme, similar to the value obtained with the yeast enzyme. A detailed interpretation of this ρ^* value is not possible due to the lack of sufficient information about the kinetic and chemical mechanism of action of yeast glyceraldehyde-3-phosphate dehydrogenase. The second-order rate constant, k_{cat}/K_M^{cor}, may not reflect a single step in the reaction. Indeed, k_{cat}/K_M^{cor} will be dependent only on the steps up to, and including, the first irreversible step in the reaction [see Northrop (1975) and Klinman (1978)]. The linear relationship between log (k_{cat}/K_M^{cor}) and σ^*, with aldehydes varying in reactivity over nearly 4 orders of magnitude, indicates that the *relative* rate constants of the steps through the first irreversible step are independent of the acyl substituent on the aldehyde (with the possible exception of D-glycer-

[2] This is similar to the situation observed by Ray et al. (1973) and Ray & Long (1976) with phosphoglucomutase. Thus, 1,4-butanediol bisphosphate binds more tightly to the dephospho form of phosphoglucomutase than does 1,4-butanediol monophosphate (by 4.6 kcal/mol). However, the free energy for binding of inorganic phosphate to the phospho form of the enzyme is only $\sim$1.4 kcal/mol. The results of binding of glucose 1,6-bisphosphate and glucose 6-phosphate are similar.

aldehyde 3-phosphate). This step (or these steps) is therefore at least 10^4 times faster with D-glyceraldehyde 3-phosphate than with the nonphosphorylated aldehydes.

The phosphate substituent also enhances both the rate constant and the specificity of the deacylation step. In experiments with the isolated acyl enzyme, and saturating amounts of NAD, it was found (She and Byers, unpublished experiments) that phosphorolysis of the phosphoglyceroylated enzyme is 750 times faster than phosphorolysis of the propionylated enzyme. Furthermore, phosphorolysis of the phosphoglyceroylated enzyme is 9×10^4 times faster than hydrolysis while phosphorolysis of the propionylated enzyme is only 560 times faster than hydrolysis. In the presence of NAD, hydrolysis of the phosphoglyceroylated enzyme is twice as fast as hydrolysis of the propionylated enzyme, but in the absence of NAD the propionylated enzyme is hydrolyzed 20 times faster than is the phosphoglyceroylated enzyme.

The phosphate substituent on the substrate, D-glyceraldehyde 3-phosphate, and on alkylating reagents alters the activity of glyceraldehyde-3-phosphate dehydrogenase. Glycidol phosphate and chloroacetol phosphate are more reactive than the corresponding nonphosphorylated analogues by 3.8 (±0.1) kcal/mol (Byers, 1977). This reflects a specific interaction of the phosphate moiety with a phosphate binding site on the enzyme and is also observed in binding of aldehydes to the enzyme. The phosphate substituent also enhances the stereoselectivity of the enzymic reaction (Byers, 1978), the conformational mobility of the protein, and the specificity of deacylation (with respect to acyl acceptors) of the acyl enzyme. A dramatic effect of the phosphate substituent is seen in the second-order rate constant (k_{cat}/K_M^{cor}) for the overall oxidative arsenolysis reaction. D-Glyceraldehyde 3-phosphate shows a rate enhancement of 10^4, compared to other aldehydes, in the overall enzymic reaction. This corresponds to a stabilizing effect of the phosphate substituent of 5.4 kcal/mol in the transition state of the rate-limiting step through the first irreversible step in the overall reaction. This suggests a more effective interaction of the phosphate substituent with the enzyme in this transition state than in the ground state. This would indicate an intrinsic binding energy of the phosphate moiety of at least 5.4 kcal/mol.

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