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Reaction of Glyceraldehyde 3-Phosphate Dehydrogenase with Aliphatic Aldehydes*

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ABSTRACT: The reaction of glyceraldehyde 3-phosphate dehydrogenase with a series of aliphatic aldehydes has been studied at 25°. A plot of log V_{max} *vs.* σ^*, the Taft substituent constant, is linear with a slope of 2.08. Thus the rate of the reaction is facilitated by electron withdrawing substituents. Steric factors are of minor importance. Increased steric bulk in the aldehyde did not in general produce significant deviations in the plot of log V_{max} *vs.* σ^*, although there was positive deviation of the points for isovaleraldehyde and isobutyraldehyde. Arsenate had no effect on the rate of the reactions. Trimethylacetyl phosphate is an inhibitor toward these substrates. This inhibition is of the noncompetitive type. Normal inhibition kinetics are observed, plots of $1/V$ *vs.* [I] being linear rather than sigmoidal as is the case when the natural substrate glyceraldehyde 3-phosphate is employed.

D-Glyceraldehyde 3-phosphate dehydrogenase (D-glyceraldehyde 3-phosphate:NAD oxidoreductase (phosphorylating), EC 1.2.1.12) is a key enzyme of carbohydrate metabolism, catalyzing several different reactions depending on the reaction conditions (Colowick *et al.*, 1966). The normal dehydrogenase reaction in the presence of P_i involves the conversion of glyceraldehyde 3-phosphate into 1,3-diphosphoglyceric acid. NAD^+ is required as a cofactor and in the reaction is converted into NADH. In addition, an acyl phosphatase activity has been noted in the presence of NAD^+ (Harting and Velick, 1954; Park and Koshland, 1958; Malhotra and Bernhard, 1968; Phillips and Fife, 1969), and esterase activity has been detected toward phenolic esters with an enzyme from which NAD^+ has been removed (Park *et al.*, 1961). The same thiol ester intermediate is apparently formed in reaction of the enzyme with acetyl phosphate and *p*-nitrophenyl acetate (Mathew *et al.*, 1967). Mechanisms have been suggested for action of the enzyme (Olson and Park, 1964), the evidence pointing strongly to involvement of a thiol group and more ambiguous evidence implicating the imidazole ring of histidine (Halcomb *et al.*, 1968; Friedrich *et al.*, 1964). However, conclusive mechanistic evidence is lacking.

The study of steric effects in α-chymotrypsin-catalyzed reactions has given results that could be directly related to the mechanism of the deacylation reaction (Fife and Milstien, 1967; Milstien and Fife, 1968). Steric effects were also studied in the acyl phosphatase reaction catalyzed by glyceraldehyde 3-phosphate dehydrogenase (Phillips and Fife, 1969), and it was found that branching in the acyl group has a profound influence on the reaction. Also, trimethylacetyl phosphate, although not a substrate, was an excellent inhibitor for both the acetyl phosphate activity and the dehydrogenase reaction involving glyceraldehyde 3-phosphate. For the latter reaction, plots of $1/V$ *vs.* [I] were sigmoidal. In continuing studies of steric effects in reactions catalyzed by this enzyme we have now employed a series of variously substituted aliphatic aldehydes as substrates to obtain information concerning the mechanism of the dehydrogenase reaction and to further ascertain

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TABLE I: Values of V_{max} and K_m for Reaction of Aliphatic Aldehyde Substrates with Glyceraldehyde 3-Phosphate Dehydrogenase at 25°.

Aldehyde	$V_{max} \times 10^7$ Moles/mg of Protein per sec	K_m (M)
Glycolaldehyde	103.1	0.408
Phenylacetaldehyde	21.07	0.185
Propionaldehyde	3.55	0.093
Butyraldehyde	7.47	0.108
Isovaleraldehyde	37.68	0.216
3,3-Dimethylbutyraldehyde	2.77	0.010
Isobutyraldehyde	13.03	0.175
Pivaldehyde	1.67	0.017

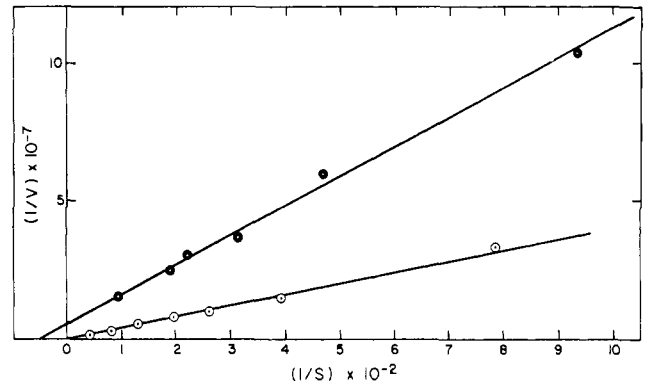


FIGURE 1: Plots of $1/V$ *vs.* 1/[S] for reaction of pivaldehyde (●) and glycolaldehyde (⊙) catalyzed by glyceraldehyde 3-phosphate dehydrogenase at pH 7.85 and 25°. Velocity is expressed in moles of NADH formed per milligram of protein per second.

the nature and scope of the acyl phosphate inhibition. Acetaldehyde, propionaldehyde, and butyraldehyde have previously been studied as substrates for the muscle enzyme (Harting and Velick, 1954). In the presence of P_i the corresponding acyl phosphate was identified as the product of the reaction.

Experimental Section

Materials. The aldehydes employed as substrates were, with the exception of 3,3-dimethylbutyraldehyde, purchased commercially (Matheson Coleman and Bell, Eastman Kodak, and K & K Laboratories) and distilled immediately prior to use. 3,3-Dimethylbutyraldehyde was synthesized by preparing the Grignard reagent from 1-chloro-2,2-dimethylpropane and magnesium and allowing this to react with triethyl orthoformate. The product after acid hydrolysis was distilled and had bp 102°, lit. (Schmerling, 1946) bp 104–105°. The 2,4-dinitrophenylhydrazone of the product melted at 146–147°, lit. (Schmerling, 1946) mp 145–146°. Glycolaldehyde (K & K Laboratories) was recrystallized from acetone.

Glyceraldehyde 3-phosphate dehydrogenase, rabbit muscle enzyme, was obtained from Worthington Biochemical Corp. The enzyme stock solution was prepared as previously reported (Phillips and Fife, 1969). For inhibition experiments Worthington enzyme Code 8GB was employed. This enzyme preparation had slightly less activity than Code 9FA which was used to determine V_{max} and K_m values. The ratio of absorbance at 280 mμ to that at 260 mμ for all enzyme preparations was 1.20 ± 0.05.

Kinetic Measurements. Each reaction was initiated by adding 1.0 ml of an aqueous solution of aldehyde in sodium barbital buffer (0.025 M) to 2.0 ml of solution in the reaction cuvet containing 1.6 ml of buffer, 0.2 ml of enzyme stock solution, 0.1 ml of a 0.4 M sodium arsenate solution, and 0.1 ml of an NAD^+ solution. The complete system contained 0.005 M EDTA and 0.005 M mercaptoethanol. The final pH of the solution was 7.85. The final concentration of enzyme was routinely 2.0 mg of protein/ml, and the added NAD^+ was 2.85×10^{-4} M. The enzyme–buffer mixture was allowed to equilibrate in the cuvet for 15 min at 25° before addition of aldehyde. In the inhibition studies, trimethylacetyl phosphate was included in the equilibrating mixture and Tris buffer was employed at pH 7.85. The reactions were followed by observation of the increase in absorbance at 340 mμ, due to conversion of NAD^+ into NADH, with a Beckman DU spectrophotometer equipped with a Gilford Model 2000 recording attachment. The initial linear portion of the tracing was used to calculate initial velocities. The concentration of NADH was determined using an extinction coefficient of 6.22×10^6 cm^2 mole^{-1} (Horecker and Kornberg, 1948). The least-squares values of the slopes and intercepts of plots of $1/V$ *vs.* 1/[S] were calculated with an Olivetti-Underwood Programma 101. At least six points were used to determine each line.

Results

Plots of $1/V$ *vs.* 1/[S] for the aldehyde substrates at 25° and pH 7.85 were nicely linear as illustrated in Figure 1 for typical examples. Values of V_{max} and K_m are given in Table I. Although 0.013 M arsenate was routinely included in the reaction mixture it was found in separate experiments that the initial velocities were independent of arsenate concentration as seen in Table II. Varying arsenate from 0 to 0.032 M had no effect on the reaction with *n*-butyraldehyde.

The data were correlated by means of the Taft (1956) equation (eq 1), where σ^* is an aliphatic substituent constant

$$\log k/k_0 = \sigma^*\rho^* + \delta E_s \quad (1)$$

reflecting inductive effects and E_s is a steric parameter. In Figure 2 is shown a plot of log V_{max} *vs.* σ^*. The correlation is quite good except for isovaleraldehyde and isobutyraldehyde which show positive deviation. The slope ρ^* is 2.08 (r = 0.982) excluding the points for isovaleraldehyde and isobutyraldehyde. Thus the reaction is enhanced by increased electron withdrawal. A plot of log V_{max} *vs.* E_s showed little correlation. Solution of the complete 4-parameter equation by an IBM 360-40 computer for values of ρ^* and δ that best fit all of the data gave $\rho^* = 1.54$ and $\delta = 0.15$, although the fit of points was not improved when the entire equation was used. The plot of log V_{max} *vs.* σ^* is similar to that obtained

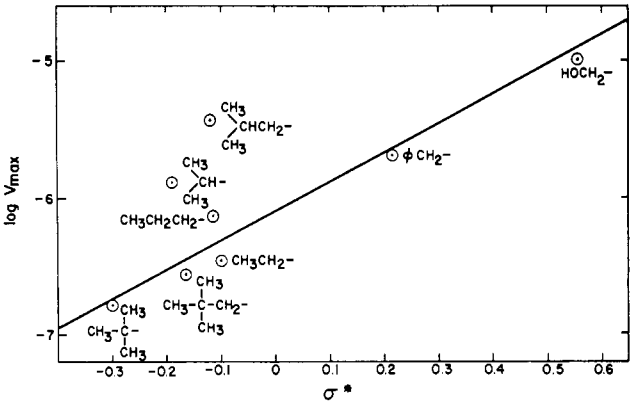


FIGURE 2: Plot of log V_{max} *vs.* σ^* for reaction of aliphatic aldehydes catalyzed by glyceraldehyde 3-phosphate dehydrogenase at pH 7.85 and 25°.

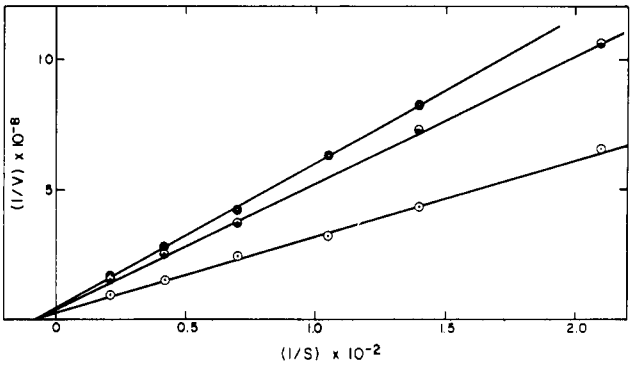


FIGURE 3: Plots of $1/V$ *vs.* 1/[S] for reaction of *n*-butyraldehyde catalyzed by glyceraldehyde 3-phosphate dehydrogenase at pH 7.85 and 25° in the presence of 0.064 M trimethylacetyl phosphate (●), 0.040 M trimethylacetyl phosphate (◒), and in the absence of inhibitor (⊙). Velocity is expressed in moles of NADH formed per milligram of protein per second.

with several of the same aldehydes for reaction catalyzed by an aldehyde dehydrogenase from bovine brain (Erwin and Deitrich, 1966), although in the present case the slope is much greater.

Trimethylacetyl phosphate was found to be an inhibitor of the dehydrogenase reaction with the aliphatic aldehyde substrates *n*-butyraldehyde and pivaldehyde. In Figure 3 is shown a plot of $1/V$ *vs.* 1/[S] for *n*-butyraldehyde at two inhibitor concentrations. The inhibition is of the noncompetitive type. The value of K_i calculated from the plot of Figure 3 is 0.069 M. With pivaldehyde as the substrate the K_i for trimethylacetyl phosphate inhibition is 0.098 M. Figure 4 shows a plot of $1/V$ *vs.* [I] at fixed concentrations of either *n*-butyraldehyde or pivaldehyde. It can be seen that the plot is linear for both substrates.

TABLE II: Effect of Arsenate on the Reaction of Aliphatic Aldehydes with Glyceraldehyde 3-Phosphate Dehydrogenase at 25°.

Arsenate (M)	*n*-Butyraldehyde[a] $V \times 10^8$ Moles/mg of Protein per sec	Propionaldehyde[b] $V \times 10^8$ Moles/mg of Protein per sec	Pivaldehyde[c] $V \times 10^8$ Moles/mg of Protein per sec
0	7.03	4.05	4.55
0.00083	6.94		
0.00167	7.58	4.06	4.28
0.0033	7.57		
0.005	7.47		4.48
0.0167	7.23	3.97	4.02
0.032	6.89		

[a] Aldehyde concentration was 0.0107 M and enzyme concentration was 2.009 mg/ml. [b] Aldehyde concentration was 0.00920 M and enzyme concentration was 2.033 mg/ml. [c] Aldehyde concentration was 0.0199 M and enzyme concentration was 2.033 mg/ml.

Discussion

The reaction scheme followed in reactions catalyzed by glyceraldehyde 3-phosphate dehydrogenase involves formation of a thiol ester intermediate (Mathew *et al.*, 1967) as in eq 2, where ES is an enzyme–substrate complex and

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES' \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P \qquad (2)$$

ES′ is the intermediate acyl-enzyme. The deacylation step is undoubtedly rate determining in the acyl phosphatase reaction with acyl phosphates following Michaelis–Menten kinetics and in the dehydrogenase reaction with D-glyceraldehyde 3-phosphate as the substrate. In both reactions arsenate has a large rate accelerating effect (Phillips and Fife, 1969). This most likely results from an enhancement in the rate of the deacylation step. The effect of steric bulk in the acyl group is quite large in the acyl phosphatase reaction as would be expected for a reaction involving hydrolysis

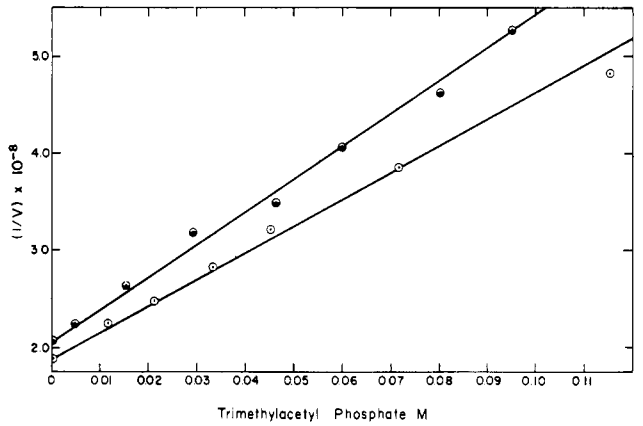
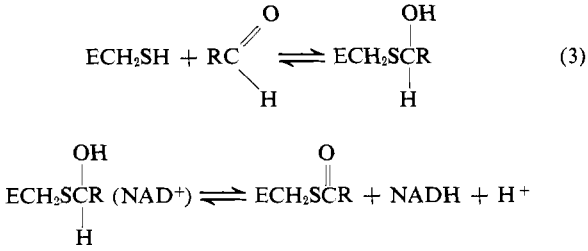


FIGURE 4: Plots of $1/V$ *vs.* trimethylacetyl phosphate concentration for reaction of 0.0134 M *n*-butyraldehyde (⊙) and 0.0106 M pivaldehyde (◒) catalyzed by glyceraldehyde 3-phosphate dehydrogenase at pH 7.85 and 25°. Velocity is expressed in moles of NADH formed per milligram of protein per second.

of an acyl-enzyme thiolester intermediate. The value of δ in a Taft steric effects plot of log k_3 *vs.* E_s was 1.1 although the highly branched acyl phosphates, trimethylacetyl phosphate and 3,3-dimethylbutyryl phosphate, could not be included since they are not substrates.

In the dehydrogenase reaction with aliphatic aldehyde substrates at the high enzyme concentration of 2 mg/ml, arsenate has no effect on the rate of the reaction. Harting and Velick (1954) previously noted that with *n*-butyraldehyde and propionaldehyde as substrates the amount of NADH measured 24 min after initiation of the reaction was increased by either arsenate or P_i. With glyceraldehyde and a high concentration of muscle glyceraldehyde 3-phosphate dehydrogenase the extent of the reaction was also found to be altered in the presence of P_i, but the initial velocity was not affected (Velick and Hayes, 1953). The identical initial velocities in the presence or absence of arsenate or inorganic phosphate are most likely due to a lack of effect of these ions on the rates of acylation.

Steric effects are of little importance in regard to the magnitude of V_{max}. This must result from the measured initial velocities being those of the acylation process. The predominant influence appears to be a polar effect. A plot of log V_{max} *vs.* σ^*, the Taft substituent constant, is linear with a slope of 2.08, indicating enhancement of the rate by increased electron withdrawal from the carbonyl group of the aldehyde. A plausible scheme is given in eq 3. The



(3)

size of the acyl group should not have great effect on the rate of formation of thiolester. It has been found that such steric effects have small influence on the equilibrium constant for addition of thiols to aldehydes (Lienhard and Jencks, 1966). However, polar effects should have considerable influence, and it would be expected that increased electron withdrawal in the R group would facilitate the addition reaction. The transfer of hydrogen from the hemithioacetal intermediate to NAD^+ would be made more difficult by increased electron withdrawal. Electron withdrawal would also hinder dehydration of the aldehyde hydrate. Thus, addition of the essential thiol to aldehyde, a reaction which would be susceptible to polar effects, is undoubtedly important in determining the magnitude and sign of ρ^*.

Trimethylacetyl phosphate is a good inhibitor toward the natural substrate glyceraldehyde 3-phosphate (Phillips and Fife, 1969) and also toward the aldehydes employed in the present study. This inhibition is of the noncompetitive type (Figure 3). However, a plot of $1/V$ *vs.* [I] with glyceraldehyde 3-phosphate was sigmoidal, implying a cooperativity effect in binding of the acyl phosphate to the enzyme. In the present case a similar plot of $1/V$ *vs.* [I] is linear with either *n*-butyraldehyde or pivaldehyde as the substrate, indicating that normal Michaelis–Menten kinetics are being followed. Thus it is evident that the different binding sites for aldehydes and acyl phosphates exert a subtle influence on each other with cooperativity effects clearly detectable only when the proper type of substrate is employed which binds to the other site. This interpretation is consistent with the fact that cooperative binding effects with an acyl phosphate like isobutyryl phosphate are not seen in the acyl phosphatase reaction for which it is a substrate but are only seen when it is employed as an inhibitor in the dehydrogenase reaction of glyceraldehyde 3-phosphate. The critical feature in promoting the sigmoidal inhibition plots may therefore be the presence of a phosphate group in the aldehyde substrate which can bind to the enzyme. It is noteworthy that maximum trimethylacetyl phosphate inhibition occurs at an inhibitor concentration of approximately 0.003 M when glyceraldehyde 3-phosphate is the substrate whereas with butyraldehyde and pivaldehyde the plot of $1/V$ *vs.* [I] is still linear at an inhibitor concentration of 0.1 M. In the reaction with glyceraldehyde 3-phosphate, deacylation of the acyl-enzyme intermediate is most likely rate limiting and is the reaction being followed at the low enzyme concentrations employed (Phillips and Fife, 1969), whereas with the present series of aldehydes it is acylation that is being observed at high enzyme concentration. Therefore, it is also possible that the different types of $1/V$ *vs.* [I] plots for trimethylacetyl phosphate inhibition are due to different kinetic effects of the inhibitor on the acylation and deacylation reactions.

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