Langford, C. H., and Gray, H. B. (1965), Ligand Substitution Processes, New York, N. Y., Benjamin, pp 1–16.
Lindskog, S. (1963), *J. Biol. Chem. 238*, 945.
Lindskog, S. (1966), *Biochemistry 5*, 2641.
Lindskog, S., and Ehrenberg, A. (1967), *J. Mol. Biol. 24*, 133.
Lindskog, S., and Nyman, P. O. (1964), *Biochim. Biophys. Acta 85*, 462.
Luz, Z., and Meiboom, S. (1964a), *J. Chem. Phys. 40*, 1058.
Luz, Z., and Meiboom, S. (1964b), *J. Chem. Phys. 40*, 2686.
Margerum, D. W., Rohrabacher, D. B., and Clarke, J. F. G. (1967), *Inorg. Chem. 2*, 667.
Margerum, D. W., and Rosen, H. M. (1967), *J. Amer. Chem. Soc. 89*, 1088.
Melson, G. A., and Wilkens, R. G. (1962), *J. Chem. Soc.*, 4208.
Navon, G., Shulman, R. G., Wyluda, B. J., and Yamane, T. (1970), *J. Mol. Biol. 51*, 15.
Peacocke, A. R., Richards, R. E., and Sheard, B. (1969), *Mol. Phys. 16*, 177.
Pearson, R. G., and Lanier, R. D. (1964), *J. Amer. Chem. Soc. 86*, 765.
Pearson, R. G., Palmer, J., Anderson, M. M., Allred, A. L. (1960), *Z. Electrochem. 64*, 110.
Pocker, Y., and Dickerson, D. G. (1968), *Biochemistry 7*, 1995.
Pocker, Y., and Stone, J. T. (1967), *Biochemistry 6*, 668.
Riepe, M. E., and Wang, J. H. (1968), *J. Biol. Chem. 243*, 2779.
Roughton, F. J. W., and Booth, V. H. (1946), *Biochem. J. 40*, 319.
Swift, T. J., and Connick, R. E. (1962), *J. Chem. Phys. 37*, 307.
Taylor, J. S., Mushak, P., and Coleman, J. E. (1970a), *Proc. Nat. Acad. Sci. U. S. 67*, 1410.
Taylor, P. W., and Burgen, A. S. V. (1971), *Biochemistry 10*, 3859.
Taylor, P. W., King, R. W., and Burgen, A. S. V. (1970b), *Biochemistry 9*, 2638.
Taylor, P. W., King, R. W., and Burgen, A. S. V. (1970c), *Biochemistry 9*, 3894.
Thorslund, A., and Lindskog, S. (1968), *Eur. J. Biochem. 3*, 117.
Verpoorte, J. A., Mehta, S., and Edsall, J. T. (1967), *J. Biol. Chem. 242*, 4221.
Wang, J. H. (1970), *Proc. Nat. Acad. Sci. U. S. 16*, 874.
Ward, R. L. (1969), *Biochemistry 8*, 1879.
Ward, R. L. (1970), *Biochemistry 9*, 2447.

Reaction of Aromatic Aldehydes with Glyceraldehyde 3-Phosphate Dehydrogenase*

Thomas H. Fife, Tadaaki Rikihisa,† and Bruce M. Benjamin‡

ABSTRACT: The reaction of glyceraldehyde 3-phosphate dehydrogenase with a series of aromatic aldehydes has been studied at 25° by following the appearance of NADH. A plot of log (V_{max}/K_m) *vs.* σ, the Hammett substituent constant, is reasonably linear with a slope of 1.24. A plot of log V_{max} *vs.* σ has a slope of 1.27. Thus, the rate of the reaction is facilitated by electron-withdrawing substituents. Trimethylacetyl phosphate is an inhibitor toward benzaldehyde. A plot of $1/V$ *vs.* (I) is linear in the presence or absence of arsenate. In the absence of arsenate, trimethylacetyl phosphate is also an inhibitor for reaction of cinnamaldehyde with the enzyme, but in that case a plot of $1/V$ *vs.* (I) is sigmoidal. In the presence of 0.013 M arsenate, trimethylacetyl phosphate is an inhibitor toward cinnamaldehyde only at concentrations greater than required for maximum inhibition in the absence of arsenate, and a plot of $1/V$ *vs.* (I) is linear.

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 inorganic phosphate involves the conversion of D-glyceraldehyde 3-phosphate into 3-phosphoglyceroyl phosphate. In the presence of arsenate the product is 3-phospho-D-glyceric 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).

Trimethylacetyl phosphate, although not a substrate, is an excellent inhibitor for the acetyl phosphatase activity and the dehydrogenase reaction involving glyceraldehyde 3-phosphate (Phillips and Fife, 1969). In the latter reaction, plots of $1/V$ *vs.* (I) were sigmoidal, but when aliphatic aldehydes were employed as substrates such plots were linear even at relatively high concentrations of inhibitor (Fife and Rikihisa, 1970). To determine the factors of critical importance in acyl phosphate inhibition and to obtain further information

* From the Department of Biochemistry, University of Southern California, Los Angeles, California. *Received December 3, 1970.* This work was supported by a research grant from the National Institutes of Health.

† Postdoctoral Fellow, Department of Biochemistry, University of Southern California.

‡ National Institutes of Health Predoctoral Fellow.

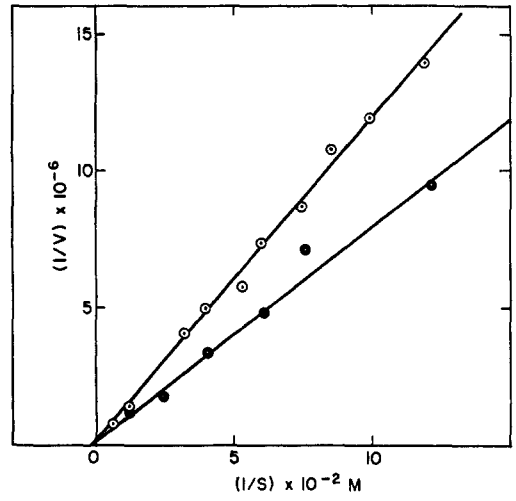


FIGURE 1: Plots of $1/V$ *vs.* 1/(S) for reaction of *p*-cyanobenzaldehyde (⊙) and *p*-nitrobenzaldehyde (●) with glyceraldehyde 3-phosphate dehydrogenase at 25°. Velocity is expressed as moles of NADH per milligram of protein per second.

concerning the mechanism of the dehydrogenase reaction, the reaction of the enzyme with a series of aromatic aldehydes has now been studied.

Experimental Section

Materials. The aldehydes employed as substrates were purchased commercially (Matheson Coleman & Bell, Eastman Kodak, and Aldrich Chemical Co.) and were distilled or recrystallized immediately prior to use. Trimethylacetyl phosphate was prepared as previously reported (Phillips and Fife, 1968).

Glyceraldehyde 3-phosphate dehydrogenase, rabbit muscle enzyme, was obtained from Worthington Biochemical Co., code 9FA. The enzyme stock solution was prepared as previously reported (Phillips and Fife, 1969). The ratio of absorbance at 280 mμ to that at 260 mμ for all enzyme preparations was 1.05.

Kinetic Measurements. In the oxidation of substituted benzaldehydes, each reaction was initiated by adding 0.1 ml of aldehyde in acetonitrile to 2.9 ml of solution in the reaction cuvet containing 2.6 ml of 0.15 M pyrophosphate buffer with 0.019 M mercaptoethanol and 0.005 M EDTA, 0.1 ml of 0.4 M arsenate in H_2O, 0.1 ml of NAD^+ solution in H_2O, and 0.1 ml of enzyme stock solution. The complete system contained 3.07×10^{-4} M NAD^+ and was 3.33% acetonitrile. The final pH was 8.54. With cinnamaldehyde as the substrate, 0.025 M sodium barbital buffer was employed. The final pH was 7.85, and the complete system contained 2.95×10^{-4} M NAD^+. Total enzyme concentration was 0.9 mg/ml.

The initial velocity of the reaction of the substituted benzaldehydes was determined by measuring the increase in absorbance at 340 mμ due to the NADH product. The extinction coefficient of NADH at 340 mμ was taken to be 6.22×10^6 cm²/mole (Horecker and Kornberg, 1948). In the case of *p*-nitrobenzaldehyde, the initial rate of NADH formation was measured at 360 mμ because of the absorption of the aldehyde substrate at 340 mμ. The extinction coefficient of NADH at 360 mμ was found to be 4.27×10^3.

With cinnamaldehyde as the substrate, the reaction was followed at 340 mμ as with the other aldehydes and also at 310 mμ where the observed absorbance change was partially due to formation of a thiol ester intermediate. Identical results were obtained at the two wavelengths. The high absorbance of cinnamaldehyde at 281 mμ, the isosbestic point of NAD^+ and NADH, precluded work at that wavelength. The absorbance maximum of cinnamoylcysteine has been found previously to be at 306 mμ (ε 22,600) and is 326 mμ for cinnamoyl-papain which is a thiol ester (Brubacher and Bender, 1966). The spectra of NADH and the cinnamoyl-enzyme therefore overlap. As a consequence, the initial velocity was calculated as the change in absorbance, which is proportional to product formed, per minute. The rate of the rapid initial increase in absorbance decreases with time. After 30–40 min when (S_0) is greater than (E_0), a slow linear increase is then obtained. Extrapolating this linear increase to zero time and employing the extinction coefficients of NADH and cinnamoylcysteine, with the assumption that the latter is the same as with the cinnamoyl-enzyme, it can be calculated that at the highest substrate concentration employed approximately 61% of an equivalent of the enzyme is being acylated in the initial reaction. When equimolar quantities of substrate and enzyme (1.5×10^{-5} M) were reacted, the slow linear increase in absorbance after the initial reaction was not observed, the absorbance becoming constant. A calculation then indicated that at completion of the reaction the ratio of acyl groups per tetramer was 1.4. Addition of 0.013 M arsenate at this point produced a slow decrease in absorbance at 310 mμ.

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

Aldehyde	$(V_{max}/K_m)^a \times 10^6$	$V_{max} \times 10^{7\,a}$	K_m (M)
p-Methoxybenzaldehyde	4.02	4.82	0.120
p-Methylbenzaldehyde	11.61	2.09	0.018
Benzaldehyde	18.67	9.71	0.052
p-Chlorobenzaldehyde	24.10	8.95	0.037
p-Cyanobenzaldehyde	84.40	81.9	0.097
p-Nitrobenzaldehyde	127.4	44.6	0.035

[a] Velocity is expressed as moles of NADH formed per milligram of protein per second.

Results

Plots of $1/V$ *vs.* 1/(S) for the aromatic aldehyde substrates at an enzyme concentration of 0.9 mg/ml were nicely linear as seen in Figure 1 for typical examples. From the least-squares values of the slopes of these plots (V_{max}/K_m), values were calculated which are reported in Table I. Also given in Table I are values of V_{max} and K_m calculated from the slopes and intercepts of the plots of $1/V$ *vs.* 1/(S). In all cases, low solubility of the substituted benzaldehydes prevented the employment of substrate concentrations greater than K_m. This problem was most serious with the *p*-chloro derivative, where the maximum substrate concentration was only 14% of the apparent K_m. With the other compounds in the series, there was good agreement between values obtained from the plots of $1/V$ *vs.* 1/(S) and plots of (S)/V *vs.* (S), where $1/V_{max}$ is obtained as the slope. Arsenate (0.013 M) was routinely included in these reactions, but it was found in separate

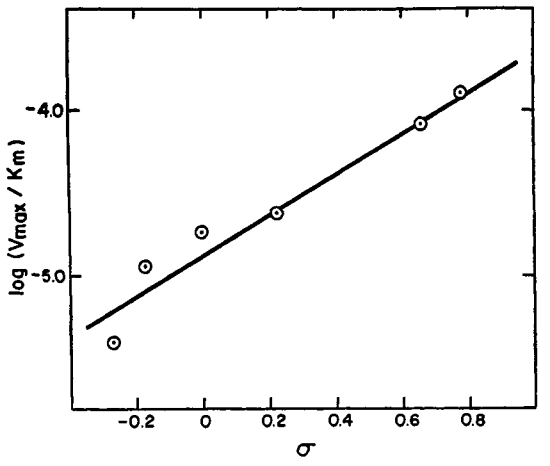


FIGURE 2: Plot of log (V_{max}/K_m) for reaction of aromatic aldehydes with glyceraldehyde 3-phosphate dehydrogenase at 25° *vs.* σ.

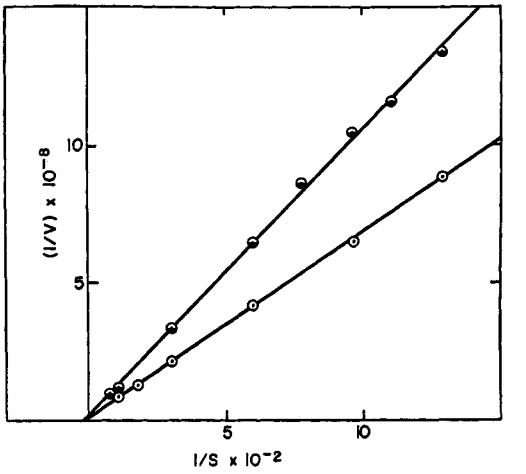


FIGURE 3: Plots of $1/V$ *vs.* 1/(S) for reaction of benzaldehyde with glyceraldehyde 3-phosphate dehydrogenase at 25° in the presence of 0.046 M trimethylacetyl phosphate (⊖) and in the absence of acyl phosphate (⊙). Velocity is expressed as moles of NADH per milligram of protein per second.

experiments that its presence had no effect on the initial velocities. In Figure 2 a plot is shown of log (V_{max}/K_m) *vs.* σ, the Hammett substituent constant (Hammett, 1940). The slope, ρ, of the least-squares line is 1.24 with a correlation coefficient of 0.974. K_m does not vary greatly in this series of para-substituted benzaldehydes. A plot of log V_{max} *vs.* σ had a slope of 1.27 with a correlation coefficient of 0.923.

The effect of varying the concentration of added NAD^+ was determined employing benzaldehyde as the substrate. With an enzyme concentration of 0.95 mg/ml and an added NAD^+ concentration of 5.13×10^{-5} M, as compared to 3.07×10^{-4} M normally employed, V_{max} was 7.51×10^{-7} mole/mg of protein per sec and the value of K_m for benzaldehyde was 5.25×10^{-2} M. These values are in reasonable accord with those obtained with the higher concentration of added NAD^+. Variation of added NAD^+ at constant benzaldehyde concentrations of 1.31×10^{-2} and 6.15×10^{-3} M gave K_m values for NAD^+ of 2.28×10^{-5} and 1.52×10^{-5} M, respectively. In these reactions, of course, the concentration of benzaldehyde was not saturating. A K_m value of 1.3×10^{-5} M has been reported for NAD^+ at pH 8.6 and 26° when glyceraldehyde 3-phosphate is the other substrate (Furfine and Velick, 1965).

Trimethylacetyl phosphate is an inhibitor for reaction of these aldehydes with glyceraldehyde 3-phosphate dehydrogenase. In Figure 3, a plot is shown of $1/V$ *vs.* 1/(S) employing benzaldehyde as the substrate with constant concentrations of trimethylacetyl phosphate inhibitor. The plot of $1/V$ *vs.* (I) at constant substrate concentration in Figure 4 is linear. Trimethylacetyl phosphate also gave a linear plot of $1/V$ *vs.* (I) in the absence of arsenate.

Cinnamaldehyde is a good substrate for glyceraldehyde 3-phosphate dehydrogenase. The increase in absorbance was followed at 310 mμ as well as at higher wavelengths at (S_0) > (E_0). This substrate will give rise to a thiol ester intermediate which should absorb strongly at 310 mμ. Employing only the initial portion of these data, initial rates were calculated which gave linear plots of $1/V$ *vs.* 1/(S) with a K_m value identical with that obtained from initial rate data at higher wavelength where the observed reaction is predominantly the conversion of NAD^+ into NADH. The value of K_m in the absence of arsenate, calculated from the plot of $1/V$ *vs.* 1/(S) in Figure 5, is 0.005 M. In the presence of 0.013 M arsenate, K_m had the value 0.0016 M. At low concentrations, arsenate exerts a small accelerating effect on the initial velocity. This effect is maximal at an arsenate concentration of approximately 0.002 M which gives a twofold enhancement in the rate when cinnamaldehyde concentration is 0.0015 M. With arsenate concentrations greater than 0.002 M an inhibitory effect could be detected.

Trimethylacetyl phosphate is also a good inhibitor of the reaction when cinnamaldehyde is the substrate. A plot of $1/V$ *vs.* 1/(S) at a constant inhibitor concentration is shown in Figure 5. This plot is linear with the line extrapolating to the same point on the abscissa axis as in the absence of inhibitor. A plot of $1/V$ *vs.* (I) at a constant substrate concentration in Figure 6 is sigmoidal with maximum inhibition at an inhibitor concentration of approximately 0.002 M. The data in Figures 5 and 6 were obtained in the absence of arsenate. However, in the presence of 0.013 M arsenate, trimethylacetyl phosphate had no detectable inhibitory effect at concentrations as low

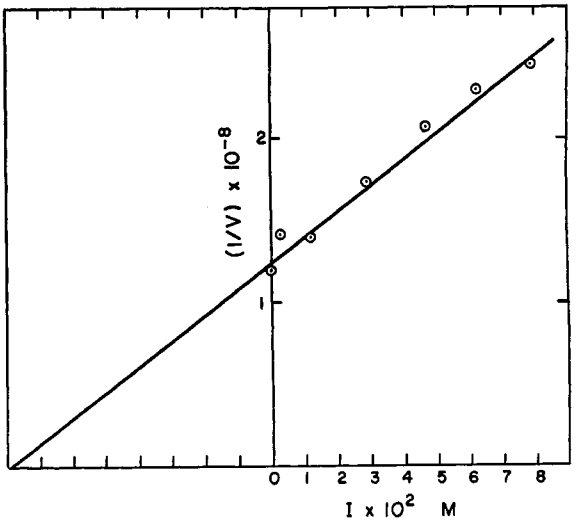


FIGURE 4: Plot of $1/V$ *vs.* trimethylacetyl phosphate concentration (M) for reaction of 5.66×10^{-3} M benzaldehyde with glyceraldehyde 3-phosphate dehydrogenase at 25°. Velocity is expressed as moles of NADH per milligram of protein per second.

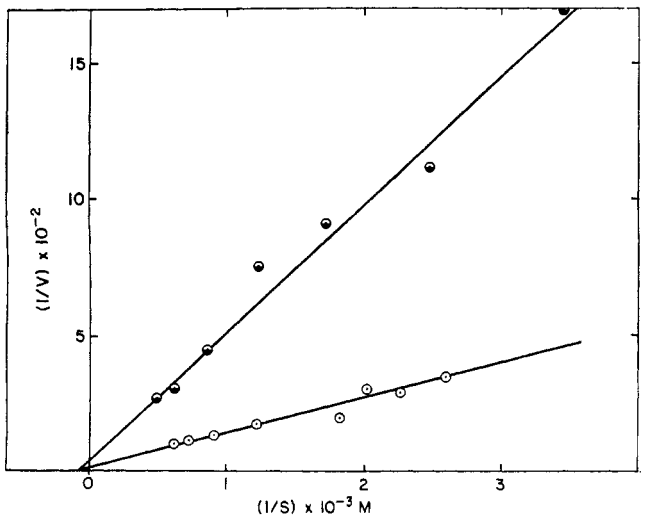


FIGURE 5: Plots of $1/V$ *vs.* 1/(S) for reaction of cinnamaldehyde with glyceraldehyde 3-phosphate dehydrogenase at 25° in the presence of 5.65×10^{-3} M trimethylacetyl phosphate (◒) and in the absence of acyl phosphate (○). Velocity is expressed as the change in absorbance at 310 mμ per minute at an enzyme concentration of 0.90 mg/ml.

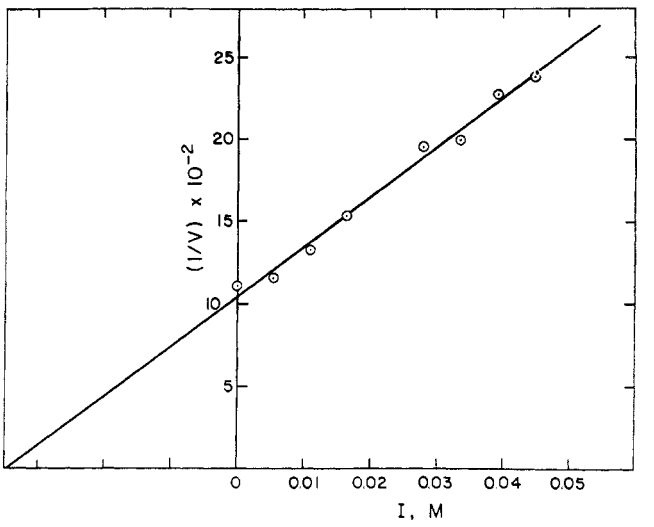


FIGURE 7: Plot of $1/V$ *vs.* trimethylacetyl phosphate concentration (M) for reaction of 3.56×10^{-4} M cinnamaldehyde with glyceraldehyde 3-phosphate dehydrogenase at 25° in the presence of 0.013 M arsenate. Velocity is expressed as the change in absorbance at 310 mμ per minute at an enzyme concentration of 0.90 mg/ml.

as 0.002 M. At higher concentrations inhibition did occur in the presence of 0.013 M arsenate, and as seen in Figure 7, a plot of $1/V$ *vs.* (I) is linear. It will be noted in Figures 6 and 7 that the velocity at zero inhibitor concentration is less in the presence of 0.013 M arsenate, illustrating the inhibitory effect of arsenate at concentrations larger than 0.002 M.

Discussion

A plot of log V_{max} for reaction of a series of aliphatic aldehydes, RCHO, with glyceraldehyde 3-phosphate dehydrogenase *vs.* σ^*, the Taft substituent constant, had a slope of +2.08 (Fife and Rikihisa, 1970). Thus, inductive electron withdrawal in the R group facilitates the reaction. Steric factors were of minor influence on V_{max}. The observed reaction at the high enzyme concentration of 2 mg/ml corresponded to oxidation of the substrate with formation of a thiol ester acyl-enzyme intermediate. It was found that K_m values for these compounds were large and were dependent on the structure of the aldehyde, the lowest being those for the highly branched compounds, pivaldehyde and 3,3-dimethylbutyraldehyde. With the present series of benzaldehydes having substituent groups in the para position, the K_m values are still fairly high but do not vary greatly as the para substituent is changed. A reasonably linear plot is obtained of log (V_{max}/K_m) *vs.* σ, the Hammett substituent constant, with a slope, ρ, of +1.24. A plot of log V_{max} *vs.* σ has a slope of 1.27. Thus, as with the aliphatic substrates, increased electron withdrawal in the R group accelerates the rate of the oxidation reaction. The most likely reaction scheme is that in eq 1.

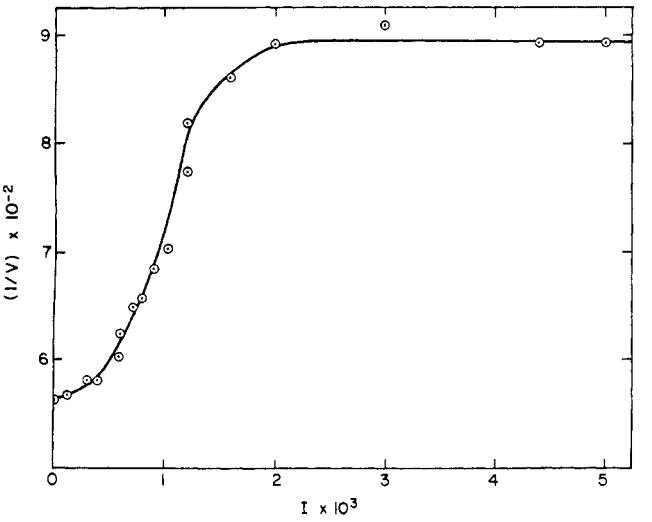
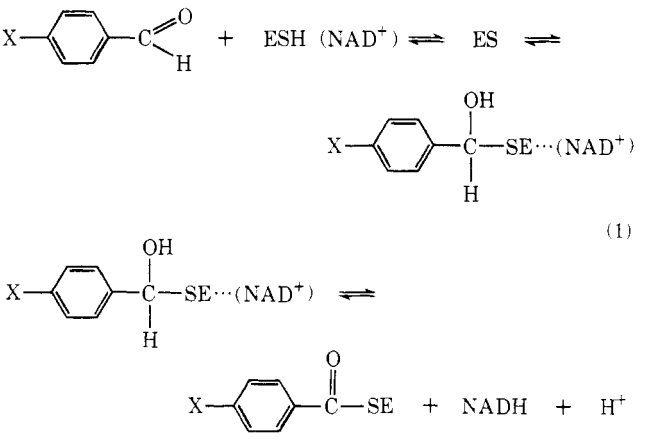


FIGURE 6: Plot of $1/V$ *vs.* trimethylacetyl phosphate concentration (M) for reaction of 3.56×10^{-4} M cinnamaldehyde with glyceraldehyde 3-phosphate dehydrogenase at 25° in the absence of arsenate. Velocity is expressed as the change in absorbance at 310 mμ per minute at an enzyme concentration of 0.90 mg/ml.



In the reaction of the aromatic aldehydes, at the high enzyme concentration of 0.9 mg/ml (6.4×10^{-6} M taking the molecular weight as 140,000 (Fox and Dandliker, 1956)) the enzyme is a stoichiometric participant in the reaction. It is unlikely in the present study with the benzaldehyde derivatives

that the initial velocity measurements are being affected by deacylation, since arsenate, which would enhance deacylation, has no effect on velocity. Arsenate at concentrations less than 0.002 M does have a small rate accelerating influence in the reaction with cinnamaldehyde, but at higher concentrations it inhibits the reaction.

Reaction of equimolar concentrations of cinnamaldehyde and enzyme when arsenate is not present produces an increase in absorbance at 310 mμ until a constant value is reached. The constancy of absorbance indicates that the acyl-enzyme is reasonably stable under these conditions. The electronically similar β-(2-furyl)acryloyl-enzyme is also reasonably stable in the absence of arsenate or phosphate (Malhotra and Bernhard, 1968). Without arsenate or other external acyl group acceptors, the initial velocity for acyl-enzyme formation should be unaffected by slow hydrolysis of the acyl-enzyme. Thus the data for cinnamaldehyde in the absence of arsenate (Figures 5 and 6) strictly relate to the oxidation reaction.

The concentration of added NAD^+ is considerably greater than its K_m. A sixfold decrease in the amount of added NAD^+ from that normally employed produced no significant change in V_{max} and K_m values for benzaldehyde. Therefore, the V_{max} and K_m values in Table I are those at saturating concentrations of NAD^+. None of the aliphatic aldehydes or substituted benzaldehydes are good substrates for the enzyme, the lowest K_m being 10^{-2} M in the aliphatic series for 3,3-dimethylbutyraldehyde and 1.79×10^{-2} M for *p*-methylbenzaldehyde among the aromatic compounds. In the case of cinnamaldehyde, however, the K_m is considerably less, 5×10^{-3} M in the absence of arsenate and 1.6×10^{-3} M in the presence of 0.013 M arsenate. Keleti (1966) previously observed that in the oxidation of glyceraldehyde the apparent Michaelis constants are influenced by the presence of arsenate.

In accord with the relative unimportance of steric effects on V_{max} values (Fife and Rikihisa, 1970), it would appear that the observed facilitation of the rate by increased electron withdrawal in the aldehyde substrate is primarily reflecting the influence of substituent groups on formation of the hemithioacetal intermediate. Steric effects in the aldehyde have small influence on the equilibrium constant for addition of thiols to aldehydes (Lienhard and Jencks, 1966), but electron withdrawal should enhance the addition reaction. On the other hand, increased electron withdrawal would make more difficult the transfer of hydrogen from the hemithioacetal to NAD^+ and would also hinder dehydration of the hydrated aldehydes.

Trimethylacetyl phosphate is an inhibitor for reaction of the enzyme with aromatic aldehydes as was previously found to be the case with aliphatic aldehydes (Fife and Rikihisa, 1970) and the natural substrate glyceraldehyde 3-phosphate (Phillips and Fife, 1969). The form of the inhibition is noncompetitive in these cases. The plot of $1/V$ *vs.* (I) (Figure 4) for inhibition of the reaction with benzaldehyde is linear. A linear plot of $1/V$ *vs.* (I) is also found for trimethylacetyl phosphate inhibition when aliphatic aldehydes are the substrates, but with glyceraldehyde 3-phosphate a sigmoidal plot was obtained with maximum inhibition at the relatively low acyl phosphate concentration of 0.003 M. In the absence of arsenate, as seen in Figure 6, when inhibitor concentration is varied at constant cinnamaldehyde concentration a plot of $1/V$ *vs.* (I) is definitely sigmoidal, showing maximum effect of inhibitor at low concentration (0.002 M).

The enzyme is a tetramer consisting of identical subunits (Harris and Perham, 1965). A possible interpretation of the sigmoidal $1/V$ *vs.* (I) plots with glyceraldehyde 3-phosphate and cinnamaldehyde therefore is that with these substrates binding of acyl phosphate to one subunit induces or stabilizes a conformation change which is unfavorable for maximum velocity and which enhances further binding of acyl phosphate until a maximum concentration is reached. With benzaldehyde and the aliphatic aldehydes, however, there is no evidence for this cooperative effect since the plots of $1/V$ *vs.* (I) are linear. Thus, the sigmoidal plots are dependent on the aldehyde substrate.

Arsenate has a pronounced effect on trimethylacetyl phosphate inhibition of the reaction with cinnamaldehyde. At an arsenate concentration of 0.013 M, the acyl phosphate is an inhibitor at concentrations greater than necessary for maximum inhibition in the absence of arsenate, but a plot of $1/V$ *vs.* (I) is linear at inhibitor concentrations as high as 0.045 M. In contrast, arsenate has little effect on inhibition of the reaction with benzaldehyde. If binding of the acyl phosphate is contributing to a conformation change, then it is clear that with cinnamaldehyde as the substrate, arsenate can alter this effect, although inhibition is not overcome. Since deacylation is enhanced by arsenate, it is possible that more rapid turnover of the enzyme could reduce the effectiveness of low concentrations of inhibitor, but this is unlikely since 0.013 M arsenate actually has an inhibitory effect on the observed initial velocity in the absence of acyl phosphate. Arsenate may have great influence on the conformation of the enzyme, thereby modifying acyl phosphate inhibition. A possibility is that in the presence of arsenate only a relatively small conformation change can be induced by binding of acyl phosphate to one subunit with cooperativity then not detectable.

In the case of glyceraldehyde 3-phosphate as the substrate, low enzyme concentrations were employed and deacylation is rate determining. It is now evident that the acylation process at high enzyme concentrations can with the proper substrate give sigmoidal plots of $1/V$ *vs.* (I). It is also clear, in view of the noncompetitive nature of the inhibition and the different types of inhibition plots which depend on the aldehyde and the presence or absence of arsenate, that the sigmoidal $1/V$ *vs.* (I) plots must result because of a subtle indirect interaction between sites.

References

Brubacher, L. J., and Bender, M. L. (1966), *J. Amer. Chem. Soc. 88*, 5871.

Colowick, S. P., van Eys, J., and Park, J. H. (1966), *Compr. Biochem. 14*, 63.

Fife, T. H., and Rikihisa, T. (1970), *Biochemistry 9*, 4064.

Fox, J. B., Jr., and Dandliker, W. B. (1956), *J. Biol. Chem. 218*, 53.

Furfine, C. S., and Velick, S. F. (1965), *J. Biol. Chem. 240*, 844.

Hammett, L. P. (1940), Physical Organic Chemistry, New York, N. Y., McGraw-Hill Book Co., Chapter VII.

Harris, J. I., and Perham, R. N. (1965), *J. Mol. Biol. 13*, 876.

Harting, J., and Velick, S. F. (1954), *J. Biol. Chem. 207*, 867.

Horecker, B. L., and Kornberg, A. (1948), *J. Biol. Chem. 175*, 385.

Keleti, T. (1966), *Acta Physiol. Acad. Sci. Hung. 29*, 101.

Lienhard, G. E., and Jencks, W. P. (1966), *J. Amer. Chem. Soc. 88*, 3982.

Malhotra, O. P., and Bernhard, S. A. (1968), *J. Biol. Chem. 243*, 1243.

Mathew, E., Meriwether, B. P., and Park, J. H. (1967), *J.*

Biol. Chem. 242, 5024.
Park, J. H., and Koshland, D. E., Jr. (1958), *J. Biol. Chem. 233*, 986.
Park, J. H., Meriwether, B. P., Clodfelder, P., and Cunningham, L. W. (1961), *J. Biol. Chem. 236*, 136.
Phillips, D. R., and Fife, T. H. (1968), *J. Amer. Chem. Soc. 90*, 6803.
Phillips, D. R., and Fife, T. H. (1969), *Biochemistry 8*, 3114.

Binding of Transition Metal Ions by Ceruloplasmin (Ferroxidase)*

David J. McKee† and Earl Frieden‡

ABSTRACT: Human ceruloplasmin (ferroxidase, EC 1.12.3.1) contains multiple binding sites for divalent transition metal ions. Equilibrium dialysis studies revealed that besides the 7 Cu ions found in the native protein, ceruloplasmin can bind an additional 10 Cu(II)'s which can be removed by Chelex. In independent and separate experiments, 3 Zn(II), 7 Ni(II), and 16 Co(II) were also bound to native ceruloplasmin. These data suggest that, in addition to the two types of copper found in the native protein, there are three other classes of binding sites in ceruloplasmin: (1) one site which is frequently occupied by Cu(II) during isolation of the enzyme; (2) two sites which serve as a substrate binding site for Fe(II) and which may bind Zn(II) and Ni(II); and (3) multiple nonspecific sites with the smallest association constants. The binding constants of these transition metal ions decrease in the order Cu(II) > Zn(II) > Fe(II) > Ni(II) > Co(II). Ferroxidase activity inhibition constants calculated for Zn(II), Ni(II), and Co(II) ions compare satisfactorily with their respective binding constants.

Ceruloplasmin (ferroxidase, EC 1.12.3.1) (Osaki *et al.*, 1966) has been known to contain copper since it was first isolated by Holmberg and Laurell (1944). The exact number of copper binding sites, however, has been in question due to the considerable variation in copper content and molecular weight reported in the past two decades (Pedersen, 1951; Kasper and Deutsch, 1963; Magdoff-Fairchild *et al.*, 1969). McDermott *et al.* (1968) defined the role of iron in ferroxidase but did not determine the binding properties of iron. This study and the investigation of the role of ferroxidase in iron mobilization (Osaki *et al.*, 1966; Ragan *et al.*, 1969; Frieden, 1971) led to the quantitative investigation of iron binding reported here.

Curzon (1960) first showed that low concentrations of Fe(II), Zn(II), Ni(II), and Co(II) activated ceruloplasmin's catalytic oxidation of *N,N*-dimethyl-*p*-phenylenediamine while higher concentrations of these ions showed marked inhibition. Huber and Frieden (1970a,b) have shown that these divalent transition metal ions similarly affected the ferroxidase activity. Both studies suggest the possibility of binding by these divalent ions to ceruloplasmin and prompted the study of the binding of these ions and a correlation with their inhibition data.

Materials and Methods

Preparation of Ceruloplasmin. Human ceruloplasmin (ferroxidase) was purified from a Cohn IV paste (Cohn *et al.*, 1946) essentially according to the method of Deutsch *et al.* (1962) using DEAE-Sephadex in the chromatography steps. The crystalline blue protein was dissolved in 0.10 M sodium acetate (pH 6.0) and showed an $A_{280}:A_{610}$ ratio of less than 23. The protein was immediately stored in acid-washed vials at −80° and rapidly thawed in water at room temperature prior to use. This treatment of the protein preparation resulted in little change in the ratio during several months storage. Upon thawing frozen ceruloplasmin, there were occasionally small amounts of a white precipitate which could be removed using a 0.45 μ Millipore filter. Only protein solutions with an $A_{280}:A_{610}$ ratio of less than 23 were used, thus indicating that the ceruloplasmin was native.

A stock solution of ceruloplasmin was prepared from frozen crystals in chelexed 0.10 M sodium acetate (pH 6.0) buffer and stored at 5°. This solution was diluted tenfold with the same buffer prior to use in equilibrium dialysis experiments. For experiments involving ^{59}Fe, all contaminating iron was removed by dialysis of the stock solution against 2% apotransferrin (Behringwerke, A.G.) for 48 hr (McDermott *et al.*, 1968). A stock solution of ceruloplasmin was routinely prepared to be 200 μM based on a molecular weight for the protein of 160,000 and $A_{610}^{1\%}$ 0.68 (Kasper and Deutsch, 1963; Kasper, 1967).

Metals, Reagents, and Glassware. All metals, acids, and other reagents used were of reagent grade purity except for ascorbic acid which was USP. Sodium acetate and ascorbate buffers were treated with Chelex-100 (200–400 mesh) (Bio-Rad) to remove any metal ion contaminants. Glassware and disposable pipets were soaked in concentrated hydrochloric acid and washed with deionized water to remove trace metals.

Equilibrium Dialysis. Plexiglas microdialysis cells (Technilab) with two 1.1-ml chambers were soaked in concentrated hydrochloric acid and washed with chelexed water to remove all traces of metal ions. Membranes for the cells were cut

* From the Department of Chemistry, Florida State University, Tallahassee, Florida 32306. *Received May 10, 1971.* Supported in part by Grant HE 08344 from the National Heart and Lung Institute. Paper no. 41 in a series from this laboratory on copper biosystems.

† Biochemistry trainee, supported by U. S. Public Health Service Training Grant No. GM 1087.

‡ To whom correspondence should be addressed.