The Inhibition of Microsomal Glucose 6-Phosphatase by Metal-Binding Agents*

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ABSTRACT: The possibility that rat liver microsomal glucose 6-phosphate phosphohydrolase (EC 3.1.3.9) is a metalloenzyme has been investigated. The inhibitory effects of a variety of classical metal-binding agents on hydrolytic and inorganic pyrophosphate–glucose phosphotransferase activities of the enzyme have been studied by both "immediate inhibition" and preincubation techniques. Inhibitions competitive with respect to glucose 6-phosphate and inorganic pyrophosphate, and noncompetitive with respect to glucose, were observed by the former technique with sodium azide, sodium cyanide, sodium oxalate, and 1,10-phenathroline. These inhibitions were prevented by titration of the metal-binding agents with divalent cations prior to exposure of the former to the enzyme. An irreversible, time-dependent inhibition was noted in preincubation experiments with 1,10-phenanthroline, sodium azide, diethyl dithiocarbamate, and 8-hydroxyquinoline. These observations suggest that glucose 6-phosphatase is a metalloenzyme, and that protein-bound metal may participate in the binding of phosphate substrates, but not glucose, to the catalyst. Inhibitions observed with a variety of other organic compounds, including *o*-tyrosine, *o*-hydroxyphenyl acetate, and cysteine, also may involve their interaction with a metal at the active enzymic site. Inhibition of enzymic activity by a variety of divalent cations, in the absence of metal-binding agents, also is described.

The effects of citrate on both hydrolase and pyrophosphate–glucose phosphotransferase activities of liver microsomal glucose 6-phosphate phosphohydrolase (EC 3.1.3.9) have been quite intensively studied in this laboratory (Nordlie and Lygre, 1966). Inhibition by this compound, which may function as a metal-binding agent (Martell and Calvin, 1952), indicated that glucose 6-phosphatase might possibly be a metalloenzyme. The effects of a variety of additional classical metal-binding agents on both hydrolytic and synthetic activities of this catalyst recently have been investigated. Results of these studies, which suggest the presence of a protein-bound metal ion at the active site of the enzyme, are described in this paper.

Materials and Methods

Sources of most chemicals were those previously described (Nordlie and Arion, 1964, 1965). Metal-binding agents were obtained from the indicated sources: sodium azide and sodium cyanide (Fisher Scientific Co., Fair Lawn, N. J.); oxalic acid and sodium diethyl dithiocarbamate (Eastman Organic Chemicals, Rochester, N. Y.); 8-hydroxyquinoline·$0.5H_2SO_4$, 1,10-phenanthroline, L-cysteine, and DL-*o*-tyrosine (Sigma Chemical Co., St. Louis, Mo.); and *o*-hydroxyphenylacetic acid (Aldrich Chemical Co., Milwaukee, Wis.). Solutions of all reagents were adjusted to pH 6.0 or 8.0, as required, with dilute NaOH or HCl solutions. Procedures for assaying glucose-6-P[1] phosphohydrolase (P_i assay or glucose[2] assay) and PP_i–glucose phosphotransferase (spectrophotometric assay) activities were as described by Nordlie and Arion (1966). The enzyme utilized in these studies was obtained by fractional ammonium sulfate precipitation of deoxycholate-dispersed rat liver microsomal suspensions as described by Nordlie and Arion (1965). Fractions precipitating between 40 and 50% ammonium sulfate saturation (calculated for 25°), having specific activities in the range 0.23–0.32 units[3]/mg of protein, were employed. The enzyme was suspended in 0.25 M sucrose solution and stored frozen at −20°.

Two methods for the study of inhibition by metal-binding agents, patterned after those employed by Adelstein and Vallee (1959) in their studies of beef liver glutamate dehydrogenase, were utilized. The first of these, termed "immediate inhibition," involved the inclusion of inhibitory compounds along with substrate(s) and buffer in assay mixtures. Reactions were inititated by the addition of 0.1-ml aliquots of enzyme preparations

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[1] Abbreviations used are found in *Biochemistry 5*, 1445 (1966).

[2] Glucose-6-P hydrolysis was followed by the glucose oxidase method (see Nordlie and Soodsma, 1966) when 1,10-phenanthroline, which interfered with the colorimetric P_i assay, was employed as inhibitor.

[3] One unit of enzymic activity = 1 μmole of glucose-6-P hydrolyzed/min at 30° in an assay mixture (pH 6.0) containing 3.3 mM glucose-6-P and 40 mM cacodylate buffer in 1.5 ml.

TABLE I: Inhibitor Constant Values for Immediate Inhibition of Phosphohydrolase and Phosphotransferase Activities by Metal-Binding Agents.[a]

Metal-Binding Agent	K_i Values[b]		
	Glucose-6-P Concn Varied	PP_i Concn Varied	Glucose Concn Varied
Azide	8.8×10^{-2}	7.8×10^{-2}	15×10^{-2}
1,10-Phenanthroline	4.1×10^{-3}	9.7×10^{-3}	22×10^{-3}
Oxalate	2.4×10^{-2}	0.95×10^{-2}	2.2×10^{-2}
Cyanide	1.7×10^{-4}	7.9×10^{-5}	6.3×10^{-5}

[a] K_i values were calculated from kinetic studies, such as those presented in Figures 1 and 2, by the method previously described by Nordlie and Lygre (1966). [b] K_i values are presented in terms of molar concentrations of metal-binding agents. Since cyanide is only partially dissociated at pH 6, K_i values for this inhibitor have been calculated in terms of the molar concentration of the fully ionized species (Plocke *et al.*, 1962).

and were terminated after 10 min by the addition of 1.0 ml of 10% (w/v) trichloroacetic acid solution (phosphohydrolase reaction, P_i assay), 0.17 ml of 6.0 M KOH solution (phosphohydrolase reaction, glucose assay, (Nordlie and Soodsma, 1966), or 0.5 ml of a 12% (v/v) aqueous perchloric acid solution (phosphotransferase assay). The second type of studies, termed "preincubation" studies, was carried out by adjusting aliquots of enzyme preparation to pH 8.0 by the addition of Tris-Cl buffer to a final concentration of 0.1 M, and then preincubating this buffered preparation at 23° for various intervals of time after adding metal-binding agents (pH 8.0). Other aliquots of buffered enzyme, which served as experimental control preparations, were supplemented with distilled water in place of metal-binding reagents and were preincubated simultaneously with the enzyme samples containing the inhibitors. Concentrations of enzyme protein in preincubation mixtures ranged from 4.8 to 10.2 mg per ml in the various experiments. After appropriate periods of preincubation, 0.1- or 0.2 ml-aliquots of these treated enzyme preparations were added to assay mixtures, and incubations were carried out for a minimum period of time (2 min). The pH of assay mixtures (pH 6.0) was unaffected by the addition of the supplemented enzyme preparations. The volume of assay mixtures was 1.5 ml and the assay incubation temperature was 30 ± 0.1° in all instances. With the exception of the studies of the kinetics of immediate inhibition, all experimental results are expressed in terms of the ratio of activity observed with inhibited preparations/activity observed with control preparations in the absence of inhibitors. Further details of the individual experiments are given in the Results section and in the legends to the figures and tables.

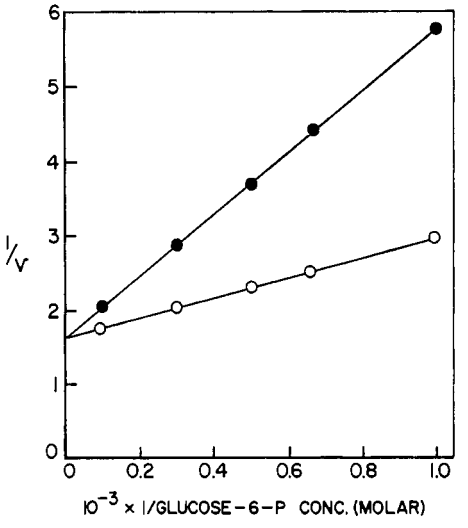

FIGURE 1: Kinetics of immediate inhibition of glucose-6-P phosphohydrolase activity by azide. Initial reaction velocities, v, were measured as functions of glucose-6-P concentrations which were varied from 1.0 to 10 mM. v was determined in the absence (○) and presence (●) of 0.2 M sodium azide. Assay mixtures (pH 6.0) also included 40 mM sodium cacodylate buffer and 0.39 mg of enzyme protein in a total volume of 1.5 ml.

Results

Immediate Inhibition

Kinetics of Inhibition by Metal-Binding Agents. The nature of inhibition of glucose-6-P phosphohydrolase and PP_i–glucose phosphotransferase activities by oxalate, azide, cyanide, and 1,10-phenanthroline were investigated by the classical kinetic approach (see Dixon and Webb, 1964). In these studies, initial reaction velocities were evaluated as a function of varied substrate concentrations without and with inhibitors included in reaction mixtures. Figure 1 depicts the results of such a study of the inhibition of glucose-6-P phosphohydrolase activity by azide; data are presented as conventional double-reciprocal plots (Lineweaver and Burk, 1934). Similar plots of data obtained in studies of the inhibition of PP_i–glucose phosphotransferase activity, in which inhibition by azide was studied with respect to both PP_i and glucose, are presented in Figure 2A,B, respectively. As indicated by the common point of convergence of the experimental plots on the y axis in

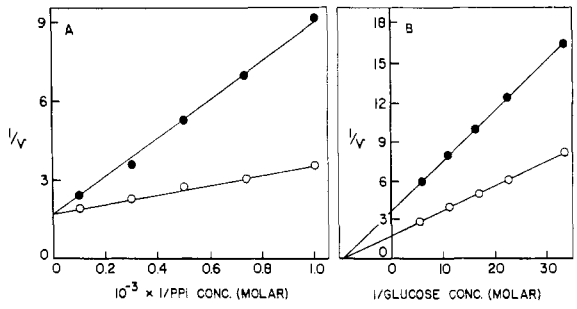

FIGURE 2: Kinetics of immediate inhibition by azide of PP_i–glucose phosphotransferase activity with respect to concentration of (A) PP_i and (B) D-glucose. Experimental details are as in Figure 1, except that varied concentrations of sodium PP_i and 0.18 M D-glucose were present in assay mixtures in A, while PP_i concentration was maintained constant at 3.0 mM and D-glucose concentration was varied from 30 to 180 mM in B. Open circles depict data obtained in the absence of azide, while closed circles indicate results obtained in the presence of 0.2 M sodium azide in both A and B.

Figures 1 and 2A, and on the x axis in Figure 2B, inhibition was competitive with respect to both glucose-6-P and PP_i, and was noncompetitive with respect to glucose. Similar patterns of inhibition were obtained in comparable studies with cyanide, oxalate, and 1,10-phenanthroline. K_i values calculated from experimental double-reciprocal plots of the data according to the method previously employed in studies of inhibition by citrate (Nordlie and Lygre, 1966) are compiled in Table I.

Prevention by Divalent Cations of Inhibition by Metal-Binding Agents. Figure 3A depicts the results of experiments in which inhibition of glucose-6-P phosphohydrolase by 40 mM oxalate was progressively diminished by increasing concentrations of $MnCl_2$. Maximal prevention of inhibition was obtained with a Mn^{2+}/oxalate ratio between 1.2 and 2. Inhibition by 4.2 mM 1,10-phenanthroline likewise could be reduced by the inclusion of Zn^{2+} in assay mixtures, as indicated by the experiment described in Figure 3B. As indicated by the *dashed lines* in Figure 3A,B, both Mn^{2+} and Zn^{2+} by themselves inhibited enzymic activity in the absence of metal-binding agents.[4] Such inhibitions by divalent cations also were observed when the ratio of cation to metal-binding agent exceeded critical values in the experiments described by the *solid lines* in Figure 3A,B. Similar inhibition of metalloenzyme activity by added cations previously has been reported for beef liver glutamate (Adelstein and Vallee, 1959; Olson and Anfinsen, 1953). Adelstein and Vallee (1959) attribute such inhibition of the dehydrogenase to the combining of divalent cations with "reactive, negative groups in the enzyme surface which are also essential for activity." Inhibition of glucose 6-phosphatase activity by divalent cations could be prevented by the inclusion of proper concentrations of metal-binding agents in assay mixtures, as indicated by the experiment depicted in Figure 4. Similar prevention of Cu^{2+}-induced inhibition of phosphotransferase activity by oxalate also was demonstrated in supplementary experiments.

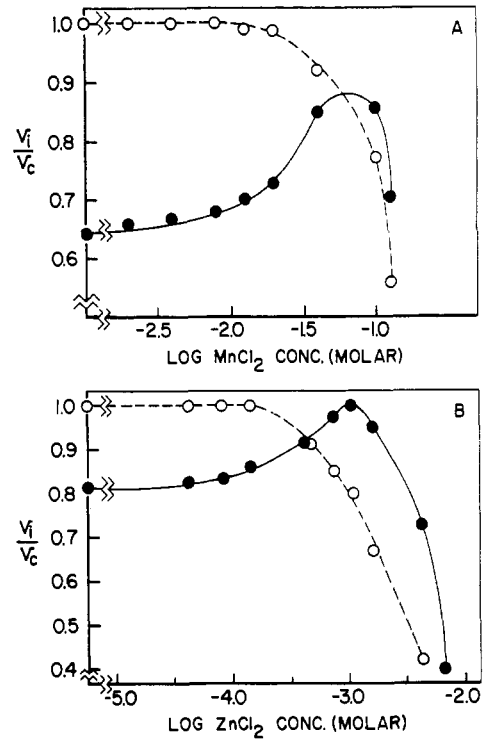

FIGURE 3: Prevention by Mn^{2+} and Zn^{2+}. (A) Prevention by Mn^{2+} of immediate inhibition of glucose-6-P phosphohydrolase activity by oxalate. Assay mixtures (pH 6.0) contained in 1.5 ml 40 mM sodium cacodylate buffer, 3.3 mM sodium glucose-6-P, 0.14 mg of enzyme protein, either no (○---○) or 40 mM (●——●) sodium oxalate, and indicated varied concentrations of $MnCl_2$. (B) Prevention by Zn^{2+} of immediate inhibition of glucose-6-P phosphohydrolase activity by 1,10-phenanthroline. Experimental conditions were as in A, except that the indicated concentrations of $ZnCl_2$ replaced $MnCl_2$, glucose-6-P concentration was 1.0 mM, and 4.2 mM 1,10-phenanthroline was employed in place of oxalate. In all instances, relative activity (v_i/v_c) = activity observed in the presence of chelator or metal (or a combination of both)/activity observed in the absence of chelator and metal salt.

Time-Dependent Inhibition

Figure 5 describes the results of experiments in which a number of metal-binding agents (azide, 1,10-phenanthroline, 8-hydroxyquinoline, and diethyl dithiocarbamate) were preincubated with enzyme preparations for periods of time ranging from 5 min to 3 hr prior to assay for glucose-6-P phosphohydrolase activity. Details of the preincubation technique employed are given in the Materials and Methods section, above. Inhibitions which increased progressively with the duration of pre-

[4] Under the conditions employed for the study of inhibition by Mn^{2+} in the absence of oxalate (see legend to Figure 3A), 50% inhibitions of hydrolase and phosphotransferase activities, respectively, were noted with the indicated concentrations of divalent cations (which were added as their chloride salts): Hg^{2+} (17 and 21 μM), Cu^{2+} (56 μM and 5.6×10^{-4} M), Zn^{2+} (3.6 and 2.8 mM), Mn^{2+} (1×10^{-1} M and 2.5 mM), Cd^{2+} (2.2×10^{-2} M and 3.2 mM), Mg^{2+} (1.8×10^{-1} and 1.0×10^{-2} M), and Co^{2+} (1.0×10^{-1} M and 2.5 mM). Inhibition of liver microsomal glucose 6-phosphatase previously has been seen with Zn^{2+} and Cu^{2+} (Beaufay *et al.*, 1954), as well as with relatively high concentrations of Mg^{2+} and Ca^{2+} (Beaufay and de Duve, 1954).

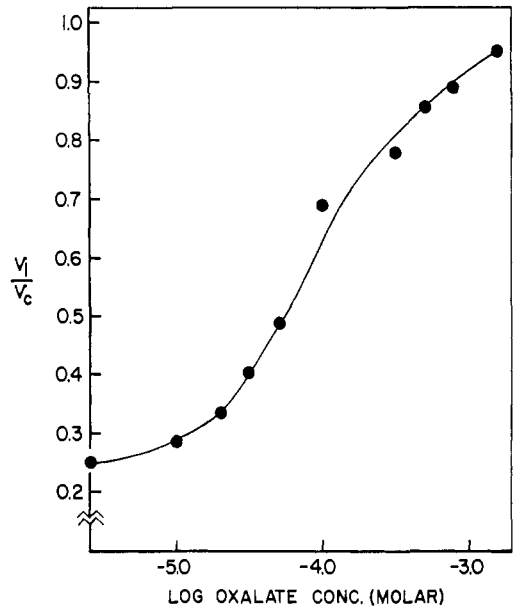

FIGURE 4: Prevention by oxalate of inhibition of glucose-6-P phosphohyrolase activity by Cu^{2+}. Assay mixtures (pH 6.0) contained, in 1.5 ml, 40 mM sodium cacodylate buffer, 3.3 mM sodium glucose-6-P, 0.10 mM $CuCl_2$, 0.22 mg of enzyme protein, and indicated varied concentrations of sodium oxalate. Relative activity (v_i/v_o) represents the ratio of activity observed in the presence of Cu^{2+} plus the indicated concentration of oxalate/activity observed in the absence of both Cu^{2+} and oxalate.

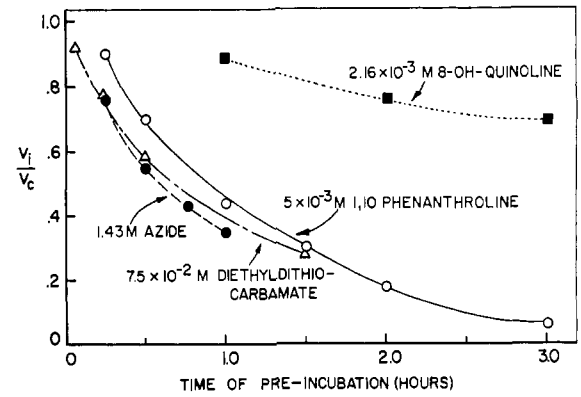

FIGURE 5: Time-dependent inhibition of glucose-6-P phosphohydrolase activity by 1.43 M sodium azide (●– – –●), 5 mM 1,10-phenanthroline (○——○), 7.5×10^{-2} M sodium diethyldithiocarbamate (△– – –△), and 2.2 mM 8-hydroxyquinoline (■· · · · ·■). The preincubation technique described in the text was employed. Periods of preincubation are indicated on the axis of abscissas. Inhibitor concentrations were diluted to either $^1/_{15}$ (diethyldithiocarbamate) or $^2/_{15}$ (azide, 1,10-phenanthroline, and 8-hydroxyquinoline) on introduction of treated enzyme preparations into assay mixtures. Assay mixtures (pH 6.0) contained, in 1.5 ml, 40 mM sodium cacodylate buffer and 3.3 mM sodium glucose-6-P. Relative velocity (v_i/v_o) represents the ratio of activities observed with enzyme preincubated with metal-binding agent/activity with enzyme preparation preincubated in the absence of such agents. See the text for further details.

incubation were noted in all instances, although the effects with 8-hydroxyquinoline were relatively small.

Inhibitions of both hydrolase and PP_i–glucose phosphotransferase activities resulting from a 1-hr preincubation of enzyme preparations with varied amounts of azide, diethyldithiocarbamate, and 1,10-phenanthroline are described in Figure 6. Inhibitions (50%) of phosphohydrolase and phosphotransferase activities, respectively, were noted under these conditions with 3 and 5 mM 1,10-phenanthroline, 70 and 73 mM diethyl dithiocarbamate, and 1.3 and 0.9 M azide. In contrast with the "immediate inhibition" described above, these time-dependent inhibitions produced by preincubation of enzyme with the metal-binding agents indicated in Figures 5 and 6 were not reversed by dilution of the inhibitor in accompanying activity assays of treated preparations.

Interestingly, oxalate, which acted as an "immediate" inhibitor, actually protected the enzyme against loss of activity due to thermal inactivation during preincubation studies, as revealed in supplementary studies not elsewhere presented. A similar stabilization against thermal inactivation (Rafter, 1960; Stetten, 1964), as well as against loss of activity on dialysis (R. C. Nordlie, unpublished observations), previously has been noted with phosphate substrates, and with the inhibitors citrate (Nordlie and Lygre, 1966; R. C. Nordlie, unpublished observation) and *o*-tyrosine (see Table II and the Discussion).

TABLE II: Immediate Inhibition of Glucose-6-P Phosphohydrolase Activity by *o*-Tyrosine and *o*-Hydroxyphenyl Acetate.[a]

Compd Added (mM)	% Inhibn of Enzymic Act.
o-Tyrosine (10)	11
o-Tyrosine (20)	23
o-Hydroxyphenyl acetate (5)	32
o-Hydroxyphenyl acetate (5) plus *o*-tyrosine (10)	39
o-Hydroxyphenyl acetate (5) plus *o*-tyrosine (20)	43

[a] Assay mixtures (pH 6.0) contained, in 1.5 ml, 40 mM sodium cacodylate buffer, 1.0 mM sodium glucose-6-P, 0.90 mg of unpurified rat liver microsomal protein, and indicated additions. Incubations were carried out for 10 min.

Discussion

The observed inhibitions of both phosphotransferase and phosphohydrolase activities of glucose 6-phosphatase by the variety of metal-binding agents described in this paper, and in addition citrate (Nordlie and Lygre, 1966), support the possibility, but do not directly prove, that this enzyme is a metalloprotein. Although all the inhibitory compounds studied have in common the ability to bind metals, it is conceivable that the observed inhibitions could involve some other common chemical property (see, for example, Fridovich, 1963). For this

reason, final unequivocal proof of the metalloenzyme nature of the catalyst must await further direct analytical studies when the enzyme can be obtained in a more highly purified form than presently is available.

The present studies do indicate that magnesium and calcium may be eliminated from consideration as metals possibly involved in the catalytic process, since 1,10-phenanthroline has little affinity for the former (Sillén and Martell, 1964), and several of the metal-binding agents tested do not interact appreciably with the latter (Sillén and Martell, 1964; Adelstein and Vallee, 1959).

Although the inhibition of a number of phosphatases by one or more metal-binding agents has been demonstrated (Schmidt and Thannhauser, 1943; Morton, 1955; Wang *et al.*, 1954; Mandl and Neuberg, 1955; Colowick, 1955; Plocke *et al.*, 1962; Mathies, 1958; Trubowitz *et al.*, 1961; Engström, 1961a; Garen and Levinthal, 1960; Belfanti *et al.*, 1935), the effects in most cases appear to involve the binding of divalent cations which are required as added cofactors. To the authors' knowledge, only four phosphatases, nonspecific alkaline phosphatases from *Escherichia coli* (Plocke *et al.*, 1962), porcine kidney (Mathies, 1958), human leukocytes (Trubowitz *et al.*, 1961), and calf intestine (Engström, 1961b), have been found by direct analytical means to contain bound metal as a functional part of their make-up.[5] All of these enzymes are inhibited by metal-binding agents including those employed in the present studies (Plocke *et al.*, 1962; Mathies, 1958; Engström, 1961a; Garen and Levinthal, 1960; Trubowitz *et al.*, 1961), even in the presence of high concentrations of added divalent cation (Mathies, 1958). It has been shown by Plocke *et al.* (1962) that the zinc metalloenzyme alkaline phosphatase from *E. coli* is affected by metal-binding agents in a manner generally quite similar to that observed with glucose 6-phosphatase in the present studies. That is, both an instantaneous, reversible inhibition and a time-dependent inhibition by a variety of metal-binding agents, including some of those employed in the present study, were observed. The alkaline phosphatase differed from glucose 6-phosphatase in that inhibition of the former by 1,10-phenanthroline could be obtained only by preincubation technics. However, sodium cyanide produced both instantaneous and time-dependent inhibitions of the alkaline phosphatase. And reversal of this instantaneous inhibition by the inclusion of Zn^{2+} in assay mixtures was similar to the partial prevention by divalent cations of immediate inhibition of glucose 6-phosphatase by oxalate and 1,10-phenanthroline observed in the present studies (see Figures 3 and 4).

Inhibitions of activities of glucose 6-phosphatase by arsenate (Swanson, 1950), molybdate (Swanson, 1950; Rafter, 1960; Nordlie and Arion, 1964), and alloxan (Beaufay and de Duve, 1954) previously have been described, as has protection of this enzyme by ethylenediaminetetraacetate and epinephrine against the effects

[5] The possible involvement of enzyme-bound magnesuim in the activity of acetyl phosphatase also has been suggested (Kielley, 1961).

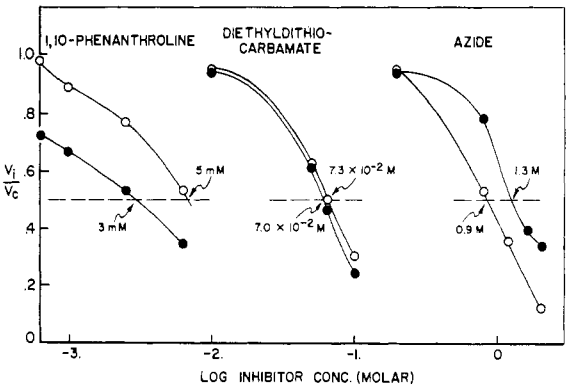

FIGURE 6: Time-dependent inhibition of glucose-6-P phosphohydrolase (●) and PP_i-glucose phosphotransferase (○) activities as a function of concentration of 1,10-phenanthroline, diethyldithiocarbamate, and azide in preincubation mixtures. Preincubations were carried out for 1 hr in all instances. Concentrations of metal-binding agents producing 50% inhibiton of enzymic activities under these conditions are indicated along with the *arrows* on the figure. Other details are as described in Figure 5.

of an inactivating system apparently present in the soluble fraction of the liver cell (Beaufay *et al.*, 1954). The effects of all of these compounds, which possess functional groups capable of binding metals, are consistent with their combination with a metal at the active site of the enzyme. It is interesting to note that oxalate (see above), citrate (Nordlie and Lygre, 1966), and phosphate substrates (Rafter, 1960; Stetten, 1964) also stabilize the enzyme against loss of activity on heating (and also dialysis; R. C. Nordlie, unpublished observation).

Some additional, initially puzzling observations which recently have been made in this laboratory also appear to be rationalizable on the basis of the interaction of certain small organic molecules with protein-bound metal at the active site of the enzyme. *o*-Hydroxyphenyl acetate inhibits glucose 6-phosphatase activity (see Table II), as Feuer *et al.* (1964a) originally reported. Further, we have found that *o*-tyrosine also inhibits (rather than reverses inhibition by *o*-hydroxyphenyl acetate, as reported by Feuer *et al.*, 1964b), either by itself or when present together with *o*-hydroxyphenyl acetate (see Table II). Other, supplementary experiments indicate that this amino acid also stabilizes the enzyme against loss of activity due to thermal denaturation at 37° (Nordlie and Johns, 1967). These observations suggest that both *o*-tyrosine and *o*-hydroxyphenyl acetate, which possess the ability to chelate metals, might bind to such a metal at the active enzymic site and thus affect activity. Similarly, inhibition which recently has been observed with L-cysteine (Nordlie and Johns, 1967; R. C. Nordlie and D. G. Lygre, unpublished observations) also may involve a similar type of interaction of the thiol compound with the enzyme. It seems probable that activities of this enzyme also may well be controlled by a variety of other compounds which possess the ability to react with the enzyme in a manner similar to that of the compounds studied (possibly by binding to enzyme-bound metal) and further,

are structurally suitable for interaction at the active site.

Mechanistic Considerations. The results of the studies on the effects of metal-binding agents on activities of glucose 6-phosphatase and associated PP_i–glucose phosphotransferase activity obtained in this study are in a number of respects quite similar to observations made by Vallee and coworkers (Vallee, 1960; Hoch *et al.*, 1958; Williams *et al.*, 1958) with the zinc metalloenzyme yeast alcohol dehydrogenase. Millimolar levels of 1,10-phenanthroline were effective in inhibiting activities of both enzymes. And with the two enzyme systems, both immediate and time-dependent inhibitions were noted. The former type of inhibition of both enzymes could be reversed by the inclusion of critical levels of certain divalent cations in assay mixtures. Vallee and coworkers (Vallee, 1960; Hoch *et al.*, 1958) have considered this immediate inhibition of the dehydrogenase to involve the reversible combination of a chelating agent with an enzyme-bound metal; a similar type of interaction of metal-binding agents with glucose 6-phosphatase also appears likely on the basis of the studies described in this paper. Also by analogy with the interpretation of Vallee and coworkers (Vallee, 1960; Williams *et al.*, 1958) regarding their observations with the dehydrogenase, the time-dependent inhibitions of activities of glucose 6-phosphatase described in Figures 5 and 6 may involve the slow, irreversible binding of a second molecule of metal-binding agent to the inhibitor–metal enzyme complex. Irreversible conformational changes in enzyme protein also may be involved in this latter type of inhibition.

Data on the kinetics of immediate inhibition studied with respect to glucose-6-P, PP_i, and glucose presented in Figures 1 and 2 are consistent with the interaction of inhibitory agents with the catalyst at or near an enzymic site involved in the binding of phosphate substrates, but not of glucose. Such an interpretation is consistent with the reaction mechanism previously proposed for this system by Arion and Nordlie (1964).[6] Patterns of inhibition analogous to those described in Figures 1 and 2 previously have been observed by Hoch *et al.* (1958) in their studies of the inhibition of the zinc metalloenzyme alcohol dehydrogenase by 1,10-phenanthroline. They interpreted their observations to indicate the involvment of enzyme-bound metal in the binding of nicotinamide–adenine dinucleotide substrates, but not ethanol or acetaldehyde. Although the data presented in the present paper are by analogy suggestive of the participation of protein-bound metal in the binding of phosphate substrates to glucose 6-phosphatase, mechanistic considerations based on such a model (including those in the preceding paragraphs) must be considered somewhat speculative until the metalloenzyme nature of this catalyst is verified by direct analytical methods after extensive purification of the enzyme.

[6] This reaction mechanism involves successively (a) formation of binary enzyme–phosphate substrate complexes, (b) dissociation of glucose (in the case of glucose-6-P) or P_i (when PP_i is substrate) from such complexes to produce a phosphoryl–enzyme intermediate, which then (c) transfers the phosphoryl group from enzyme either to water (phosphohydrolase) or to glucose (phosphotransferase).

References

Adelstein, S. J., and Vallee, B. L. (1959), *J. Biol. Chem. 234*, 824.

Arion, W. J., and Nordlie, R. C. (1964), *J. Biol. Chem. 239*, 2752.

Beaufay, H., and de Duve, C. (1954), *Bull. Soc. Chim. Biol. 36*, 1525.

Beaufay, H., Hers, H. G., Berthet, J., and de Duve, C. (1954), *Bull. Soc. Chim. Biol. 36*, 1539.

Belfanti, S., Contardi, A., and Ercoli, A. (1935), *Biochem. J. 29*, 834, 1491.

Colowick, S. P. (1955), *Methods Enzymol. 2*, 598.

Dixon, M., and Webb, E. C. (1964), Enzymes, New York, N. Y., Academic, p 315.

Engström, L. (1961a), *Biochim. Biophys. Acta 52*, 49.

Engström, L. (1961b), *Biochim. Biophys. Acta 52*, 36.

Feuer, G., Goldberg, L., and Gibson, K. I. (1964a), *Biochem. J. 93*, 5P.

Feuer, G., Goldberg, L., and Gibson, K. I. (1964b), *Biochem. J. 93*, 6P.

Fridovich, I. (1963), *J. Biol. Chem. 238*, 592.

Garen, A., and Levinthal, C. (1960), *Biochim. Biophys. Acta 38*, 470.

Hoch, F. L., Williams, R. J. P., and Vallee, B. L. (1958), *J. Biol. Chem. 232*, 453.

Kielley, W. W. (1961), *Enzymes 5*, 149.

Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56*, 658.

Mandl, I., and Neuberg, C. (1955), *Methods Enzymol. 2*, 577.

Martell, A. E., and Calvin, M. (1952), Chemistry of the Metal Chelate Compounds, Englewood Cliffs, N. J., Prentice-Hall.

Mathies, J. C. (1958), *J. Biol. Chem. 233*, 1121.

Morton, R. K. (1955), *Methods Enzymol. 2*, 533.

Nordlie, R. C., and Arion, W. J. (1964), *J. Biol. Chem. 239*, 1680.

Nordlie, R. C., and Arion, W. J. (1965), *J. Biol. Chem. 240*, 2155.

Nordlie, R. C., and Arion, W. J. (1966), *Methods Enzymol. 9*, 619.

Nordlie, R. C., and Johns, P. T. (1967), *Biochem. J. 104*, 37P.

Nordlie, R. C., and Lygre, D. G. (1966), *J. Biol. Chem. 241*, 3136.

Nordlie, R. C., and Soodsma, J. F. (1966), *J. Biol. Chem. 241*, 1719.

Olson, J. A., and Anfinsen, C. B. (1953), *J. Biol. Chem. 202*, 841.

Plocke, D. J., Levinthal, C., and Vallee, B. L. (1962), *Biochemistry 1*, 373.

Rafter, G. W. (1960), *J. Biol. Chem. 235*, 2475.

Schmidt, G., and Thannhauser, S. J. (1943), *J. Biol. Chem. 149*, 369.

Sillén, L. G., and Martell, A. E. (1964), Stability Constants of Metal-Ion Complexes, London, The Chemical Society.

Stetten, M. R. (1964), *J. Biol. Chem. 239*, 3576.
Swanson, M. (1950), *J. Biol. Chem. 184*, 647.
Trubowitz, S., Feldman, D., Morgenstern, S. W., and Hunt, V. M. (1961), *Biochem. J. 80*, 369.
Vallee, B. L. (1960), *Proc. 4th Intern. Congr. Biochem., Vienna, Austria, 8*, 138.
Wang, T. P., Shuster, L., and Kaplan, N. O. (1954), *J. Biol. Chem. 206*, 299.
Williams, R. J. P., Hoch, F. L., and Vallee, B. L. (1958), *J. Biol. Chem. 232*, 465.

Glucose Dehydrogenase Activity of Yeast Glucose 6-Phosphate Dehydrogenase. I. Selective Stimulation by Bicarbonate, Phosphate, and Sulfate*

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ABSTRACT: Glucose dehydrogenase (β-D-glucose:NADP oxidoreductase) activity associated with commercial highly purified yeast glucose 6-phosphate dehydrogenase has been studied. The anions bicarbonate, phosphate, and sulfate were found to exert a marked, selective stimulatory effect on glucose dehydrogenase, although they inhibited activity with glucose 6-phosphate as substrate; bicarbonate > sulfate > phosphate in effectiveness as activators of glucose dehydrogenase. Stimulatory concentrations of anions also lowered the pH optimum of glucose dehydrogenase. For example, maximal activity was observed at pH 9.4 without bicarbonate, at pH 8.8 in the presence of 10 mM bicarbonate, and at pH 8.2 when 50 mM bicarbonate was present. In the absence of bicarbonate, glucose dehydrogenase activity at pH 8.4 was <1% of that observed with glucose 6-phosphate as substrate; however, as assayed in the presence of 50 mM sodium bicarbonate, the former activity was increased to 20% of that observed with the sugar phosphate ester as substrate. These observations suggest the possibility of a significant metabolic role for glucose dehydrogenase activity, and point up the need for caution when glucose 6-phosphate dehydrogenase is used as an analytical tool for the measurement of glucose 6-phosphate production in assay mixtures containing high concentrations of glucose and certain anions.

During the past 6 years, the catalytic properties of two multifunctional enzymes, microsomal glucose 6-phosphatase (see, for example, Nordlie and Arion, 1964; Nordlie and Soodsma, 1966) and bacterial alkaline phosphatase (Anderson and Nordlie, 1967), have been extensively studied in this laboratory. Commercial yeast glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate:NADP[1] oxidoreductase, EC 1. 1. 1. 49) has been used routinely as an analytical tool for the assay of glucose 6-phosphate in these investigations. This dehydrogenase previously had been shown by Colowick and Goldberg (1963) to exhibit glucose dehydrogenase activity. Similar observations also have been made by Kuby and Noltmann (1966) and by Salas *et al.* (1965). During the course of our studies of inorganic pyrophosphate–glucose phosphotransferase activity of alkaline phosphatase, we also noted that a glucose-dependent reduction of NADP was catalyzed by yeast glucose 6-phosphate dehydrogenase preparations when the concentration of glucose was very high (Anderson and Nordlie, 1967). A variety of factors were observed to affect this activity which was detected with phosphotransferase control assay mixtures upon the addition of the dehydrogenase preparation. Because of our previous interest in carbohydrate metabolism and in multifunctional enzyme systems, we initiated detailed studies of factors affecting both glucose dehydrogenase (β-D-glucose:NADP oxidoreductase) and glucose 6-phosphate dehydrogenase activities of yeast glucose 6-phosphate dehydrogenase. This paper describes the selective stimulation of glucose dehydrogenase activity associated with the enzyme by the anions bicarbonate, sulfate, and phosphate.

Materials and Methods

Purified yeast glucose 6-phosphate dehydrogenase (140 units/mg of protein) was obtained from Boehringer Mannheim Corp., New York. The sodium salts of glucose-6-P and NADP, and also α- and β-D-glucoses, and crystalline bovine serum albumin were obtained from

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