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EFFECTS OF SULFHYDRYL REAGENTS ON SYNTHETIC AND HYDROLYTIC ACTIVITIES OF MULTIFUNCTIONAL GLUCOSE-6-PHOSPHATASE*

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SUMMARY

1. Comprehensive, systematic studies of inhibitions of both Glc-6-*P* phosphohydrolase and carbamyl-*P*:glucose phosphotransferase activities of rat liver microsomal glucose 6-phosphatase (EC 3.1.3.9) by three classes of sulfhydryl reagents—mercaptideforming reagents, thiol-oxidants, and thiol-alkylating agents—have been carried out.

2. Essentially equal, parallel patterns of time-dependent inhibitions of both activities were noted with all enzyme preparations tested—microsomal suspensions, detergent-dispersed microsomal preparations, and partially purified preparations—indicating that inhibitions by relatively low concentrations of thiol reagents may be produced regardless of the physical state of this membrane-bound enzyme.

3. Inhibitions were dependent on (a) the nature of the inhibitor (*p*-chloromercuriphenyl sulfonate > Hg^{2+} > *N*-ethylmaleimide > iodosobenzoate > iodoacetate), (b) duration of exposure of the enzyme to inhibitor, and (c) reaction pH. Various thiol compounds—L-cysteine, GSH, and β-mercaptoethanol—partially reversed inhibitions, while phosphate substrates (but not D-glucose) slowed the rate of reaction of the enzyme with inhibitor.

4. Exposure of the enzyme simultaneously to inhibitor and substrate for short periods of time produced kinetics resembling those of inhibition competitive with respect to phosphate substrates. Unit change in the slope of Dixon plots near pH 8.7 also implicates enzyme sulfhydryl in Glc-6-*P* binding.

5. These observations, *in toto*, support the presence of functional sulfhydryl group or groups in glucose 6-phosphatase–phosphotransferase. Further, they suggest that such sulfhydryl may be located at or near the active site, and possibly might be involved in enzyme–phosphate–substrate binding.

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid.

* A preliminary report covering some of this work has appeared[1].

INTRODUCTION

The multifunctional nature of microsomal glucose 6-phosphatase (D-Glc-6-*P* phosphohydrolase; EC 3.1.3.9) has been extensively described in the literature (see recent reviews by Nordlie[2,3]). During the past 8 years the comparative effects of a variety of inhibitors and other modifiers on synthetic and hydrolytic activities have been studied with the objective of gaining added insight regarding the mechanism of action of this complex enzyme system[2].

Only brief or preliminary mention of the effects (or lack of effect) of one such significant group of potential inhibitors—the sulfhydryl reagents—on one or more of these activities is made in the literature. Swanson[4] was unable to detect inhibition of Glc-6-*P* phosphohydrolase activity by iodoacetate concentrations as high as 50 mM. Ashmore and Weber[5] citing unpublished observations, and Wallin and Arion[6] in a brief communication, indicate inhibition of Glc-6-*P* hydrolysis by certain sulfhydryl reagents, while Nordlie and Johns[7] mention briefly in an abstract their observed inhibitions of both phosphohydrolase and PP_i–glucose phosphotransferase activities by several sulfhydryl inhibitors.

Systematic, comparative studies of the effects of a number of classical sulfhydryl reagents on both phosphohydrolase (Eqn 1) and carbamyl-*P*:glucose phosphotransferase (Eqn 2) activities of this multifunctional enzyme in a variety of types of preparations have now been carried out. Results of these studies, which indicate extensive, generally parallel patterns of time-dependent inhibition of both activities and which *in toto* support the involvement of a thiol group or groups in this multifunctional catalyst, are described in this paper.

$$\text{Glc-6-}P + H_2O \rightarrow \text{glucose} + P_i \quad (1)$$

$$\text{Carbamyl-}P + \text{glucose} \rightarrow \text{Glc-6-}P + \text{carbamate} \quad (2)$$

MATERIALS AND METHODS

Chemicals

Sources of most reagents and methods of assay of substrates were as previously described[8,9]. $HgCl_2$ ("ACS grade") was a product of Merck. *N*-Ethylmaleimide, iodosobenzoate, *p*-chloromercuriphenyl sulfonate, sodium iodoacetate, and ribonuclease A (Type IA; 5 times recrystallized; protease-free) were obtained from Sigma Chemical Co., St. Louis, Mo. This ribonuclease was used as a dispersion of 1 mg ribonuclease per ml of 0.1 M EDTA (pH 7.1).

The commercial sodium iodoacetate was further purified by classical techniques. Iodine impurities in the salt were removed by several washings with chloroform, and the chloroform-washed salt was dissolved in water and titrated with 3 M HCl until pH 3.5. The acidified solution was extracted with chloroform to partition the free acid into the organic layer. The solvent was removed by evaporation, and the free acid was used for the inhibition studies after adjustment to desired pH.

Enzyme preparations

Several types of enzyme preparations were employed in various studies, all of them somewhat crude since glucose-6-phosphatase has as yet defied all attempts at extensive purification[2]—(a) microsomal preparations, (b) deoxycholate supple-

mented (to 0.2%, w/v) microsomal suspensions, (c) partially purified preparation obtained by the method of Nordlie and Arion[9] ("partially purified preparation I"), and (d) partially purified preparation prepared by modifications of the method used by Ward and Pollak[10] to obtain "reticulosomes" ("partially purified preparation II").

In all instances, microsomal fraction was first isolated from liver homogenates of young, albino, male rats (Sprague–Dawley, Madison, Wisc.) by differential centrifugation as described previously[11,12].

Partially purified preparation II was obtained by a slight modification of the procedure of Ward and Pollak[10], including all those steps in their preparation of reticulosomes preceding gradient density centrifugation in CsCl solution. Unfractionated microsomal suspension rather than rough microsomal fraction[10] was employed as starting material. Such microsomal preparations were washed by suspension in 5 vol. of 0.01 M Tris buffer (pH 7.1), also containing 0.001 M KCl, per g original liver. The wash solution was removed by centrifugation at 30 000 rev./min (78 480 × *g*) for 45 min in the No. 30 rotor of the Beckman Model L preparative ultracentrifuge and discarded. The resulting pellet was dispersed in enough of the above Tris–KCl solution to give a protein concentration of 20 mg/ml and stored overnight frozen at −20 °C.

The frozen dispersion was thawed and treated with a ribonuclease preparation (1 mg ribonuclease per ml of the above preparation) for 1 h at 0 °C. The ribonuclease treated dispersion was then diluted with enough of the Tris–KCl solution so that when 0.11 vol. of 2.6% deoxycholate (w/v) was added, the deoxycholate/protein ratio was 1.2. This mixture was inverted 8 times, and the dispersion was recentrifuged in the No. 30 rotor at 30 000 rev./min (78 480 × *g*) for 80 min. The resulting pellet, after decantation of supernatant, was redispersed in 1 ml of the Tris–KCl solution for each g of original liver. This preparation was stored frozen at −20 °C. It was thawed at 0 °C, and used immediately in the inhibition studies as required.

Typically, such preparations represent an increase of from 5–7-fold in specific activity of glucose 6-phosphatase over that of deoxycholate-supplemented (to 0.2%, w/v) microsomal suspensions. A representative preparation catalyzed the formation of 1.1 μmoles of Glc-6-*P* *via* carbamyl-*P*:glucose phosphotransferase activity, or the hydrolysis of 1.4 μmoles of Glc-6-*P*, per min per mg protein under the assay conditions described immediately below. Such preparations, possessing the highest specific activities of any preparation of glucose 6-phosphatase–phosphotransferase yet available, were employed in the bulk of the studies described below.

Assays of enzymic activities

Enzymic activity assays routinely were carried out at 30 ± 0.1 °C in a thermostatically-regulated water bath equipped with a mechanism for shaking reaction tubes. All assay mixtures, pH 7.0, contained in 1.5 ml, 40 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES) buffer, substrates (6 mM sodium Glc-6-*P* for phosphohydrolase; 6 mM carbamyl-*P* and 120 mM D-glucose for phosphotransferase), and sufficient supplemental NaCl such that ionic strength was 0.1. Incubation time routinely was 3 or 4 min (assays in the time-dependent inhibition studies) or 10 min (other studies). Synthetic and hydrolytic activities of the enzyme were measured by the previously described procedures[11], phosphohydrolase as P_i produced and phosphotransferase as Glc-6-*P* formed. Hg^{2+} have been shown to interfere with

the spectrophotometric assay of Glc-6-*P* under certain conditions[13] by complexing with NADH or NADPH and thus depressing absorbance at 340 nm. However, it was demonstrated in supplemental studies that at the concentrations of Hg^{2+} involved in the present studies no interference was produced. Linearity of enzymic activity with time of incubation and enzyme protein concentration also was established in preliminary, supplemental experiments.

Studies of time-dependent inhibitions by thiol reagents

Studies were carried out by incubating the enzyme preparation with indicated concentrations of sulfhydryl reagents at either 0 or 30 °C at designated pH for specified periods of time. Such incubation mixtures routinely contained between 0.1 (partially purified preparation II) and 0.4 mg (microsomes) of protein per ml. Unless otherwise explicitly stated, such incubations were carried out at 0 °C and pH 6.0. Seven volumes of original enzyme suspension in 0.01 M Tris–KCl buffer were diluted with 7 vol. of 0.1 M sodium cacodylate buffer, pH 6.0, and 1 vol. of inhibitor solution (or distilled water in the case of controls) was then added to attain these conditions. Incubations were then carried out, with gentle shaking, and 0.15-ml aliquots were withdrawn at designated intervals and immediately added to assay mixtures (see above) at 30 °C and pH 7.0 for assay of enzymic activities.

These manipulations produced a 10-fold dilution in sulfhydryl reagent concentration in assay mixtures compared with original enzymic incubation mixtures. Initially, control assay mixtures were supplemented to contain these lesser concentrations of such sulfhydryl reagents; aliquots of enzyme preparations previously incubated at 0 °C concomitantly with experimental preparations but in the absence of sulfhhydryl reagents served as experimental controls and were assayed in these sulfhydryl reagent-supplemented assay mixtures. Later, it was found that such supplementation of assay mixtures with sulfhydryl reagents was unnecessary, as no discernible inhibitions were produced by the very low levels of sulfhydryl reagents under our conditions of brief exposure to enzyme during assay.

Experimental data are expressed as the ratio, v_i/v_0, where v_i is the reaction velocity observed with enzyme preparations initially incubated with inhibitor while v_0 is the corresponding reaction velocity obtained with control enzyme preparations initially incubated in the absence of such sulfhydryl reagents.

Additional, supplemental details and modifications of these basic procedures, as necessary, are given in various places in Results.

RESULTS

Time-dependent inhibitions by sulfhydryl reagents

Susceptibility of various types of enzyme preparations to inhibition. The inhibitory effects of both $HgCl_2$ and *p*-chloromercuriphenyl sulfonate on Glc-6-*P* phosphohydrolase and carbamyl-*P*:glucose phosphotransferase activities of a variety of different types of enzyme preparations were studied by the time-dependent technique described above. Inhibitions of both activities progressive with time of exposure of enzyme to inhibitor were observed with all preparations tested—freshly prepared microsomal suspensions, such suspensions which had been supplemented to 0.2% (w/v) with deoxycholate, and partially purified enzyme preparation. Data obtained

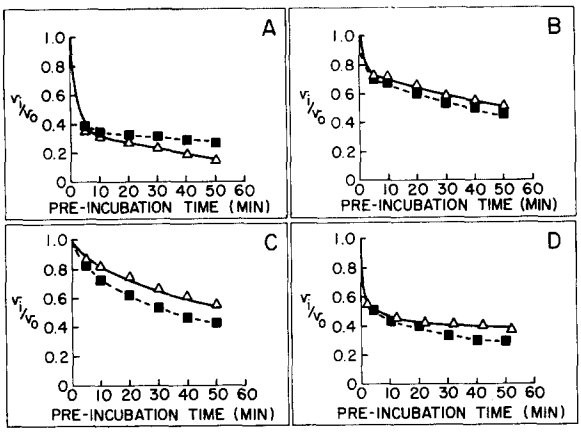

Fig. 1. Time-dependent inhibition by *p*-chloromercuriphenyl sulfonate (A–C) or Hg^{2+} (D) of Glc-6-*P* phosphohydrolase (■) and carbamyl-*P*:glucose phosphotransferase (△) activities of a variety of enzyme preparations. Included are: A, microsomal suspensions; B and D, microsomal preparations supplemented to 0.2% (w/v) with sodium deoxycholate; and C, partially purified preparation II. *p*-Chloromercuriphenyl sulfonate concentration was 0.05 mM (A) or 0.1 mM (B and C). In D, $HgCl_2$, 0.1 mM, was employed. Enzyme preparations were incubated with inhibitor for designated periods of time at 0 °C and pH 6.0. The expression, v_i/v_0, is as defined in the text.

in typical studies with *p*-chloromercuriphenyl sulfonate are presented in Figs 1A–1C. Generally similar patterns of results also were obtained in studies with 0.1 mM Hg^{2+} (see, for example, Fig. 4D). Total inhibition could be attained within 50 min with more elevated levels of these sulfhydryl reagents.

It is obvious from these studies that activities of a variety of preparations of the enzyme, detergent-activated and partially purified as well as crude microsomal suspensions, which probably represent several physical states[2,14–16] of membrane-bound[17] lipoprotein-involving[14–18] catalyst, are all sensitive in varying degrees, to inhibitions by sulfhydryl reagents. Partially purified preparation II was chosen for succeeding studies, as it is the most highly purified form of the enzyme presently available[2].

Effects of various sulfhydryl reagents. That a variety of sulfhydryl reagents all are effective inhibitors of both Glc-6-*P* phosphohydrolase and carbamyl-*P*:glucose phosphotransferase is indicated by the experiments depicted in Figs 2A and 2B. Essentially parallel patterns of inhibition of both activities increasing with duration of time of exposure of the enzyme to inhibitor were observed with iodoacetate, iodosobenzoate, *N*-ethylmaleimide, Hg^{2+}, and *p*-chloromercuriphenyl sulfonate. These latter two compounds were most potently effective, while iodoacetate, even at the 10 mM level, produced but modest, although significantly reproducible, effects.

Effects of Hg^{2+} concentration and incubation pH. Figs 3A and 3B, describe various relevant characteristics of time-dependent inhibitions by $HgCl_2$. Increasing rates of inhibition of both activities progressive with elevations in concentrations of $HgCl_2$ were observed, as indicated in Fig. 3A.

The variations in rate of inhibition by 0.05 mM $HgCl_2$ with alterations in pH of initial incubation mixtures are indicated in the experiments depicted in Fig. 3B. In these studies, the pH of enzyme preparation was adjusted by diluting 1 vol. of enzyme in Tris–KCl buffer with 1 vol. of 0.1 M sodium cacodylate buffer (pH 5.5 or

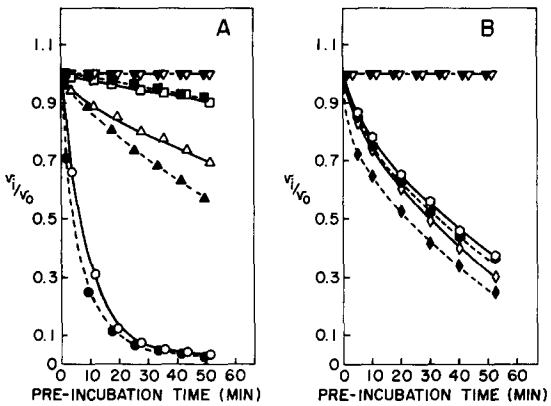

Fig. 2. Time-dependent inhibition of Glc-6-*P* phosphohydrolase (solid symbols) and carbamyl-*P*:glucose phosphotransferase (open symbols) by a variety of sulfhydryl reagents. Incubations were carried out at pH 9.0 and 30 °C; partially purified preparation II was employed. The pH of enzyme preparations was adjusted to pH 9.0 by titration with 1 M NH_4OH at room temperature immediately prior to initial incubation. Inhibitors included 0.05 mM *p*-chloromercuriphenyl sulfonate (○, ●), 1 mM iodosobenzoate (△, ▲), 10 mM iodoacetate (□, ■), 0.05 mM $HgCl_2$ (◇, ◆) or 1 mM *N*-ethylmaleimide (⬡, ⬢). Control values (see text) are given by ▽ and ▼. The ratio, v_i/v_0, is defined in the text.

6.0), 0.1 M HEPES buffer (pH 7.0), 0.1 M Tris–HCl buffer (pH 8.0), or 0.1 M glycine buffer (pH 9.0). The rate of inhibition of both activities of the enzyme increased generally as pH was lowered from 9.0 to 5.5. No studies at lower pH were performed due to the acid-lability of the enzyme[19]. This inverse relationship between reaction pH for exposure of enzyme to inhibitor and rate of inhibition by mercurials often has been seen with other sulfhydryl enzymes, as pointed out by Webb[20].

Reversal of inhibition by sulfhydryl compounds and protection by phosphate substrates. The partial reversal by various thiol compounds, 10 mM L-cysteine, GSH, or β-mercaptoethanol, of inhibitions by Hg^{2+} of both hydrolase and phosphotransferase

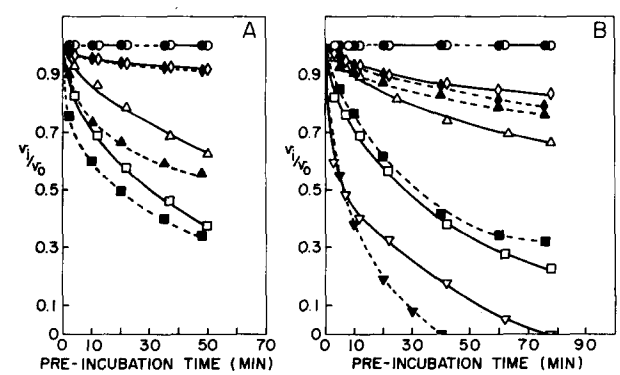

Fig. 3. Effects of inhibitor concentration (A) and pH (B) on time-dependent inhibition by Hg^{2+} of Glc-6-*P* phosphohydrolase (closed symbols) and carbamyl-*P*:glucose phosphotransferase (open symbols). Partially purified preparation II was employed in these studies carried out at 0 °C. In A, pH was 6.0; $HgCl_2$ concentration was zero (○, ●), 0.005 mM (◇, ◆), 0.02 mM (△, ▲), or 0.05 mM (□, ■). In B, $HgCl_2$ was in all cases 0.05 mM, and pH for exposure of enzyme to inhibitor was 9.0 or 8.0 (◇, ◆) 7.0 (▽, ▲), 6.0 (□, ■), or 5.5 (▽, ▼). The ratio, v_i/v_0, is defined in the text

activities was demonstrated (see Fig. 4A). Enzyme was first incubated with 0.1 mM $HgCl_2$ for 22 min and thiol compounds were then added as indicated. Highly-significant reversals of inhibitions of both activities were effected by all three of the thiols tested.

A partial protection by phosphate substrates, but not by glucose, against the time-dependent inhibition by Hg^{2+} also was noted (see Fig. 4B). In these studies, aliquots of enzyme were incubated with 0.05 mM $HgCl_2$ and either 60 mM D-glucose, 3 mM carbamyl-*P*, 3 mM Glc-6-*P*, or supplemental distilled water (experimental controls) as indicated. The rate of inhibition by Hg^{2+} was significantly lowered by either of the two phosphate substrates, but not by glucose, suggesting an involvement of enzyme sulfhydryl in phosphate substrate binding.

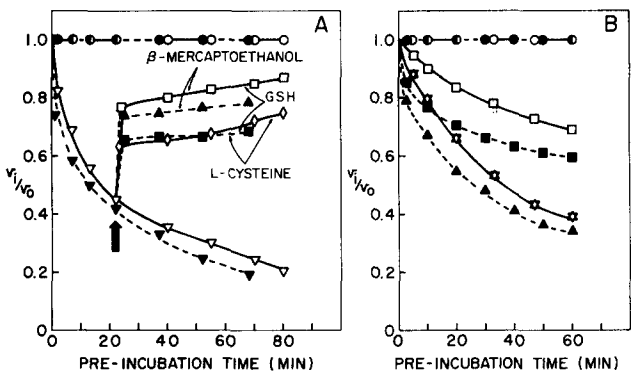

Fig. 4. Reversal of Hg^{2+}-effected inhibition by sulfhydryl compounds (A), and protection against such inhibition by phosphate substrates (B). Partially purified preparation II was employed in all studies, which were carried out at 0 °C and pH 6.0. Closed symbols depict Glc-6-*P* phosphohydrolase activity and open symbols indicate carbamyl-*P*:glucose phosphotransferase in both A and B. ○ and ●, depict experimental control values in the absence of inhibitor. In A, enzyme preparations were incubated with 0.10 mM Hg^{2+} for the indicated periods of time. At 20 min (arrow), the indicated thiol compound was added to a final concentration of 10 mM; distilled water was added to one set of inhibited controls (▽, ▼). In B, enzyme preparations were incubated with 0.05 mM Hg^{2+}, and were supplemented with 60 mM D-glucose (△), 3 mM Glc-6-*P* (■), 3 mM carbamyl-*P* (□), or distilled water (▽, ▲). The ratio, v_1/v_0, is defined in text.

Kinetics of immediate inhibition by mercurials*

Studies of the kinetics of inhibition by certain mercurials of both Glc-6-*P* phosphohydrolase and phosphotransferase activities also were carried out in which inhibitor ($HgCl_2$ or *p*-chloromercuriphenyl sulfonate) was included along with substrates and buffer in assay mixtures, untreated enzyme was added, and the reaction was allowed to procede for 10 min at 30 °C. Some representative data, obtained in a study of the inhibition by 0.01 mM or 0.04 mM $HgCl_2$ of Glc-6-*P* hydrolysis, are

* Immediate inhibition, as employed here, refers simply to inhibition seen in studies carried out by the usual kinetic method wherein inhibitor is included along with substrates in assay mixtures and enzyme preparation then added to initiate reaction, which is then allowed to proceed for a relatively short period of time before termination. Intersection of double-reciprocal plots, ± inhibitor, on the *y*-axis as seen here (Fig. 5, for example) is interpreted as indicative of an initial competition of phosphate substrate and mercurial for the enzyme. With prolonged duration of incubation, complete inhibition by reaction of the "irreversible" inhibitor with enzyme will, of course, be seen.

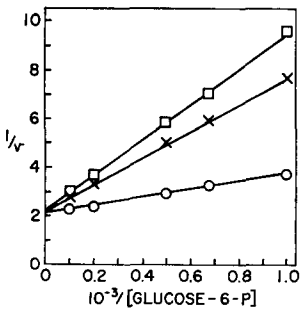

Fig. 5. Lineweaver–Burk plot[21] of results of studies of "immediate" inhibition by Hg^{2+} of Glc-6-*P* phosphohydrolase activity. Assay mixtures, pH 6.0, contained in 1.5 ml, 40 mM cacodylate buffer, between 1 and 10 mM Glc-6-*P* (see figure), and either no (○), 0.01 mM $HgCl_2$ (×) or 0.04 mM $HgCl_2$ (□). Incubations were for 10 min at 30 °C; *v* is expressed as μmoles Glc-6-*P* hydrolyzed. K_i for Hg^{2+}, calculated for classical competitive inhibition[22], was 4.4 μM.

presented in Fig. 5 as conventional Lineweaver–Burk double-reciprocal plots[21]. Generally similar competitive patterns of inhibition by these mercurials also were observed with respect to the phosphate substrates carbamyl-*P* or PP_i in phosphotransferase reactions, although curvilinearity appeared in double-reciprocal plots at lower levels of substrates. Inhibitions studied with respect to glucose in the phosphotransferase reactions were either of classical non-competitive type, or midway between non-competitive and un-competitive.

Studies of the pH kinetics of Glc-6-P phosphohydrolase

A Dixon plot[23] of data obtained in extensive studies of the variations of K_m values for Glc-6-*P* as a function of assay pH is presented as Fig. 6. K_m values were

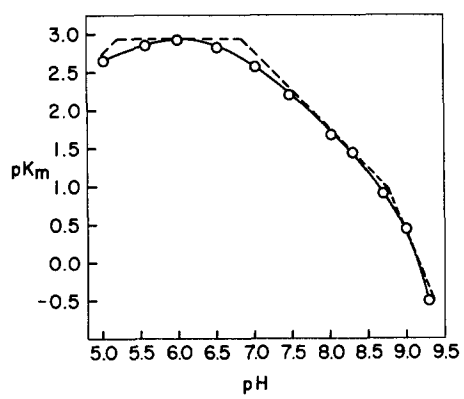

Fig. 6. Variation of Michaelis constant for Glc-6-*P* with pH. K_m (Glc-6-*P*) values were determined as described in the text, and negative logarithms (pK_m) plotted *vs* pH according to the method of Dixon[23]. — — —, slopes of +1,0, −1, and −2. Assay mixtures contained, in 1.5 ml, 40 mM buffer, partially purified preparation II, Glc-6-*P* concentrations ranging between 2 and 20 mM, and sufficient supplemental NaCl such that ionic strength was 0.1. Incubation was for 10 min at 30 °C. Buffers employed were as follows: pH 5.0, 40 mM acetate buffer; pH 5.5, 20 mM acetate + 20 mM cacodylate buffer; pH 6.0 or 6.5, 40 mM cacodylate; pH 7.0, 20 mM cacodylate + 20 mM HEPES; pH 7.5, 40 mM HEPES; pH 8.0, 20 mM HEPES + 20 mM Tris–HCl; pH 8.3 or 8.7, 40 mM Tris–HCl; pH 9.0 or 9.3, 20 mM Tris–HCl + 20 mM glycine. The pH was determined with a Beckman research model meter before and after incubation and was found unchanged.

estimated by the statistical method of Wilkinson[24] from data obtained at 0.5-unit increments of pH between pH 5.0 and 9.3. Experimental details are given in the legend to Fig. 6. The two one-unit changes in slopes of tangents to the Dixon plot between pH 5.6 and 7 may be attributed to ionizations in the substrate and an imidazolium group of enzyme-bound histidine, as indicated previously[25]. The change in slope from -1 to -2 at approx. pH 8.7, as indicated by the results of the present studies carried out under conditions of carefully controlled ionic strength, suggests the involvement of an enzyme sulfhydryl group in substrate binding[26].

DISCUSSION

The experiments described in this paper constitute the first systematic, comprehensive study of the effects of a variety of sulfhydryl reagents on activities of the multifunctional catalyst glucose 6-phosphatase. *In toto*, results of these studies rather strongly suggest* the involvement of functional sulfhydryl group(s) at or near the active site of the enzyme. This conclusion is based on the following considerations: (a) Both hydrolytic and synthetic activities of the enzyme were extensively inhibited by relatively low concentrations of a variety of types of sulfhydryl inhibitors[29], mercaptide-forming mercurial reagents (Hg^{2+}, *p*-chloromercuriphenyl sulfonate), thiol-oxidizing reagent (iodosobenzoate), and thiol-alkylating reagents (iodoacetate, *N*-ethylmaleimide) (Fig. 2); (b) inhibitions observed were dependent on duration of exposure of enzyme to sulfhydryl reagents (Figs 1–4), concentrations of such reagents (Fig. 3A), and reaction pH (Fig. 3B) in the manner previously observed with other sulfhydryl enzymes[20]; (c) such inhibitions were extensively reversed by various supplementary sulfhydryl compounds (see Fig. 4A); (d) phosphate substrates (but not glucose) slowed down the rate of time-dependent inhibition by thiol inhibitors (Fig. 4B), and appear also from studies in which enzyme was exposed simultaneously to substrates and inhibitor (see Fig. 5 and text) to compete with such inhibitors for enzyme; and (e) the existence of enzyme sulfhydryl group or groups involved in the binding of phosphate substrates is independently supported by pH kinetic studies (Fig. 6, for example).

It thus appears to the authors that although interaction of certain of the studied inhibitors with enzyme functional groups other than thiols is possible, all of these observations taken together constitute a rather imposing body of evidence supporting the involvement of enzyme sulfhydryl group(s) in glucose 6-phosphatase–phosphotransferase action. Evidence to date thus implicates enzyme-bound divalent cation[7,30], histidine–imidazolium group[25,31], and now enzyme–thiol in enzyme–substrate interactions.

The possibility that such a thiol group might be used for binding of the metal ion to the enzyme, as in homogentisate oxidase[32], may be eliminated as a mechanistic possibility as added thiol compounds by themselves reverse the inhibition by mercurials (Fig. 4A).

The present studies also reveal that inhibitions by low concentrations of thiol reagents are observed with both synthetic and hydrolytic activities of the enzyme,

* Webb[27], citing Boyer[28], has indicated that in no case has it been established with absolute certainty that essential sulfhydryl groups are directly involved in substrate binding.

that such inhibitions are essentially parallel in all cases, and that inhibitions are manifest regardless of the physical state of this membrane-bound enzyme. As indicated in Figs 1A–1D, such inhibition patterns were observed with freshly prepared microsomal preparations (Fig. 1A) and also with preparations in which membrane integrity had been destroyed through preliminary exposure to detergents (Figs 1B and 1D) or detergent exposure *plus* additional purification procedures (Figs 1C and 2–5). Although the first such preparation appears somewhat more sensitive to inhibitor action than do the latter two, both carbamyl-*P*:glucose phosphotransferase and Glc-6-*P* phosphohydrolase activities are in all cases inhibited parallelly and equally.

The observations presented in this paper, indicating generally demonstrable inhibitions by sulfhydryl reagents of both hydrolase and phosphotransferase activities of glucose 6-phosphatase with enzyme preparations in a variety of states of "disruption", would, on first consideration, appear to contradict Wallin and Arion[6] who have concluded with respect to inhibition by sulfhydryl reagents of Glc-6-*P* phosphohydrolase activity that "...a requirement for an intact membrane structure in the action of these reagents is indicated".

It appears to us that the conflict, if indeed it exists, relates to the extrapolation by Wallin and Arion[6] of certain of their experimental observations, made under very special conditions, to reach the above general conclusion. Indeed, as they indicate (see p. 697 in ref. 6), inhibition by sulfhydryl reagents of Glc-6-*P* phosphohydrolase activity of both "intact" and disrupted microsomal preparations was observed unless a precisely defined set of experimental conditions was employed (for example, a 2.5-fold excess of EDTA (0.5 mM), a powerful chelating agent, was required to be present along with Hg^{2+} (0.2 mM) if inhibition of activity of this metaloenzyme[30], with "intact" microsomes, but not with taurocholate-disrupted microsomal preparations, was to be observed).

We conclude that multifunctional glucose 6-phosphatase, with all its ramifications, is generally susceptible to inhibition by sulfhydryl reagents. Certain precisely defined conditions may be arrived at under which susceptibility to such inhibition is interrelated with the physical state of this membrane-bound enzyme. The latter phenomenon in turn conceivably may be related to the existence of a phosphate–substrate-specific permease as suggested by Wallin and Arion[6], it may be a reflection of substrate and/or activity discriminant conformational modifications of the multifunctional enzyme as generally conceived by Nordlie and coworkers (see ref. 2), or it may well involve yet other still-undefined factors.

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