Dimerization of Brain Hexokinase Induced by Its Regulator Glucose 6-Phosphate*

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SUMMARY

Bovine brain mitochondrial hexokinase type I, undergoes a concentration-dependent dimerization in presence of its product inhibitor glucose 6-phosphate. The effectiveness of this ligand in inducing the aggregation of brain hexokinase closely parallels its kinetic behavior as an inhibitor of this enzyme. ATP and inorganic phosphate known to antagonize the inhibitory effect of glucose 6-phosphate also cause a reversal of this dimerization process. ADP, another inhibitor of brain hexokinase, however, has no effect on the sedimentation behavior of the enzyme. It is suggested that the conformational alteration underlying the formation of hexokinase dimer in presence of glucose 6-phosphate has physiological significance.

The inhibitory and regulatory effects of glucose 6-phosphate in the mammalian hexokinase reaction have been well documented (1–5). The importance of hexokinase as a control point in the utilization of glucose in mammalian systems derives largely from these effects (3–5). Even so, the molecular mechanism underlying the inhibitory and regulatory functions of this ligand have not been identified so far. In a previous communication from this laboratory (6), we reported the changed reactivity of sulfhydryl residues of brain hexokinase in the presence of glucose 6-phosphate and postulated a conformational change induced in the enzyme by this ligand. We also made a brief mention of the fact that the sedimentation behavior of brain hexokinase changes significantly in presence of glucose 6-phosphate. In the present paper, we present detailed evidence to suggest that the interaction of brain hexokinase with glucose 6-phosphate leads to a conformationally altered enzyme capable of concentration-dependent dimerization. This conformational alteration in the enzyme as detailed by its ability to dimerize, appears to be physiologically significant.

**MATERIALS AND METHODS**

Chemicals—Glucose 6-phosphate dehydrogenase, aldolase, pyruvate kinase, NADP, NADPH, NAD, NADH, ATP, ADP, glucose 6-phosphate, and fructose 6-phosphate were obtained from Boehringer Mannheim, West Germany. Crystalline bovine albumin was obtained from Nutritional Biochemicals Corporation. 2-Mercaptoethanol was a product of Koch-Light Laboratories, Colnbrook, Bucks, England. Analar grade EDTA was a product of BDH division of Glaxo Laboratories, Bombay, India. Bovine brain hexokinase was prepared by the procedure of Redkar and Kenkare (6).

Enzyme Assays—Hexokinase was assayed as described previously (6). Pyruvate kinase and aldolase were assayed on an Eppendorf fluorimeter as described by Maitra and Lobo (7).

Protein Concentration—Protein concentration was measured by the method of Lowry et al. (8), with bovine plasma albumin as the standard.

Ultracentrifugal Studies—Sedimentation velocity studies were carried out on a Beckman model E analytical ultracentrifuge equipped with a rotor temperature indicator and a control unit, using AN-D rotor for high concentrations of protein and AN-E rotor for low concentrations. Schlieren optics was employed throughout. Sedimentation experiments with AN-D rotor were carried out at speeds of 50,780 rpm, using a 12-mm 4° sector cell with an aluminum centerpiece. Sedimentation experiments with AN-E rotor were performed at a speed of 50,740 rpm using a 30-mm, 4° sector cell with an aluminum centerpiece. The sedimentation coefficients were corrected to a value corresponding to a solvent with the viscosity and density of water at 20°C (s20,w).

The solvent system for the ultracentrifuge runs was Tris-HCl buffer, pH 8.0, containing 5 mM EDTA, 5 mM 2-mercaptoethanol, and 100 mM KCl with such other additions as indicated. The areas under the schlieren peaks were determined using a planimeter. Base-line was obtained by extrapolation.

Glycerol density gradient centrifugation studies were performed essentially by the sucrose density gradient procedure of Martin and Ames (9). The gradient was linear between 10 and 30% (v/v) glycerol, dissolved in 50 mM Tris-HCl buffer, pH 8.0, containing 5 mM EDTA, 5 mM 2-mercaptoethanol, and 0.1 mM glucose 6-phosphate (when added). Pyruvate kinase with s20,w value of 10.8 (10) and aldolase with s20,w value of 7.35 S (11) were used as the marker proteins. The enzyme solution together with marker enzymes, in 1.0 ml of the buffer in which the gradient was formed, was layered on top of the gradient. The centrifugation was carried out at 40,000 rpm for 24 hours at 4°C in the Beckman model L2-65B ultracentrifuge using an SW 41 rotor. About 70 5-drop fractions were collected and these were assayed for various proteins as described above.

**RESULTS AND DISCUSSION**

The sedimentation behavior of bovine brain mitochondrial hexokinase in presence of increasing concentrations of the ligand, glucose 6-phosphate, is shown in Fig. 1. The ultracentrifugal patterns clearly show that glucose 6-phosphate brings about a change in the sedimentation coefficient of the enzyme from about 6 to 8 S. Increase in sedimentation coefficient of a protein can...
obtained for slow and fast moving zones. In the presence of varying concentrations of glucose 6-phosphate, the solvent system employed and other details are as described under "Materials and Methods." Pictures were taken after the rotor reached the top speed of 59,780 rpm and sedimentation runs were performed at average temperatures varying in the range -20°C. Frames A to C were obtained with a protein concentration of 5.5 mg per ml and Frames D to F were obtained with a protein concentration of 2.7 mg per ml. Bar angles used, glucose 6-phosphate concentrations employed, sedimentation coefficients are given in that order for each picture. A, 45°, nil, 5.8 S, 100%; B, 40°, 0.05 mM, 5.2 S and 7.8 S, 28 and 72%; C, 45°, 0.1 mM, 5.4 S and 7.9 S, 16 and 84%; D, 40°, nil, 6 S, 100%; E, 40°, 0.01 mM, 5.6 S and 8 S, 47 and 53%; F, 40°, 1 mM, 7.5 S, >55%.

The effectiveness of extremely low concentrations of glucose 6-phosphate, i.e., 0.01 mM, in causing dimerization of the enzyme can be correlated with the $K_d$ of the enzyme for glucose 6-phosphate which is of the order of 0.01 mM (17). The almost complete conversion of hexokinase to the dimer form at 1 mM glucose 6-phosphate (Fig. 1F) also corresponds to the almost complete inhibition of hexokinase in presence of 1 mM glucose 6-phosphate. The reversibility of the glucose 6-phosphate effect is shown by an experiment in which the glucose 6-phosphate-treated enzyme is exhaustively dialyzed to remove the ligand. The dialyzed enzyme then showed the sedimentation behavior characteristic of the native enzyme (figure not shown). A close parallel between the ability of glucose 6-phosphate to dimerize the enzyme and its kinetic effects on the enzyme reaction is thus evident.

The variation of sedimentation coefficient of hexokinase with concentration plotted in Fig. 2 shows that native hexokinase does not apparently undergo any concentration-dependent dimerization. (However, see the text relating to Fig. 3B.) The phenomenon of dimerization of the enzyme in presence of glucose 6-phosphate is thus a result of its interaction with this ligand. There is also no evidence of dissociation of the enzyme at low protein concentrations. This result is fully in accord with our observations that the enzyme consists of a single polypeptide chain. Easterby and O'Brien (18) and Chou and Wilson (19) results either from decrease in its frictional ratio or increase in its molecular weight. The latter possibility is favored in the present case because it is difficult to explain such a large increase in sedimentation coefficient merely on the basis of change in shape of the molecule brought about by its interaction with the ligand. The concentration dependence of this phenomenon (see below) also supports the hypothesis that an increase in sedimentation coefficient reflects an increase in molecular weight of the enzyme.

The value of the sedimentation coefficient of the enzyme in presence of various concentrations of glucose 6-phosphate leads us to conclude that hexokinase tends to dimerize in presence of glucose 6-phosphate. Such a conclusion, however, calls for some comment. On the basis of Gilbert's theory (12, 13), a monomer-dimer system should result in a single peak in the ultracentrifuge unless the rate of equilibration between the two species is very slow compared to the rate of sedimentation. The sedimentation coefficient of such a peak would be a weighted average of the sedimentation coefficients of the monomer and the dimer. On the other hand, monomer-dimer reactions mediated by a tight binding small molecule would result in a double boundary despite rapid equilibration between the monomer and the dimer, if the concentration of the ligand is of the same order of magnitude as the concentration of the protein (14, 15). Results from Rose's laboratory indicate that the interaction between glucose 6-phosphate and type I hexokinase is rapid and not time-dependent (16). However, the binding of glucose 6-phosphate to the enzyme is tight, the $K_d$ being of the order of about 0.01 mM (17). Also the concentrations of glucose 6-phosphate employed are comparable to those of the enzyme for cases represented by Fig. 1, B, C, and E. The appearance of a double boundary for hexokinase in presence of glucose 6-phosphate thus can be understood.

In cases like this the sedimentation coefficients pertaining to the two peaks do not faithfully represent the sedimentation coefficient of monomer and dimer, respectively. Since the faster moving peak may contain varying concentrations of the monomer, its sedimentation coefficient would be less than that of a dimer (14). This can perhaps explain why the sedimentation coefficients of the faster moving peak are somewhat less than would be expected of a dimer of hexokinase of molecular weight around 200,000. The sedimentation patterns obtained in our experiments also render unlikely the possibility of an equilibrium between monomer and trimer existing in the ultracentrifuge cell. Although such a situation can also give rise to two peaks according to Gilbert's theory (12, 13), the faster moving peak obtained in these cases would have a sedimentation coefficient more than that corresponding to a dimer. As pointed out above, in our experiments the sedimentation coefficient of the faster moving peak is about equal to or slightly less than that corresponding to a dimer. Hence the polymerization process in presence of glucose 6-phosphate does not appear to proceed beyond dimerization.

The arguments presented above do not constitute a proof that a trimer of hexokinase is not formed in the presence of glucose 6-phosphate or that change in sedimentation constant is not solely due to change in frictional coefficient. But considering that these are very remote possibilities, the conclusions already drawn appear to be valid.

It also follows from the above discussion that the areas under the monomer and dimer peaks would not strictly correspond to the concentrations of monomer and dimer present in the solution. No attempt is made, therefore, to use the data to calculate the binding constant between glucose 6-phosphate and hexokinase.

The effectiveness of extremely low concentrations of glucose 6-phosphate in causing dimerization of the enzyme can be correlated with the $K_d$ of the enzyme for glucose 6-phosphate which is of the order of 0.01 mM (17). The almost complete conversion of hexokinase to the dimer form at 1 mM glucose 6-phosphate (Fig. 1F) also corresponds to the almost complete inhibition of hexokinase in presence of 1 mM glucose 6-phosphate. The reversibility of the glucose 6-phosphate effect is shown by an experiment in which the glucose 6-phosphate-treated enzyme is exhaustively dialyzed to remove the ligand. The dialyzed enzyme then showed the sedimentation behavior characteristic of the native enzyme (figure not shown). A close parallel between the ability of glucose 6-phosphate to dimerize the enzyme and its kinetic effects on the enzyme reaction is thus evident.
FIG. 2. Variation of sedimentation coefficients of brain hexokinase with concentration. For details, see "Materials and Methods."

FIG. 3. Sedimentation behavior of low and high concentration of brain hexokinase on a glycerol gradient in presence and absence of glucose 6-phosphate. For details of procedure, see "Materials and Methods." Corrections are made for slight differences in the number of fractions collected per tube. Fractions are numbered from bottom to top. A, protein concentration, 0.5 mg per ml; C---O, without glucose 6-phosphate; ●---●, with 0.1 mM glucose 6-phosphate; ---, sedimentation behavior of marker proteins with arrowheads indicating their positions. B, protein concentration, 10 mg per ml; O---O, without glucose 6-phosphate; ●---●, with 0.1 mM glucose 6-phosphate; ---, sedimentation behavior of marker proteins with arrowheads indicating their positions. It should be noted that the presence of glucose 6-phosphate in the gradient at 0.1 mM concentration did not interfere with the hexokinase assay based on the use of glucose 6-phosphate dehydrogenase. Whatever little glucose 6-phosphate was introduced into the assay mixture was removed rapidly and did not affect the rate.

also have shown, respectively, that type I hexokinases from pig heart and rat brain contain a single polypeptide chain.

At this stage it was of interest to find out whether the inhibitory role of glucose 6-phosphate in the brain hexokinase reaction could be ascribed to the dimerization of the enzyme in the reaction mixture. To establish such a correlation the effect of glucose 6-phosphate had to be studied at low enzyme concentrations as normally employed in an assay system (about 50 μg per ml). This effect was studied by velocity sedimentation on a glycerol density gradient using a preparative ultracentrifuge. The results are presented in Fig. 3A. The sedimentation profiles show that, at very low enzyme concentrations, the sedimentation coefficient of the enzyme in the presence and absence of glucose 6-phosphate is almost the same (about 5.8 S), indicating that glucose 6-phosphate has no demonstrable effect on the sedimentation property of hexokinase at low protein concentrations.

Since the above result strongly indicated that the glucose 6-phosphate-mediated dimerization of the enzyme was concentration-dependent, a study was carried out on the ultracentrifugal behavior of the enzyme at different protein concentrations in presence of 0.1 mM glucose 6-phosphate. Fig. 3 presents such a study. The ultracentrifugal patterns included in the figure show that at relatively high enzyme concentration, most of the enzyme sediments as a fast moving peak (Fig. 3A). The relative area under the fast moving peak decreases as enzyme concentration decreases (Fig. 3B). As concentration of the enzyme
fast moving peaks or of the major peak, runs was 45°. Protein concentration was 5.5 mg per ml in all
inorganic phosphate, 10 mM; glucose, 1 mM. Bar angle in all
picture.

7.9 S, 16 and 84%; B, 40°, 3.6 mg per ml, 7.9 S, 70%; C, 40°, 1.8
mg per ml, 6.5 S at midpoint of the peak, areas not calculated
because of inadequate resolution of the peaks; D, 30°, 1.0 mg per
ml, 6.2 S, 100%. All pictures were taken 36 min after rotor
reached the top speed of 59,780 rpm. Temperature, 19-20°.

Fig. 4 (left). Effect of glucose 6-phosphate on the sedimenta-
tion velocity patterns of brain hexokinase at different protein
concentrations. For details of procedure see "Materials and
Methods." Glucose 6-phosphate was added at 0.1 mM concen-
tration. Bar angles, protein concentrations, sedimentation coeffi-
cients of slow and fast moving peaks or of the major peak, and
approximate per cent areas under the respective peaks are given
in that order for each picture. A, 45°, 5.5 mg per ml, 5.9 S and
7.9 S, 16 and 84%; B, 40°, 3.6 mg per ml, 7.9 S, 70%; C, 40°, 1.8
mg per ml, 6.5 S at midpoint of the peak, areas not calculated
because of inadequate resolution of the peaks; D, 30°, 1.0 mg per
ml, 6.2 S, 100%. All pictures were taken 36 min after rotor
reached the top speed of 59,780 rpm. Temperature, 19-20°.

Fig. 5 (right). Sedimentation velocity patterns of brain hexo-
kinase in the presence and absence of various metabolites. For
details of procedure, see "Materials and Methods." Concentra-
tions employed were: glucose 6-phosphate, 0.1 mM; ATP, 5 mM;
inorganic phosphate, 10 mM; glucose, 1 mM. Bar angle in all
runs was 45°. Protein concentration was 5.5 mg per ml in all
cases. Additions made, sedimentation coefficients of slow and
fast moving peaks or of the major peak, and the approximate per
cent areas under respective peaks are given in that order for each
picture. A, no addition, 5.6 S, 100%; B, glucose 6-phosphate,
5.4 S and 7.9 S, 16 and 84%; C, glucose 6-phosphate + ATP, 5.3 S,
100%; D, glucose 6-phosphate + inorganic phosphate, 5.8 S,
100%; E, glucose 6-phosphate + glucose; 5.3 S and 8.3 S, 20 and
is still lowered the slow and the fast moving peaks do not resolve
very well (Fig. 4C). At very low concentrations of the enzyme
(1.0 mg per ml), there is no noticeable difference between the
sedimentation behavior of the enzyme in presence and absence
of glucose 6-phosphate (Fig. 4D). This last result is as expected
on the basis of the experiment presented in Fig. 3A. No further
decrease in sedimentation coefficient was observed at concentra-
tions of the enzyme lower than 1 mg per ml (patterns not shown).

The concentration dependence of the dimerization process
was further confirmed by running high concentrations of the
enzyme on a glycerol gradient in presence of glucose 6-phosphate.
The results presented in Fig. 5B show that whereas in absence
of glucose 6-phosphate the enzyme sediments principally with a
sedimentation coefficient of about 5.7 S, in the presence of glu-
cose 6-phosphate the enzyme sediments as a diffuse boundary
with a mean sedimentation coefficient of about 7.3 S. The
diffuse boundary which was invariably observed in this experi-
ment probably indicates that the lighter and the heavier species
of hexokinase are not properly resolved during the run. A close
examination of the sedimentation profile of the enzyme at high
concentration, in the absence of glucose 6-phosphate shows that
a small fraction of the native enzyme sediments in the position
of the dimer. The significance of this fact is discussed later on.
Easterby (20) has reported a similar aggregating tendency in
pig heart hexokinase.

The above experiments led us to the conclusion that, in pres-
ence of glucose 6-phosphate, brain hexokinase exists in a type of
conformation, with a strong tendency to dimerize. The ques-
tion that remained was whether this conformational change
would have any physiological significance. This question could
be answered by studying the response of this dimerizing system
to those metabolites which are known to relieve the inhibition
of hexokinase by glucose 6-phosphate. Such metabolites are
ATP (5, 17, 21-23) and inorganic phosphate (5, 16, 24, 25).
The effect of these substances on the glucose 6-phosphate-hexo-
kinase system are shown in Fig. 5. In Fig. 5A the sedimenta-
tion behavior of native hexokinase is presented. Fig. 5B shows
that brain hexokinase exists mostly as a dimer in the presence of
0.1 mM glucose 6-phosphate. If as shown in Fig. 5, C and D,
ATP and inorganic phosphate are added to this system at concen-
trations of 5 mM and 10 mM respectively, the enzyme reverts
to its normal sedimentation behavior. On the other hand glu-
cose, known to be ineffective in reversing the inhibition of hexo-
kine by glucose 6-phosphate (2, 21), also failed to reverse the
hexokinase reaction (2, 23), has no comparable effect on the
regularization behavior of brain hexokinase (experiment not
shown). As shown earlier (6), fructose 6-phosphate is also
without any significant effect on the reactivity of sulphydryl
residues of the enzyme toward 5,5'-dithiobis(2-nitrobenzoic
acid). The result indicates the specificity of the glucose 6-phos-
phate effect and points to its physiological significance. It is
interesting to note that ADP, also a product of the hexokinase
reaction which has been implicated in its regulation (26, 27)
does not modify the sedimentation behavior of the enzyme even
at 10 mM concentration.

In considering the physiological significance of the above re-
80%. All pictures were taken 36 min after rotor reached top
speed of 59,780 rpm. Temperature, 19-20°.
these metabolites were obtained at concentrations of the enzyme phosphate is the result of a conformational change induced by it used in the assay system (about 50 pg per ml). Although it is reasonable to suppose that the physical effects of this ligand on the enzyme, described above, have a bearing on the kinetic effects of glucose 6-phosphate in the enzyme molecule, such a conformational change has not been directly shown in the present case at low enzyme concentrations. In spite of this limitation of our studies, it is tempting to speculate that the physical effects of this ligand on the enzyme, concentrations. In spite of this limitation of our studies, it is tempting to speculate that the physical effects of this ligand on the enzyme, described above, have a bearing on the kinetic effects of glucose 6-phosphate in the brain hexokinase reaction.

At this stage it is necessary to comment on our observation that at high enzyme concentrations a small fraction of the enzyme is present in a dimeric state even in the absence of glucose 6-phosphate (Fig. 3B). This may mean that in the native state, a conformer of the enzyme exists (albeit at very low concentrations) which has a strong tendency to dimerize. The glucose 6-phosphate-induced dimerization may then merely mean that the ligand binds to this conformer preferentially, and this shifts the equilibrium in its favor. The present data is not sufficient to distinguish between this possibility and its alternative that the glucose 6-phosphate itself induces a conformational change in the enzyme which makes it susceptible to dimerization.

Glucose 6-phosphate-mediated dimerization of brain hexokinase reported here may be apparently related to the aggregation of glucose 6-phosphate-solubilized brain hexokinase observed by Craven and Basford (28). The validity of the latter observation is, however, somewhat in doubt in view of the report by Wilson (29) that some proteins behave anomalously when subjected to chromatography on Sephadex G-200 under the conditions used by Craven and Basford (28).

The conformational alteration imposed on the enzyme by glucose 6-phosphate also suggests that its locus of action as a regulator of brain hexokinase may be at an allosteric site as suggested by Kosow and Rose (30). But any unequivocal conclusion in this respect would have to await detailed investigations on the binding behavior of glucose 6-phosphate towards this enzyme. Such studies are currently in progress in our laboratory.

Note Added in Proof—Wilson (31) has recently reported a concentration-dependent aggregation of rat brain hexokinase in presence of glucose 6-phosphate. In their system, they have been able to show a small increase in sedimentation coefficient in presence of glucose 6-phosphate even at low concentrations of the enzyme.

REFERENCES