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Allosteric Inhibition of Brain Hexokinase by Glucose 6-Phosphate in the Reverse Reaction[1]

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A study of the reverse reaction of rat brain hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) has been performed using a photometric method based on a mutarotase-glucose oxidase-peroxidase-chromogen system to trap and visualize glucose, plus a glycerol kinase-glycerol system to trap ATP. Glucose 6-phosphate or 2-deoxyglucose 6-phosphate were used as phosphoryl donors at different concentrations of ADP. Variation of glucose 6-phosphate concentrations resulted in a biphasic curve from which apparent K_m and K_i values of ca. 0.2 mM were calculated. In contrast, variation of 2-deoxyglucose 6-phosphate concentrations resulted in Michaelian kinetics with an apparent K_m of 2 mM. The K_m value for MgADP was 16 mM irrespective of the nature and concentration of the hexose 6-phosphate substrate. These results are fully consistent with an allosteric site for glucose 6-phosphate as an explanation for the inhibition of animal hexokinases by glucose 6-P and further indicate that the maximal rate is the parameter affected. From these observations and previous knowledge, the possible occurrence in animal hexokinases of a regulatory site for ATP to account for the competition between glucose 6-phosphate and ATP in the forward reaction is postulated.

The first postulate of a specific regulatory site in an enzyme was formulated by Crane and Sols in 1954 (1) on the basis of the differential specificity of brain hexokinase for analogs of glucose as substrates and for analogs of Glc-6-P[5] as inhibitors. They concluded that . . . "brain hexokinase possesses, in addition to the binding sites for substrates and adenosine-triphosphate, a third specific binding site for Glc-6-P . . . for the control of the rate of the hexokinase reaction". This postulate was an obvious precursor of what, after Monod and co-workers (2), has come to be known as an "allosteric site" for metabolic regulation. The criteria for postulating allosteric inhibition by a primary product and the characteristics of such regulatory mechanism were later developed by Sols (3). Physiologically, inhibition of animal hexokinases by Glc-6-P has obvious significance as a feedback mechanism in the glucose phosphorylation pathway (4). Strong evidence for the operation of Glc-6-P inhibition in animal cells *in vivo* (5) and *in situ* (6) has been presented.

The presence of an allosteric site as an explanation for the inhibition of animal hexokinases by Glc-6-P has been challenged by Fromm and co-workers after

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[5] Abbreviations used: Glc-6-P, glucose 6-phosphate; dGlc-6-P, 2-deoxyglucose 6-phosphate.

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their finding that this inhibition is competitive *versus* ATP (7). They suggested that the phosphate of Glc-6-P competes with the γ-phosphoryl group of ATP at the ATP site on the enzyme while also hinting that an adjacent site for the hexose moiety of Glc-6-P different to that for glucose may exist (8–10).

Early studies on the binding of Glc-6-P to animal hexokinases by Ellison *et al.* (11) and by Wilson and co-workers (12, 13) were interpreted as indicating only one binding site. However, Lazo *et al.* (14) have recently shown that two nonoverlapping binding sites for Glc-6-P exist in brain hexokinase. This conclusion has been disputed by Solheim and Fromm (15) on the basis of kinetic observations of the reverse reaction giving a K_m value for Glc-6-P almost as low as the K_d reported by Lazo *et al.* for the site with the specificity corresponding to the putative allosteric site.

Evidence is now reported here that Glc-6-P, but not dGlc-6-P, inhibits its own utilization as substrate for the reverse reaction by brain hexokinase, in contrast to the behavior of Glc-6-P with yeast hexokinase. Moreover, it has been found that the inhibition of the reverse reaction by excess Glc-6-P is noncompetitive with respect to MgADP. These observations rule out the hypothesis that Glc-6-P inhibition of the forward reaction is due to steric hindrance with respect to the γ-phosphate of ATP, and lead to the suggestion that the relief of Glc-6-P inhibition by ATP may involve a regulatory site for ATP different from the substrate site.

EXPERIMENTAL PROCEDURES

Enzyme purification. Hexokinase A was purified from rat brain mitochondria using the procedure of Chou and Wilson (16) with the following modifications (suggested by John E. Wilson, personal communication): (a) the sucrose solution was replaced throughout by 0.01 M glucose, 0.01 M thioglycerol; (b) the pH of the original homogenate and of the resuspended particles was adjusted to pH 8.2 before each centrifugation; and (c) solubilization by 1 mM Glc-6-P was performed at pH 8.2 for 30 min at room temperature. Therefore, the enzyme preparation can be considered as the highly bindable form of hexokinase described by Polakis and Wilson (17). The purified preparation after the DEAE-cellulose step had a specific activity of 46 units/mg protein and was stored at 4°C in 0.01 M Tris, 0.01 M thioglycerol, 0.001 M EDTA, pH 7. Prior to use, aliquots were filtered through small Sephadex G-25 columns in order to remove thioglycerol, which interferes in the assay to be described below.

Assay of hexokinase in the reverse reaction. The assay for the reverse hexokinase reaction was patterned after the system used by DelaFuente and Sols (18) and is based on the following coupled reactions:

$$\text{Glc-6-P} + \text{MgADP} \xrightarrow{\text{hexokinase}} \text{glucose} + \text{MgATP} \quad [1]$$

$$\alpha\text{-Glucose} \xrightarrow{\text{mutarotase}} \beta\text{-glucose} \quad [1b]$$

$$\beta\text{-Glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{glucono-}\gamma\text{-lactone} + H_2O_2 \quad [2]$$

$$H_2O_2 + \text{reduced chromogen} \xrightarrow{\text{peroxidase}} \text{oxidized chromogen} \quad [3]$$

$$\text{MgATP} + \text{glycerol} \xrightarrow{\text{glycerol kinase}} \text{MgADP} + \textit{sn}\text{-glycerol 3-P.} \quad [4]$$

The assay mixture contained, in a final volume of 0.5 ml, 40 mM Tris-maleate buffer, pH 7, 10 μg peroxidase, 50 units glucose oxidase, 150 μg 2,2′-azino-di-(3-ethylbenzthiazolinesulfonic acid), 10 units mutarotase, 0.5 unit glycerol kinase, 10 mM glycerol, MgADP, either Glc-6-P or dGlc-6-P at the indicated concentrations, and 0.3–0.6 unit (forward reaction) of brain hexokinase. In a few experiments, the yeast enzyme was used for comparison. Mg^{2+} concentrations were adjusted in each case according to the concentration of ADP so that a level of free divalent ion of 1 mM was always present. Blanks with either ADP or hexose 6-phosphate omitted were routinely included. Under the described conditions, variation of the amount of hexokinase in the mixture [up to 0.8 unit (forward reaction), the highest amount used] resulted in linear variation of the amount of oxidized chromogen (not shown). Although the mixture (minus substrates and hexokinase) was stable when stored at 4°C, it was usually freshly prepared when used.

The reaction was followed at 420 nm in a Gilford Model 2400 spectrophotometer at room temperature (ca 25°C). A lag of about 5 min was usually observed. Continuous recording of absorbance was routinely followed for 30 min.

Assay of hexokinase in the forward reaction. During the purification of brain hexokinase and for routine control measurements, formation of Glc-6-P was followed spectrophotometrically at 340 nm by coupling the reaction to the formation of NADPH by Glc-6-P dehydrogenase. The reaction mixture contained, in a final volume of 0.5 ml, 40 mM Tris-maleate buffer, pH 7, 0.5 mM $NADP^+$, 0.5 unit of

Glc-6-P dehydrogenase, 1 mM glucose, 5 mM ATP, 6 mM $MgCl_2$ and hexokinase.

Protein determination. Protein was measured by the procedure of Lowry *et al.* (19).

Chemicals. Glucose oxidase and peroxidase were the products of Boehringer. Mutarotase, glycerol kinase, yeast hexokinase (C-300), ATP, ADP, $NADP^+$, Glc-6-P, dGlc-6-P, and 2-2'-azino-di-(3-ethylbenzthiazolinesulfonic acid) were purchased from Sigma Chemical Company. Other chemicals were of the highest purity commercially available.

RESULTS

The glucose oxidase-dependent assay for the reverse reaction of hexokinase permits the comparative study of Glc-6-P and dGlc-6-P as alternative substrates. The latter is a product of the forward reaction which is not inhibitory for the brain enzyme (1). The marked contrast in their behavior for the reverse reaction is shown in Fig. 1. With Glc-6-P as the variable substrate, a biphasic curve showing marked inhibition by "excess substrate" was obtained at 5 mM (Fig. 1) or 20 mM MgADP (not shown). In contrast, the curve obtained with dGlc-6-P as variable substrate shows hyperbolic kinetics without inhibition by excess substrate.[6] Apparent K_m and K_i values for Glc-6-P (obtained from double-reciprocal plots) were about the same, ca. 0.2 mM. The K_m value for dGlc-6-P was 2 mM.

The specificity of the effect of Glc-6-P on the rate of the reverse reaction of brain hexokinase can be clearly seen using yeast hexokinase, with which a hyperbolic curve was obtained (Fig. 1), confirming previous results (18).

Saturation curves for MgADP (Fig. 2) indicate hyperbolic kinetics with either 10 mM dGlc-6-P or Glc-6-P up to 2.5 mM. The pattern obtained was of the noncompetitive type. K_m values of 16 mM were obtained in either case.

DISCUSSION

The results presented in this paper, i.e., a biphasic curve with strong inhibition of the reverse reaction rate of brain hexokinase by increasing levels of Glc-6-P, but not by dGlc-6-P, plus the fact that the former has a "normal" behavior vis-à-vis yeast hexokinase, are fully consistent with the occurrence in animal hexokinases of the regulatory site for Glc-6-P postulated by Crane and Sols in 1954 (1) and supported by the binding studies of Lazo *et al.* (14).

The fact that increasing the concentration of Glc-6-P leads to complete inhibition of the reverse reaction rules out the possibility of a site at which Glc-6-P would sterically compete with either the γ phosphate of ATP or with some other part of the molecule (in which case it would also compete with ADP, which is not the case as shown in this paper). Therefore, the mechanism of the specific inhibition of animal hexokinase by Glc-6-P must involve a regulatory site different from the product site (1, 14), and also a conformational change (21).

Our results do not indicate a major difference in the values of the relative affinities of the two sites for Glc-6-P. This is at variance with the suggestion of Lazo *et al.* (14) that the high-affinity binding

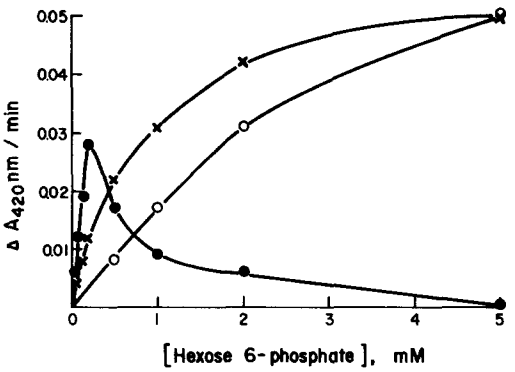

FIG. 1. Effect of the nature and concentration of the hexose 6-phosphate substrate on the rate of the reverse reaction of brain hexokinase, assayed as described under Experimental Procedures; MgADP concentration was 5 mM; 0.4 unit (forward reaction) of brain hexokinase was used. For comparison, parallel measurements using 0.35 unit of a commercial preparation of yeast hexokinase (Sigma C-300), and Glc-6-P as the variable substrate, are also included. ●, Glc-6-P, and ○, dGlc-6-P, with brain hexokinase; ×, Glc-6-P with yeast hexokinase.

[6] A very preliminary, and rather crude, experiment showing about the same results was performed several years ago by one of us (A.S.), and was reported in sketchy form (20).

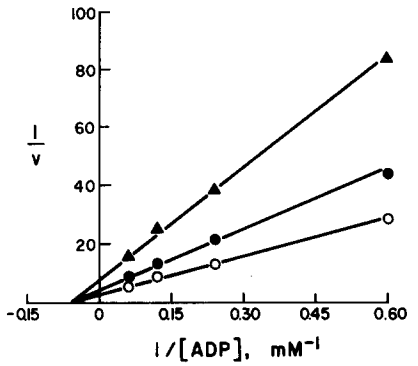

FIG. 2. Effect of the concentration of MgADP on the rate of the reverse reaction of brain hexokinase with either dGlc-6-P or two different concentrations of Glc-6-P; assayed as described under Experimental Procedures, except for substrates as indicated: ○, 10 mM dGlc-6-P; ●, 0.1 mM Glc-6-P; ▲, 2.5 mM Glc-6-P; 0.8 unit (forward reaction) of brain hexokinase was used.

site for Glc-6-P corresponds to the allosteric site. Also, from kinetic observations, Solheim and Fromm (15) concluded that the high-affinity site for Glc-6-P was the active site. It is very difficult to account for differences in the reported values of apparent affinities since different approaches, assay conditions, and enzyme preparations have been involved in the actual measurements of those values. On the other hand, it seems important to emphasize the qualitatively different behavior of Glc-6-P or anhydroglucitol-6-P, which bind easily and inhibit brain hexokinase, contrasted with that of mannose-6-P and dGlc-6-P, which are at least a hundred fold less efficient (1, 14).

Inhibition by excess Glc-6-P of the reverse reaction of brain hexokinase is noncompetitive with MgADP (Fig. 2), a surprising fact since inhibition of the forward reaction by Glc-6-P is known to be competitive with ATP (7). Since different mechanisms for inhibition by Glc-6-P in the forward and reverse reactions are unlikely, we propose, as an alternative, more plausible possibility, that the allosteric inhibition by Glc-6-P of the forward V_{max} of the hexokinase reaction can be counteracted by ATP acting on a regulatory site. These interactions would appear as competition by Glc-6-P versus the nucleotide substrate in the forward, but not in the reverse, reaction.

Mechanistically, i.e., antagonistic pairs of allosteric effectors, the proposal has many precedents (22). From the physiological point of view, the short-term feedback regulation of the glucose phosphorylation pathway by its end-product, Glc-6-P, would be modulated by the end-product of the overall glycolytic pathway (4). Thus, a substantial hexokinase activity may be operating at Glc-6-P concentrations high enough to favor glycogen synthesis, but this gate of energy expenditure would be closed, through Glc-6-P inhibition, in the event that cellular ATP concentrations decrease.

Most phosphofructokinases have two sites for ATP, the substrate site and a regulatory site, with the latter displaying a narrower specificity than the former (22). Although differential specificity is not a prerequisite for allosteric sites, we tried to find such a phenomenon in brain hexokinase through the use of an alternative phosphoryl donor. Magnani *et al.* (23) reported that erythrocyte hexokinase can use MgITP as substrate. However, at 5 mM MgITP, brain hexokinase showed only about 1% of the rate with ATP (data not shown), thus precluding its use as an alternative substrate.

The postulated regulatory site for ATP could eventually be ascertained by binding studies. Baijal and Wilson (24) studied the binding of nucleotides by brain hexokinase and tentatively interpreted their findings as indicative of one single site for ATP. However, multiplicity of binding sites has not been unequivocally ruled out. We predict the presence of two binding sites for ATP, although whether the affinity of the second would be high enough for easy determination is not foreseeable. If two sites were found, it should be expected that dGlc-6-P would displace ATP from the active site, that 1,5-anhydroglucitol-6-P would displace ATP from the allosteric site, and that Glc-6-P would be able to displace ATP from both sites. Also, binding of 1,5-anhydroglucitol-6-P

at the Glc-6-P allosteric site would likely be antagonized by ATP but not by ADP.

The demonstration of two sites for ATP in animal hexokinases would be a strong argument in support of the possibility that the allosteric regulation of this enzyme was developed by evolution of one of the two active sites resulting from gene duplication and fusion, from a hexokinase with a molecular weight of ca. 50,000 (as yeast hexokinase or liver glucokinase) to the ca. 100,000 of mammalian hexokinases (25-28).

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