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Rat brain hexokinase: A kinetic comparison of soluble and particulate forms

The previous suggestion[1] for the involvement of the soluble–particulate distribution in regulating brain hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) activity may be stated as:

(a) The relative distribution of the enzyme between soluble and particulate (bound to the mitochondria) forms is regulated by the intracellular level of metabolites affecting this distribution (*e.g.* glucose-6-*P*, ATP, P_i).

(b) During times of high energy requirement, these metabolite levels change in a manner conducive to increasing the relative amount of hexokinase bound to the mitochondria.

(c) In comparison with the soluble form, the particulate enzyme is more active due to an increased affinity for ATP and (or) a decreased susceptibility to inhibition by glucose-6-*P*.

That physiological concentrations of various metabolites can indeed influence the soluble–particulate distribution, at least *in vitro*[1,2], and that the concentrations of these metabolites do change in the expected manner during times of increased glycolytic rate[3,4] has been clearly demonstrated. The third point in the above proposal, *i.e.* that kinetic differences exist between the soluble and particulate forms, was based on extant kinetic studies of soluble and particulate hexokinases from ascites tumor[5], bovine brain[6–8] and frog skeletal muscle[9]. The purpose of the present investigation was to extend this kinetic comparison to the soluble and particulate forms of rat brain hexokinase. Further kinetic studies with the soluble and particulate hexokinase of ascites tumor have also been reported[10].

The rats used were adult males, Sprague–Dawley strain (175–250 g), maintained on stock laboratory animal diet. Brains were removed immediately after decapitation and chilled on ice. After homogenization in 10 vol. of cold 0.25 M sucrose, the samples were centrifuged at 1000 × *g* for 20 min at 0–4° and the pellet discarded. The suspension was then centrifuged at 40 000 × *g* for 20 min. The hexokinase in the supernatant will be referred to as the "soluble" enzyme. The pellet, resuspended in 4 vol. of 0.25 M sucrose (based upon original brain weight), is defined as the "particulate" activity. In some experiments, purified mitochondrial preparations were obtained by centrifugation through 1.2 M sucrose (40 000 × *g* for 1.5 h), but this resulted in no apparent change in kinetic properties and therefore was not routinely done.

Hexokinase was routinely assayed at 25° by coupling to the glucose-6-*P* dehydrogenase reaction and following the increase in absorbance at 340 nm due to NADPH formation. The reaction medium contained 50 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 0.3 mM $NADP^+$, the indicated amounts of ATP and glucose, and 1.0 I.U. of glucose-6-*P* dehydrogenase in a total volume of 1.0 ml. Appropriate controls verified the absence of a significant rate in the absence of added hexokinase and the linear response of the assay to added enzyme. Negligible solubilization (< 10%) of the particulate enzyme occurred during the time required to make initial velocity measurements. Alternatively, the glucose-6-*P* produced was determined directly by using [^{14}C]glucose as substrate. The reaction was run at 25° in 20 μl of solution containing 20 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM glucose, 5.0 mM ATP, 0.1 μC

of uniformly labeled D-[^{14}C]glucose, and the indicated additions. The reaction was stopped by heating in a boiling-water bath for 20 sec, carrier glucose-6-*P* added, and aliquots spotted on Whatman No. 1 paper. After electrophoresis in pyridine–acetic acid–water (100:4:900, by vol.; pH 6.5) (ref. 11) at 2000 V for 1.5 h, the paper was dried at 170° for approx. 1 h. Glucose and glucose-6-*P* spots could then be seen as fluorescent areas under ultraviolet light. (The reaction responsible for the production of fluorescence with these compounds is not known but was found to be time and temperature dependent.) Alternatively, the radioactive areas were located with a Packard Radiochromatogram Scanner. The spots were cut out and radioactivity determined in a Packard Liquid Scintillation Counter. The [^{14}C]glucose used contained only one detectable contaminant (about 0.01%), which moved identically with authentic glucose-6-*P*. All values have been appropriately corrected.

The K_m (average of 3–5 experiments, ± average deviation) for glucose (at either 4 mM or 8 mM ATP) was 0.029 ± 0.006 mM for the soluble enzyme and 0.053 ± 0.007 mM for the particulate enzyme. The K_m values for ATP (at either 2 mM or 25 mM glucose) were 0.24 ± 0.01 mM and 0.20 ± 0.03 mM for the soluble and particulate forms, respectively. These values are in reasonable agreement with those found at 37° using a 150-fold purified preparation of the soluble enzyme[12].

N-Acetylglucosamine inhibited competitively with glucose. The soluble and particulate enzymes were equally sensitive to this inhibitor, with apparent K_i values of 0.17 mM and 0.15 mM, respectively.

Glucose-6-*P* inhibition and its reversal by P_i were studied with the assay employing radioactive glucose. The reaction proceeded linearly for the first 1.5–2 min, then slowly departed from linearity due to the buildup of inhibitory concentrations of glucose-6-*P*; linearity could be restored by the addition of $NADP^+$ and glucose-6-*P* dehydrogenase to the reaction mixture. Virtually identical results were reported by KOSOW AND ROSE[10] who used a very similar assay technique to study the soluble and particulate hexokinases of ascites tumor. Relative reaction rates during the first minute of reaction, when the departures from linearity are minimal, are given in Table I. Clearly the soluble enzyme is appreciably more susceptible to inhibition by glucose-6-*P*. Define the dissociation constants

$$K_{is} = \frac{[S]\,[I]}{[SI]} \text{ and } K_{ip} = \frac{[P]\,[I]}{[PI]}$$

for the complexes of the inhibitor (I), glucose-6-*P*, with the soluble (S) and particulate (P) forms of the enzyme, respectively. The assumption that the enzyme–inhibitor complexes (SI and PI) are inactive is compatible with the competitive nature of the inhibition of ATP utilization by glucose-6-*P* (refs. 6, 8, 12). With this assumption, one may take the observed activity in the presence of the inhibitor as a measure of the amount of free enzyme. Using the data in Table I, one may calculate (since $[S]$, $[P] \ll [I]$)

$$\frac{K_{ip}}{K_{is}} = \frac{(37)(0.25)}{(63)} \cdot \frac{(90)}{(10)(0.25)} = 5.3$$

Thus we estimate that the particulate form is approx. 5 times less sensitive to glucose-6-*P* inhibition, as measured by K_i values. This is quite comparable to the differences observed between soluble and particulate hexokinases from other

TABLE I

EFFECT OF P_i ON INHIBITION OF HEXOKINASE BY GLUCOSE-6-*P*

The assay employing [^{14}C]glucose was run as described in the text. The data represent the relative amounts of glucose-6-*P* produced during the first minute of the reaction. Equal amounts (6.7 munits) of either the soluble or particulate enzyme were used. The reaction rates with no additions were, as expected, virtually identical and corresponded to the incorporation of 4000 counts/min into glucose-6-*P*

Additions	*Relative hexokinase activity*	
	Soluble	*Particulate*
None	100	100
0.25 mM glucose-6-*P*	9.6	37
0.25 mM glucose-6-*P*, 0.5 mM P_i	14	45
0.25 mM glucose-6-*P*, 2.5 mM P_i	28	69

sources[8–10,13]. Although some solubilization of the particulate enzyme undoubtedly occurs during the assay, this could only have the effect of lessening the observed differences. Thus, the actual differences between the soluble and particulate forms are at least as great as indicated from these results.

The present results give no indication of any appreciable difference in the affinity (as inferred from the K_m) of the soluble and particulate forms for the substrates, ATP and glucose, but they do suggest that the soluble enzyme is indeed more susceptible to glucose-6-*P* inhibition than is the particulate form. These results are therefore consistent with the previously proposed regulatory mechanism[1].

It seems to be generally agreed that regulation of hexokinase activity in a variety of mammalian tissues is due to alterations in the degree of inhibition resulting from changes in intracellular glucose-6-*P* levels[14–18]. The proposed involvement of the soluble–particulate distribution in the regulation may be considered simply as a means of amplifying the regulatory signal represented by the intracellular glucose-6-*P* level.

The dissociation of hexokinase from the mitochondria can be represented as[19]:

$$MS \leftrightarrows M + S$$

where MS is the particulate form, S is the soluble enzyme, and M is the vacated binding site on the mitochondrial membrane. If the solubilizing action of glucose-6-*P* and ATP is due to the "pulling" of the reaction to the right by complexing with the enzyme, it is apparent that the binding constants for the complexing of S with ATP and glucose-6-*P* must be considerably greater than those for the particulate enzyme. The kinetic experiments (refs. 6, 8, 10, 13 and this paper) are in accord with the view that the soluble form does indeed interact more strongly with glucose-6-*P*. Furthermore, P_i reverses both the inhibition and the solubilization activity[1,19] of glucose-6-*P*. Finally, glucose-6-*P* is a competitive inhibitor of ATP in both the catalytic[6,8,12] and solubilization[1] reactions. These results would be consistent with the view that both solubilization and inhibition are the result of the binding of glucose-6-*P* to a single site on the enzyme, with the soluble enzyme having greater affinity for glucose-6-*P*.

In contrast, the K_m values suggest no difference in the affinity of the soluble and particulate forms for ATP. It is of course quite possible that the kinetic factors

determining K_m are such that this kinetic constant is *not* a valid indication of the substrate binding constant. Alternatively, solubilization may be the result of binding ATP to a site other than the catalytic site.

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