PURIFICATION OF NONBINDABLE AND MEMBRANE-BINDABLE MITOCHONDRIAL HEXOKINASE FROM RAT BRAIN

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SUMMARY: $MgCl_2$-induced binding of glucose-6-P solubilized rat brain hexokinase to rat liver mitochondria has been found to be markedly diminished by increasing ionic strength. Using a modified assay of binding ability, it has now been possible to demonstrate that purified preparations of brain hexokinase do retain appreciable ability to bind to mitochondria. A slight modification of the previous DEAE-cellulose chromatography procedure (4), permits resolution of the hexokinase into two major components designated as Type I_b and Type I_n based on their ability to bind and not bind, respectively, to mitochondria. I_b and I_n appear to be identical in molecular size and subunit composition, but differ slightly in net charge.

It has been demonstrated (1-3) that the Type I isozyme of hexokinase, as found in brain, selectively binds to the outer mitochondrial membrane, presumably indicating some specific component (or components) of this membrane which selectively interacts with the enzyme. In order to better understand the nature of the interaction between hexokinase and the outer mitochondrial membrane it would be advantageous to purify the enzyme and the requisite component(s) of the membrane. Although the enzyme has been purified to homogeneity (4), it was not previously possible to demonstrate that the purified enzyme retained the ability to interact with the mitochondrial membrane. It is the purpose of this communication to describe a method for resolution of purified bindable and nonbindable forms of hexokinase and to indicate some of the reasons why this was not observed earlier.

MATERIALS AND METHODS

Chemicals and rats were obtained from commercial sources given earlier (4,5). Hexokinase was assayed as described previously (4) except that the NADP and glucose-6-P dehydrogenase concentrations were doubled. Rat liver mitochondria were prepared according to the method of Sottocasa et al. (6).

Three times washed rat brain particles (5) and glucose-6-P solubilized hexokinase (7) were obtained as previously described. Rat brain hexokinase was purified according to Chou and Wilson (4) except for slight modification in the DEAE-cellulose column procedure (see Fig. 3).

Binding ability was assayed by incubation of the enzyme with excess mitochondrial binding sites. Except where noted (Fig. 1), the conditions were as described previously (5) with the following modification: a) hexokinase preparations were diluted with 0.25 M sucrose such that the total ionic strength in the incubation medium did not exceed 0.005 M, b) rat brain particles were used in place of rat liver mitochondria, and c) incubation was at 25° rather than 0°. These modifications resulted from observations that rat brain mitochondria bound an appreciably greater proportion of the hexokinase activity than did liver mitochondria, and the binding was less susceptible to inhibition by increasing ionic strength (see Fig. 1); with brain particles (but not with liver mitochondria), binding was also enhanced by increasing the temperature to 25°. Binding assays were done

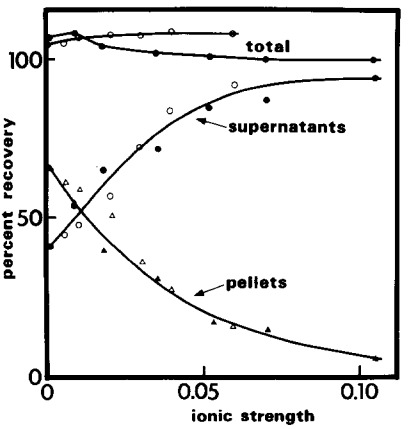

Fig. 1. Inhibition of Binding by Increasing Ionic Strength. Binding ability of glucose-6-P solubilized hexokinase was assayed as described previously (5), using rat liver mitochondria. The indicated salt concentrations were added to the tubes before initiating binding with $MgCl_2$. Open symbols (o,Δ) represent KCl and closed symbols (●,▲) potassium phosphate (pH 7.0).

in polypropylene microcentrifuge tubes (Brinkmann Instruments) which had been dipped in 2% bovine serum albumin (BSA) solution then dried at approx. 60°; coating with BSA was found to prevent artifacts due to adsorption of the hexokinase to polypropylene (unpublished observation).

RESULTS

From Fig. 1, it is clear that low ionic strength severely inhibits the $MgCl_2$ - induced binding of glucose-6-P solubilized hexokinase to liver mitochondria, which have previously been routinely used for the binding assay (5). Fifty percent inhibition occurs at 0.02-0.03 M ionic strength.

Using a modified binding assay (see Methods), it has been possible to show for the first time that purified hexokinase can bind to rat brain particles (Fig. 2). In this experiment more than 50% of the enzyme was bound when excess binding sites were present.

When the conditions for DEAE-cellulose column chromatography used by Chou and Wilson (4) are slightly modified by using a shallower gradient of KCl, elution patterns such as those shown in Fig. 3 are obtained. Under these conditions, two peaks of hexokinase, one at about 0.065 M KCl and the other at about 0.075 M KCl, have been reproducibly resolved (eight experiments).

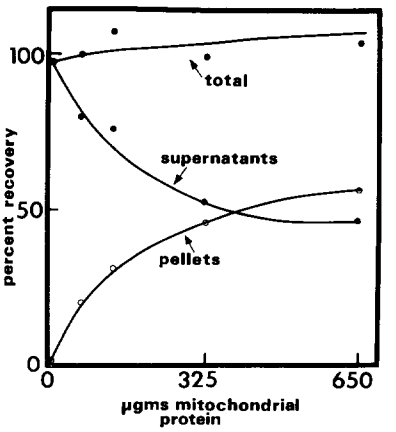

Fig. 2. Binding of Pure Hexokinase. Hexokinase (0.065 units, purified according to Chou and Wilson (4)) was incubated with brain particles as described in Methods. Total volume was 0.25 ml.

The relative amounts of activity in the two peaks have, however, varied from about 75%:25% to 25%:75%; the reasons for this remain under investigation.

Fig. 3 also indicates that the enzyme eluted at higher KCl concentrations is at least partly bindable to brain particles, while hexokinase eluted at lower salt concentrations is completely nonbindable. For this reason we refer to these two forms of the enzyme as Type I_b for the bindable enzyme, and Type I_n for nonbindable. The asymmetry of the eluted peaks, together with preliminary analytical isoelectric focusing experiments, suggest that

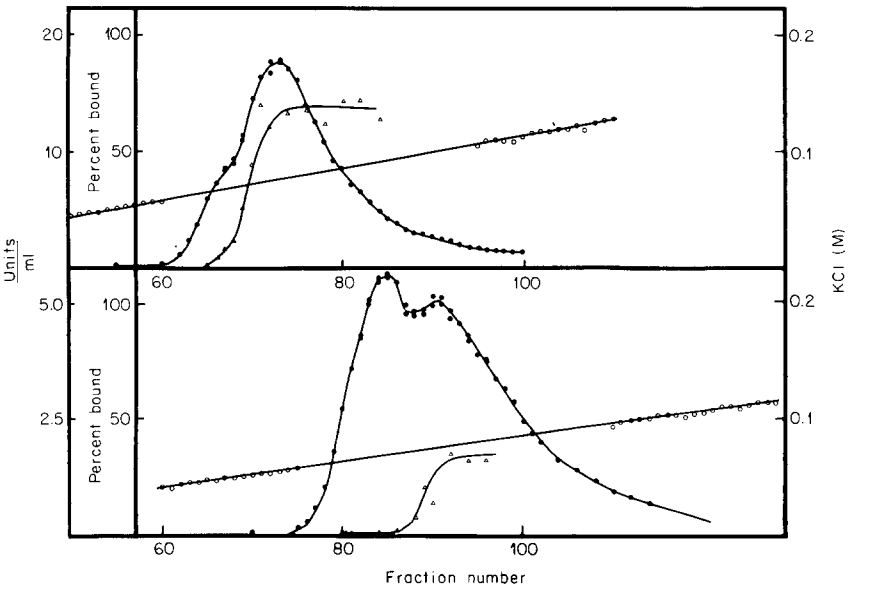

Fig. 3. DEAE-Cellulose Column Chromatography of Rat Brain Hexokinase. Chromatography was performed as previously described (4) except that shallower salt gradients were used. In the upper elution pattern a 600 ml linear gradient from 0.0 to 0.20 M KCl in column buffer was used, collecting 3.8 ml fractions. The lower elution pattern was done exactly the same except the gradient went from 0.0 to 0.15 M KCl. The KCl concentration (o) was determined by conductivity measurements on the fractions. Hexokinase activity (●) and percent bound (Δ) were measured as described in the Methods section. In both experiments bindable enzyme was found only in the high salt peak (0.075 M KCl). The proportion of the enzyme in the second peak which could be bound was, however, found to vary in different experiments e.g. in the lower profile, only 35% of the activity in the second peak was bindable, whereas in the experiment shown in the upper profile, 70% was bindable.

Type I_n and Type I_b are in turn composed of more subforms (at least six total). Variation in the percentage bindability of Type I_b (see Fig. 3) also suggests that some of these subforms are nonbindable.

DISCUSSION

It had been previously observed that high ionic strength could cause solubilization of mitochondrial hexokinase (1, 8, 9). In contrast, low ionic strengths actually appeared to slightly enhance the strength of association of the enzyme with the membrane (8). In view of this observation, it was presumed that low to moderate ionic strengths would have little or no effect on the $MgCl_2$-induced rebinding of the enzyme to mitochondria. The results presented here show that this presumption was incorrect. Furthermore, we have also found that (for reasons which remain under investigation) binding of hexokinase by brain mitochondria is less susceptible to the ionic strength effect than is binding by liver mitochondria which have, for reasons of practicality (5), previously been routinely used in assays of binding ability. Using this new information, and making appropriate modifications of the binding assay, it has now been possible to demonstrate that purified brain hexokinase can bind to mitochondria.

Furthermore, slight modification of the previously described DEAE-cellulose chromatography procedure (4) has permitted the demonstration of a previously undetected heterogeneity of the enzyme. Two major forms, designated Type I_n and Type I_b have been resolved. Although I_n and I_b are readily distinguished by the difference in their ability to interact with the mitochondrial membrane, the physical or chemical basis for this distinction remains under investigation. Since mitochondrial enzyme (presumably all Type I_b) serves as the starting point for the purification, it seems quite probable that Type I_n represents, at least to some extent, an artifactual form produced in variable amounts (see Fig. 3) during purification. The enzyme does not contain detectable carbohydrate (10, and J. E. Wilson unpublished observations) or lipid (5), so the difference between I_b and I_n

is not likely to be due to such factors. Partial proteolysis during purification of the enzyme has not been entirely ruled out, but seems unlikely in that I_b and I_n have not been found to differ in N-terminal amino acid (by the dansylation technique) or apparent molecular weight (98,000 by SDS-gel electrophoresis). [1] The latter results suggest that the principle difference between I_b and I_n lies in a modification affecting their net charge, with I_b being more negatively charged (based on its greater affinity for DEAE-cellulose) than is I_n. Such modification might include phosphorylated and dephosphorylated forms e.g. perhaps I_b is a phosphorylated form which is (enzymatically?) converted to the dephosphorylated (and thus less negative) I_n during purification with resulting loss of binding ability.

ACKNOWLEDGEMENT

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[1] These results are consistent with earlier studies using the enzyme prepared according to the original Chou and Wilson (4) procedure. These enzyme preparations probably were, in view of the present observations, mixture of I_b and I_n, but were found to be homogeneous with regard to molecular weight (e.g., SDS-gels or centrifugal methods).