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GLUCOSE 6-PHOSPHATE-DEPENDENT BINDING OF HEXOKINASE TO MEMBRANES OF ASCITES TUMOR CELLS

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SUMMARY

A pH-dependent, saturable binding of hexokinase isozyme I from Ehrlich ascites carcinoma to plasma membrane and microsome preparations from the same tissue is demonstrated. This binding is enhanced by glucose 6-phosphate and may be considered as the sum of a glucose 6-phosphate-dependent binding and an independent binding. The half saturation concentration of hexokinase is about 0.4 unit per ml for both types of binding, and a maximal binding of 0.5–2.0 units per mg membrane protein is observed for both, although the pH optimum of the independent binding (5.4) is lower than that of the dependent binding (5.9). The half saturation concentration of glucose 6-phosphate required for the dependent binding is 0.05 mM at pH 6.1. 2-Deoxyglucose 6-phosphate competatively reverses the effect of glucose 6-phosphate on binding but does not diminish its inhibition of hexokinase activity.

INTRODUCTION

In 1954 Crane and Sols [1] noted that a substantial fraction of the hexokinase in homogenates of mammalian cells was associated with the particulate fraction. Later work [2–4] supported the conclusion that this particulate bound hexokinase resided in the mitochondria, and the binding to mitochondria from sarcoma 37 ascites tumor cells was characterized in some detail by Rose and Warms [5], who reported that the enzyme was specifically eluted from the binding sites by low concentrations of glucose 6-phosphate (< 0.1 mM) and higher concentrations of the nucleoside triphosphates. Although most attention has been directed toward the mitochondria, reports of binding to other subcellular fractions have also appeared. Emmelot and Bos [6] found that while plasma membranes from normal liver cells contained no hexokinase, those from a rapidly proliferating hepatoma bound substantial amounts of the enzyme. This observation was confirmed with another strain of hepatoma [7], but membranes from "minimal deviation" hepatomas showed little binding [8, 9].

The suggestion that malignant cells might possess a specialized mechanism for localizing hexokinase on cellular membranes prompted us to examine the binding of this enzyme to membranes of Ehrlich ascites carcinoma. J. Molnar had noted

earlier that plasma membrane preparations from these cells usually contained some hexokinase activity (personal communication, 1968). The work reported below describes a pH-dependent, saturable binding of hexokinase isozyme I of ascites tumor cells to plasma membranes or microsome preparations from the same cells; this binding is shown to be specifically enhanced by low levels (< 0.1 mM) of glucose 6-phosphate.

METHODS

Preparation of enzyme and membranes. A hyperdiploid strain of Ehrlich ascites carcinoma was grown in CF-1 mice for 7 days, harvested, and washed in 0.25 M mannitol containing 5 mM EDTA, pH 7.4. Cells were resuspended in 0.25 M sucrose containing 20 mM Tris · HCl, pH 7.2, 5 mM $MgCl_2$, 2 mM EDTA, and 0.5 mM dithioerythritol, homogenized three times by sudden decompression from 800 lb/inch^2 N_2 [10, 11] and fractionated by differential centrifugation. Mitochondria, washed three times in 0.25 mM sucrose, were treated with 1 mM glucose 6-phosphate to release the bound hexokinase, as described by Chou and Wilson [12]; the enzyme, in the presence of 5 mM phosphate, pH 7.2, 2 mM EDTA, 0.5 mM dithioerythritol, and 10 mM glucose, was purified by KCl gradient chromatography on DEAE-cellulose [12]. The major peak of activity, eluting at about 0.1 M KCl, corresponded to isozyme I and contained 10–15 units of hexokinase per mg protein. (A unit, as used here, is defined as μmol of glucose phosphorylated per min at 23 °C; protein was determined by the method of Lowry et al. [13]). The plasma membrane fraction was separated from the crude microsomal preparation on a discontinuous sucrose density gradient, as described by Molnar et al. [10]. Electron microscopy revealed smooth membranes with little ribosomal contamination. Membrane preparations were diluted in homogenizing medium.

Estimation of binding. In the typical binding experiment, a membrane preparation equivalent to 0.5 mg protein was suspended in 1 ml containing 10 mM Tris, 5 mM succinate, 0.5 mM dithioerythritol, 2 mM EDTA, 2.5 mM $MgCl_2$, and 0.5 unit hexokinase. The enzyme was passed through a low molecular weight exclusion polyacrylamide gel with a volume of a Tris/dithioerythritol/EDTA buffer immediately before use to remove the glucose, phosphate, and KCl components from the enzyme solution obtained in the purification procedure. The suspension was incubated for 30 min at 23 °C and centrifuged at $100\,000 \times g$ for 60 min. The supernatant was separated and the pellet was rinsed once with a layer of 0.1 ml incubation medium (without enzyme). Inclusion of sucrose in the first supernatant and analysis of sucrose in the pellet after the rinsing procedure indicated negligible contamination of the pellet with the original incubation medium. Hexokinase activity was assayed by NADP reduction, measured at 340 nm, in the presence of 100 mM Tris · HCl, pH 7.4, 8 mM glucose, 2 mM ATP, 10 mM $MgCl_2$, 0.5 mM NADP, and 2 units/ml glucose-6-phosphate dehydrogenase.

RESULTS AND DISCUSSION

The dependence of binding on hexokinase concentration

Fig. 1 illustrates the dependence of binding on the concentration of hexokinase

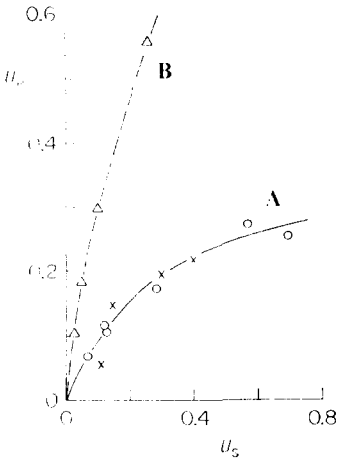

Fig. 1. Binding of hexokinase to membranes as a function of hexokinase concentration. U_p = units bound/0.5 mg protein; U_s = units/ml in solution. Curve A illustrates binding to plasma membrane (0) and crude microsome (×) fraction at pH 6.10. Curve B shows binding to plasma membrane at pH 5.68 in the presence of 1 mM glucose 6-phosphate.

(units/ml). Curve A represents the binding to both plasma membrane and unfractionated microsomes at pH 6.10: curve B indicates the increase in binding at lower pH (5.68) in the presence of 1 mM glucose 6-phosphate. The curves appeared to approach some saturation value, and double reciprocal plots of these and other similar data yielded linear relationships with half saturation values ranging from 0.2 to 0.5 units of hexokinase per ml. Comparable values were obtained in presence or absence of glucose 6-phosphate at pH values varying from 5.4 to 6.1, and all the curves could be approximated with the equation:

$$U_p = \frac{BU_S}{K_H + U_S} \tag{1}$$

where U_p = units bound/0.5 mg membrane protein; U_s = units of soluble hexokinase/ml; and K_H = the mean binding constant, 0.4 unit/ml.

Effect of pH and glucose 6-phosphate on binding

The constant B, which represents the maximal binding under the conditions used, proved to be highly sensitive to pH and was substantially increased by the presence of glucose 6-phosphate. For convenience, the maximal binding was resolved into two components:

$$B_T = B_O + B_G$$

Where B_O = binding in the absence of glucose 6-phosphate; B_T = total binding capacity; and B_G = change in binding caused by glucose 6-phosphate. These three parameters are shown as a function of pH in Fig. 2. The actual binding capacity varied from one membrane preparation to another, ranging from B_O = 0.17 to 0.94 unit/0.5 mg protein at pH 6.10; hence, all experiments were arbitrarily adjusted

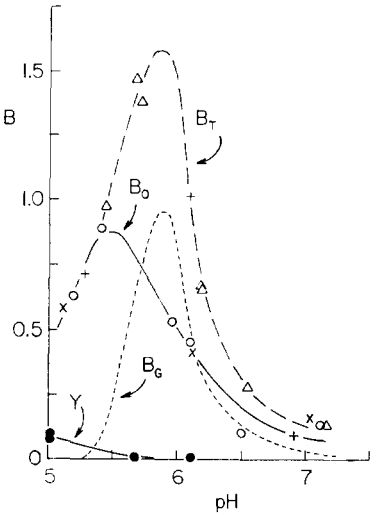

Fig. 2. Hexokinase binding capacity of membranes as a function of pH. Maximal binding (B) in units/0.5 mg protein was calculated from measured U_p and U_s values and a hexokinase binding constant (K_H) of 0.4 unit/ml; results from different experiments were adjusted to a B_O of 0.43 at pH 6.10 for comparison. Curve (B_T) gives the total binding to plasma membrane (Δ) and microsomes (+) in the presence 1 mM glucose 6-phosphate; curve (B_0) shows binding to plasma membrane (○) and microsomes (×) in the absence of glucose 6-phosphate; curve (B_G) is the difference between B_T and B_O; and curve (Y) indicates the binding of yeast hexokinase.

to a B_O of 0.43 at pH 6.10 for comparison. B_G varied proportionally with B_O, indicating that the same sites were involved with both types of binding. The glucose 6-phosphate-dependent binding, B_G, clearly reaches a maximum at a higher pH (5.9) than the independent binding (5.4). Most points in Fig. 2 were derived from experiments with plasma membrane fractions, but a few were derived from bulk microsomal preparations, and these fall on the same curves; moreover, the actual (unadjusted) binding capacity of different microsome preparations were in the same range as the capacity of the plasma membranes, indicating that the hexokinase binding properties are not peculiar to the plasma membrane. Yeast hexokinase (curve Y, Fig. 2) remains essentially unbound.

Factors influencing the glucose 6-phosphate-dependent binding

The extent of glucose 6-phosphate-dependent binding as a function of glucose 6-phosphate concentration is shown in Fig. 3. Half saturation of the dependent binding is reached at about 0.05 mM glucose 6-phosphate. The presence of 5 mM ATP has no effect on binding in either the presence or absence of glucose 6-phosphate, 10 mM phosphate decreases the independent binding somewhat, and deoxyglucose 6-phosphate has a more pronounced effect on the glucose 6-phosphate-dependent binding (Table I). A substantial elution of the enzyme with 100 mM KCl is also evident; in this regard, the hexokinase binding to the ascites tumor membranes more closely resembles the binding to mitochondria than the binding to hepatoma plasma membranes. Emmelot and Bos [6, 9] noted that hexokinase bound to the plasma membranes was not removed by washing in isotonic saline, but a number of reports

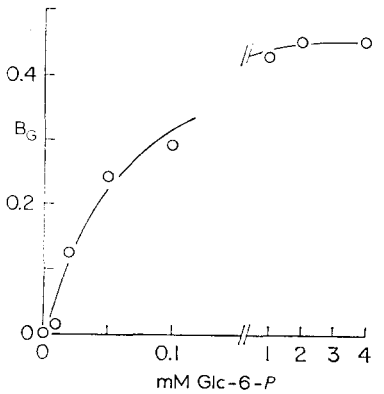

Fig. 3. Glucose 6-phosphate-dependent binding of hexokinase by plasma membranes as a function of glucose 6-phosphate concentration. All values were adjusted to pH 6.10 and a B_O of 0.43.

TABLE I

EFFECT OF PHOSPHATE, ATP, AND dGlc-6-*P* ON RELATIVE BINDING CAPACITIES
Adjusted to pH 6.10; P_i, inorganic orthophosphate; dGlc-6-*P*, 2-deoxyglucose 6-phosphate.

Additions	B_O	B_T +1 Mm Glc-6-*P*	B_G (B_T–B_O)
None	1.00	2.21	1.21
10 mM P_i	0.74	1.85	1.11
5 mM ATP	1.01	2.18	1.17
2.5 mM dGlc-6-*P*	0.97	1.61	0.64
100 mM KCl	0.23	–	–

[5, 14, 15] indicate that either NaCl or KCl will elute the enzyme from mitochondria. NaCl and KCl are equally effective on brain mitochondria [14]. Studies on heart mitochondria with KCl [15] and tumor mitochondria with NaCl [5] indicate that more than 0.2 M salt is required to elute appreciable amounts of enzyme at neutral pH but that elution is greatly enhanced below pH 7. Clearly, more information concerning the influence of pH on this salt effect will be needed before further comparisons are possible.

The pronounced lowering of B_G by 2-deoxyglucose 6-phosphate (Table I) suggested a specific interference with the glucose 6-phosphate-dependent binding. A double reciprocal plot of B_G against glucose 6-phosphate in the presence of 2.5 mM 2-deoxyglucose 6-phosphate is consistent with a competitive inhibition and implies an association constant, or K_I, for 2-deoxyglucose 6-phosphate of about 0.06 mM (Fig. 4). In contrast deoxyglucose 6-phosphate has no observable effect on the enzymatic activity in either the presence or absence of glucose 6-phosphate at pH 6.1 (Table II). Calculation of the effect expected if deoxyglucose 6-phosphate had influenced the reaction in the same way as the binding reveals that the concentration used (0.5 mM) would have decreased the inhibitory effect of glucose 6-phosphate by a significant and easily measured amount. This strongly implies that the site which

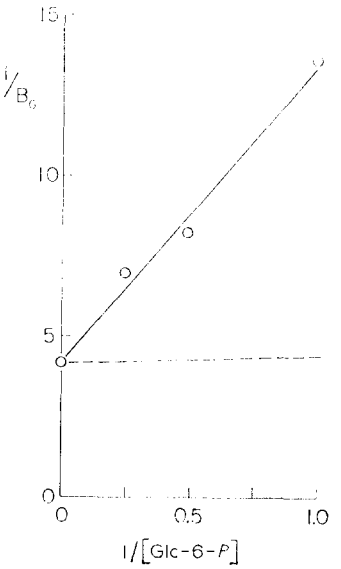

Fig. 4. Effect of 2.5 mM 2-deoxyglucose 6-phosphate on the glucose 6-phosphate-dependent binding of hexokinase to plasma membranes. Dashed line shows the calculated relationship between the reciprocals of B_G and glucose 6-phosphate concentration in the absence of 2-deoxyglucose 6-phosphate.

TABLE II

EFFECT OF dGlc-6-*P* ON THE INHIBITION OF HEXOKINASE ACTIVITY BY Glc-6-*P*

(mM) Glc-6-*P*	Relative velocity observed	
	Control	+ dGlc-6-*P*
0	1.00	0.99
0.08	0.64	0.65
0.16	0.43	0.42

dGlc-6-*P*, 0.5 mM 2-deoxyglucose 6-phosphate.

responds to glucose 6-phosphate by increasing the hexokinase binding to the membranes is separate and distinct from the site which regulates enzyme activity.

Magnitude of binding in intact cells

Molnar et al. [10] have determined that 1 ml of packed cells contains about 20 mg membrane (bulk microsomal) protein, of which 6 mg is plasma membrane protein. These cells contain about 5 units of hexokinase per ml (unpublished observations); using the values for B_O and B_T at pH 7.0 shown in Fig. 2, it may be estimated that the microsomal membranes could bind 70 % of the hexokinase in the absence of glucose 6-phosphate and 90 % in its presence. The plasma membrane would contribute 30 % of this binding capacity. Below pH 7, the binding would increase, and con-

sidering that tumor cells may often exist at a pH less than 7 because of their extensive aerobic glycolysis, such membrane binding may have a significant effect on both the distribution and regulation of hexokinase.

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