PURIFICATION AND PROPERTIES OF RAT SKELETAL MUSCLE HEXOKINASE

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1. Introduction

Rat tissues contain four hexokinase isoenzymes which are present in differing proportions in the various tissues. Each of the low-K_M hexokinase types (EC 2.7.1.1), designated I, II and III, has been partially purified from one or more rat tissues [1] but of these only type I from rat brain has been purified to homogeneity [2]. Recently a low yield preparation has been reported of rat hepatic glucokinase (EC 2.7.1.2), sometimes also called type IV hexokinase, which exhibits a high K_M for glucose and a narrower substrate specificity than the other hexokinases [3]. The purification of hexokinase types II–IV by conventional methods has proved to be difficult due to the small quantities available from the tissues and their inherent instability.

A high yield preparation of homogeneous glucokinase from rat liver has recently been achieved by exploiting affinity chromatography on an agarose-*N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose matrix [4–6]. In this report we describe how such an affinity chromatographic method can be utilized along with more conventional techniques in order to prepare homogeneous type II hexokinase from rat skeletal muscle in high yield. The enzyme has a polypeptide chain mol. wt of 96 000. Amino acid analysis reveals similarities between the compositions of the hexokinase isozymes types 1, II and IV (glucokinase) which may have evolutionary significance.

2. Materials and methods

Hexokinase activity was determined as described by Holroyde et al. [5]. A unit of hexokinase activity is defined as that which catalyses the formation of 1 μmol of glucose 6-phosphate/min at 30°C. Determination of protein concentration was by the turbidimetric microtannin method of Mejbaum-Katzenellenbogen and Dobryszycka [7]. The enzyme was concentrated by ultrafiltration at 4°C using an Amicon TCF-10 ultrafiltrator fitted with a UM 20E membrane.

2.1. *Preparation of muscle extracts*

Minced rat muscle from 50 rats (1250 g) was homogenized for 2 min at 4°C with 2 volumes of 20 mM potassium phosphate buffer, pH 7.0, containing 10 mM glucose, 0.5 mM EDTA, 0.5 mM dithiothreitol and 5% (v/v) glycerol. The extract was centrifuged at 22 000 *g* for 30 min and the supernatant filtered through glass-wool and re-adjusted to pH 7.0 with 2 N KOH.

2.2. *Preparation of affinity column*

N-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose was prepared [5] and coupled to CNBr-activated Sepharose-4B essentially as described by Axen et al. [8] under the conditions given by Trayer et al. [9] at a ligand concentration of 8 μmol ligand per g wet packed gel. This was diluted one-fold with unsubstituted Sepharose-4B prior to use.

2.3. *Ultracentrifugation*

Sedimentation equilibrium studies were performed in a Spinco Model E ultracentrifuge equipped with Rayleigh interference optics. The high speed method of Yphantis [10] was used. Relatively high rotor speeds (27 670 rev/min) and low protein concentrations (0.3 mg per ml) were used to ensure that the meniscus concentration was effectively zero. All runs

were performed in 20 mM triethanolamine–HCl buffer, pH 7.0, containing 0.3 M KCl, 1 mM EDTA, 0.5 mM dithiothreitol, 50 mM glucose and 5% (v/v) glycerol.

2.4. *Polyacrylamide gel electrophoresis*

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate was carried out by the method of Weber and Osborn [11].

3. Results and discussion

3.1. *Purification of hexokinase type II*

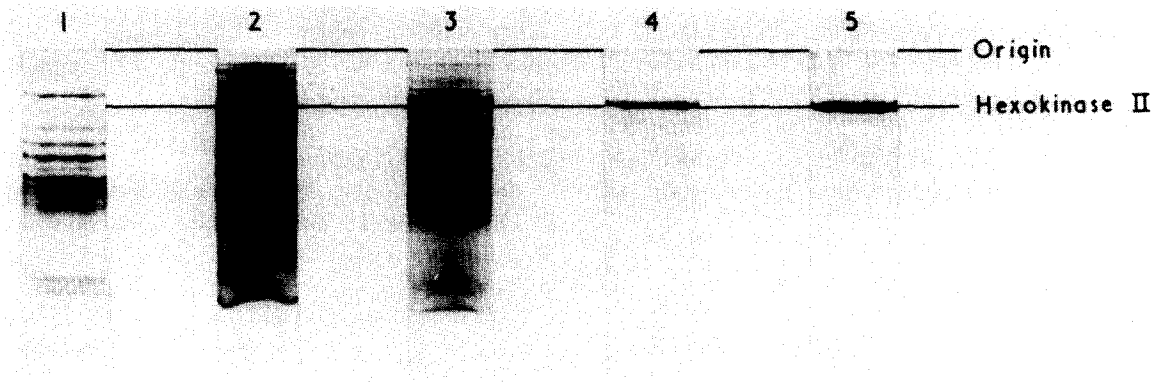
Table 1 summarizes the purification procedure used. The crude muscle extract, after centrifugation, was absorbed batchwise on the DEAE-cellulose which was collected and washed on a Buchner funnel under suction before packing into a column. Fig.1(a) describes how this column was developed. The active fractions eluting under the KCl gradient were pooled and applied to DEAE-Sephadex operated as shown in fig.1(b). This second anion exchange column offered an additional five-fold purification. The key to the purification scheme, however, was affinity chromatography on the Sepharose-glucosamine derivative (fig.2). This resulted in another 50-fold purification (table 1) and the polyacrylamide gels (run in the presence of sodium dodecyl sulphate) show the hexokinase as the major component for the first time. The concentration of KCl used to wash this column (120 mM) is fairly critical and dependent upon the ligand concentration attached to the Sepharose. If too high a concentration of KCl in the wash or too low

Table 1
Purification scheme for rat skeletal muscle hexokinase II

Step	Stage	Volume	Protein concentration	Activity	Total activity	Yield	Specific activity	Fold purification
		(ml)	(mg/ml)	(units/ml)	(units)	(%)	(units/mg of protein)	
1	Extraction and centrifugation	2300	66	0.42	1000	100	0.007	
2	DEAE-Cellulose	356	4.7	2.75	980	98	0.6	90
3	DEAE-Sephadex	280	0.8	2.50	700	70	3.1	470
4	Affinity column	138	0.03	4.35	600	60	145	21,000
5	Sephadex G-200	146	0.01	3.37	490	49	210	30,000
6	Concentration	16.0	0.14	30	480	48	210	30,000

The results presented below are taken from a typical preparation starting with the skeletal muscle of 50 rats.

Sodium dodecyl sulphate – polyacrylamide gel electrophoresis after Step:



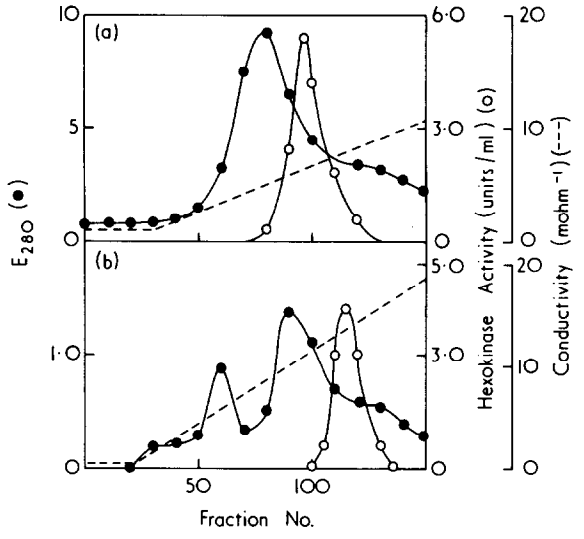


Fig.1. (a) Chromatography of extract on DEAE-cellulose. After absorption on to the cellulose, the column material was packed into a column (25 X 4.5 cm) and washed with the running buffer, i.e. 20 mM potassium phosphate pH 7.0, containing 100 mM glucose, 1 mM EDTA, 0.5 mM dithiothreitol and 5% (v/v) glycerol. The column was developed by a linear gradient from 0 to 0.5 M KCl over a total volume of 2 litres. (b) Chromatography on DEAE-Sephadex equilibrated in 50 mM potassium phosphate buffer, pH 7.0, containing 50 mM glucose, 1 mM EDTA, 0.5 mM dithiothreitol and 5% (v/v) glycerol. The active fractions from the DEAE-cellulose column were concentrated by ultrafiltration, dialysed against the above buffer and applied to a DEAE-Sephadex column (20 X 4.5 cm). After adequate washing this column was developed with a potassium phosphate gradient from 50 to 500 mM (total volume 2 litre). Both columns were run at 40 ml/h and 8 ml fractions were collected.

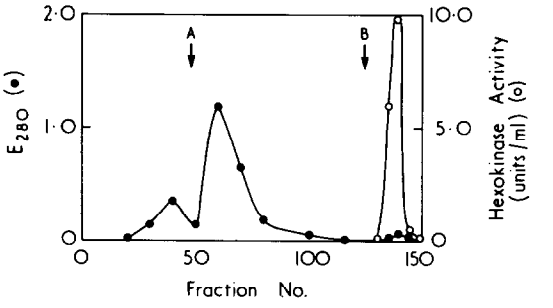


Fig.2. Affinity chromatography on Sepharose-*N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose (4 μmol/g wet wt. of Sepharose 4B). The active fractions from the DEAE-Sephadex column were concentrated by ultrafiltration and equilibrated with 20 mM triethanolamine-HCl buffer, pH 7.0, containing 50 mM KCl, 4 mM EDTA, 7.5 mM $MgCl_2$, 0.5 mM dithiothreitol and 5% (v/v) glycerol by passing through Sephadex G-25. This pool was applied directly to the affinity column (35 X 3 cm) equilibrated in the above buffer and washed through with a further 250 ml of this buffer. At A, the KCl concentration in this buffer was raised to 120 mM and at B, 200 mM glucose was also included. The column was operated at 40 ml/h and 6 ml fractions were collected.

a ligand concentration on the column is chosen then the enzyme will begin to leak from the column prior to introducing the glucose. The need for adjustment of ligand concentration on the Sepharose support to achieve maximum purification of hepatic glucokinase has already been discussed [5,6] and the same principles also apply to this related enzyme.

The active fractions emerging from the affinity column were concentrated on a small DEAE-cellulose column [6] prior to gel filtration on Sephadex G-200. This column (90 X 3 cm) was operated in 20 mM triethanolamine–HCl buffer, pH 7.2, containing 100 mM KCl, 100 mM glucose, 1 mM EDTA, 0.5 mM dithiothreitol and 5% (v/v) glycerol. The enzyme, essentially homogeneous at this stage (table 1), could be stored at 4°C in this buffer, after concentration on a small DEAE-cellulose column as above, for several months without appreciable loss of activity.

3.2. *Properties of hexokinase type II*

Polyacrylamide gel electrophoresis of the hexokinase in the presence of sodium dodecyl sulphate showed a single component migrating with a mol. wt of 98 000 when compared to known standards. Sedimentation equilibrium measurements of the native enzyme in the presence of glucose gave a mol. wt. of 96 000. This value is the same as that obtained for the hexokinase type I isolated from rat brain [2], rat kidney (M. J. Holroyde and I. P. Trayer, unpublished observations) and pig heart [13] but is twice that obtained for the rat liver type IV (glucokinase) isoenzyme [6]. No evidence of high mol. wt. species was seen under the conditions used in these studies on the type II isoenzyme, even after prolonged ultracentrifugation (48 h). Glucose 6-phosphate, however, has been shown to favour dimer formation of the pig heart type I hexokinase [14].

Initial velocity experiments of the muscle hexokinase indicated that the Michaelis constants for

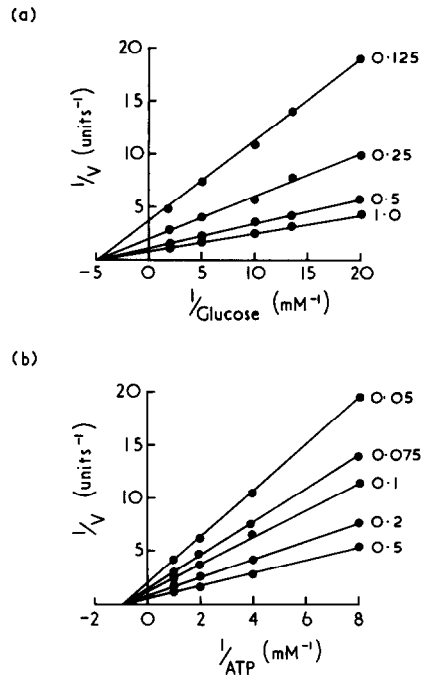


Fig.3. Plots of the reciprocal initial velocity (v) with respect to (a) reciprocal of concentration of glucose in the presence of varying fixed concentrations (mM) of Mg·ATP^{2-} (concentrations indicated by appropriate line) and (b) reciprocal concentrations of Mg·ATP^{2-} in the presence of varying fixed concentrations (mM) of glucose (concentrations indicated by appropriate line).

Mg·ATP^{2-} and glucose were 1.11 mM and 0.2 mM respectively (fig.3). The slightly different assay conditions almost certainly account for the minor differences noted between our figures and those reported by others for impure preparations of the enzyme [1, 15].

In table 2 the amino acid composition of rat muscle hexokinase, type II, is compared with published values for the rat brain type I isoenzyme [2] and rat hepatic glucokinase [6]. The similarities are quite striking and are emphasized when the 'difference indices of compositional relatedness' of Metzger et al. [16] are calculated. Despite the obvious limitations inherent in this type of analysis, it is tempting to speculate that the high mol. wt., low-K_M mammalian hexokinases may have arisen by partial gene duplication from some primitive presumptive glucokinase-type enzyme. Any evolutionary relationships in this class of enzymes must, however, await sequence analysis.

Table 2
Amino acid composition of mammalian hexokinases

Amino acid	Rat brain type I[a]	Rat skeletal muscle type II[b]	Rat liver type IV[c]
Aspartic acid	98	92	92
Threonine	50	54	48
Serine	48	52	62
Glutamic acid	88	104	118
Proline	36	26	28
Glycine	80	86	80
Alanine	48	66	60
Valine	64	66	64
Methionine	30	30	36
Isoleucine	50	36	34
Leucine	84	92	82
Tyrosine	8	16	16
Phenylalanine	36	36	36
Histidine	18	23	22
Lysine	60	52	50
Arginine	54	56	56

Amino acid analyses were performed as described by Wilkinson et al. [12]. Duplicate samples were hydrolysed in 6 N HCl under vacuum for 24 and 72 h. All figures are calculated as residues per 96 000 g. (NB mol. wt of the liver type IV isoenzyme (glucokinase) is 48 000 [6]). The Metzger 'difference indices of compositional relatedness' [16] between these enzymes are as follows: types I and II, 5.5; types II and IV, 3.4; and types I and IV, 4.5.
[a]From Chou and Wilson [2].
[b]From this study.
[c]From Holroyde et al. [6].

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