THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 255, No. 4, Issue of February 25, pp. 1752-1756, 1980
Printed in U.S.A.

Rabbit Red Blood Cell Hexokinase

PURIFICATION AND PROPERTIES*

(Received for publication, July 19, 1979)

Mauro Magnani, Marina Dachà, Vilberto Stocchi, Paolino Ninfali, and Giorgio Fornaini

From the Istituto di Chimica Biologica, Università degli Studi di Urbino, Via Saffi, 2, 61029, Urbino, Italy

Rabbit red blood cell hexokinase (EC 2.7.1.1.) has been purified 300,000-fold by a combination of ion exchange chromatography, affinity chromatography, and preparative polyacrylamide gel electrophoresis. The hexokinase activity has been isolated in 35% yield as a protein that is homogeneous by polyacrylamide and sodium dodecyl sulfate gel electrophoresis. The highest specific activity obtained was 145 units/mg of proteins. The native protein has a molecular weight of 110,000 by gel filtration on Ultrogel AcA 44 and 112,000 by sedimentation velocity on sucrose density gradients. Sodium dodecyl sulfate-polyacrylamide gels gave a molecular weight of 110,000, indicating that hexokinase is a monomer.

The enzyme had a pI of 6.20 to 6.30 pH units by isoelectric focusing. The enzyme was specific for Mg•ATP and Mg•ITP as the nucleotide substrates. Several hexoses could be phosphorylated by hexokinase with different affinities.

The hexokinase (EC 2.7.1.1) reaction in red blood cells has the lowest turnover with respect to all of the other erythrocyte enzymes (1). This enzyme is markedly suppressed by its product (2-7) and, as it was shown by mathematical models (2) and free energy changes (8), has a higher control strength on erythrocyte glycolysis. Many authors have found a marked decrease in the enzyme level during cell aging (9-11), changes in the isozyme pattern, and the formation of "secondary" isozymes (9). Recently, we have shown in pig, rabbit, and human erythrocytes the presence of a glucose phosphorylating activity with low affinity for glucose (12, 13). This last activity appears during red cell aging (14, 15) and is probably a post-translational modification of the native hexokinase. The mature red cell is lacking in protein synthesis and glycolysis is the only energy supply; therefore, the age-dependent modifications at the hexokinase level can severely affect the erythrocyte metabolism.

Preliminary investigations on the age-dependent modifications of this enzyme have shown changes in its kinetic and physical properties (16). In order to compare the native enzyme and the senescent protein, it is necessary to obtain pure hexokinase from red blood cells of different ages; however, until now, no homogeneous purification procedures have been reported.

In this paper we report the purification to homogeneity of rabbit red blood cell hexokinase and some of its properties.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

EXPERIMENTAL PROCEDURES

Materials—Coenzymes, enzymes, substrates, phenazine methosulfate, and MTT[1] were obtained from Sigma. Activated CH-Sepharose 4B, DEAE-Sephadex A-50, Sephadex G-25, and blue dextran 2000 were purchased from Pharmacia. Ultrogel AcA 44 and Ampholines (pH range, 3.5 to 10, 4 to 6, and 5 to 8) were from LKB. *N,N,N′,N′*-tetramethylethylenediamine, bis, and acrylamide were from Bio-Rad. All other reagents were of an analytical grade.

Hexokinase Assay—Hexokinase activity was measured at 37°C spectrophotometrically in a system coupled with glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44). The assay mixture contained, in a total volume of 1 ml, 0.135 M glycylglycine (pH 8.1), 5 mM glucose, 5 mM ATP·$MgCl_2$, 0.5 mM $NADP^+$, 5 mM $MgCl_2$, 0.05 IU of glucose-6-phosphate dehydrogenase, and 0.05 IU of 6-phosphogluconate dehydrogenase.

Initial rate measurements were performed by following the reduction of $NADP^+$ at 340 nm with a Beckman spectrophotometer model 25. For each molecule of glucose utilized, 2 molecules of $NADP^+$ are reduced. One unit of hexokinase activity is defined as the amount of enzyme which catalyzes the formation of 1 μmol of glucose 6-phosphate/min at 37°C.

In the sugar specificity studies the initial rates of ADP production were measured in a coupled enzyme system with pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.28). The assay mixture contained, in a total volume of 1 ml, 0.25 M Tris-HCl (pH 8.1), 1.5 mM EDTA, 5.0 mM $MgCl_2$, 5.0 mM ATP·$MgCl_2$, 0.75 mM phosphoenolpyruvate, 0.2 mM NADH, 1 IU of pyruvate kinase, 1 IU of lactate dehydrogenase, and sugar substrate.

Hence, 1 unit of hexokinase activity is defined as the amount of enzyme which catalyzes the production of 1 μmol of ADP/min at 37°C.

Protein Estimation—In the hemolysate, hemoglobin concentration was determined spectrophotometrically at 540 nm with Drabkin's solution as described by Beutler (17). During the purification procedure, protein was determined according to Lowry *et al.* (18), with bovine serum albumin as a standard, or spectrophotometrically at 280 nm.

Preparation of Sepharose-N-Aminohexanoyl Glucosamine—The activated CH-Sepharose 4B was swollen in 1 mM HCl. The gel was washed with distilled water on a sintered glass filter using approximately 300 ml/g of dry powder. Four milligrams of D(+)-glucosamine hydrochloride/mg of dry powder was dissolved in the coupling solution (0.1 M $NaHCO_3$ containing 0.5 M NaCl), of which there were 5 ml/g of dry powder, and mixed with the gel. Excess ligand was removed by washing with the coupling solution and the remaining active groups blocked with ethanolamine, 1 M, pH 9.

The product was washed with three cycles of alternating pH, consisting of a wash at pH 4 (0.1 M acetate buffer and 1 M NaCl) followed by a wash at pH 8 (0.1 M Tris-HCl and 1 M NaCl). The product was stored at +4°C in 5 mM sodium potassium phosphate buffer, pH 7.5, containing 3 mM KF and 3 mM 2-mercaptoethanol.

Sodium Dodecyl Sulfate-Polyacrylamide Disc Gel Electrophoresis—Electrophoresis was carried out in 7.5% polyacrylamide gels (0.4 × 6.5 cm) containing 0.1% SDS according to Weber *et al.* (19). Protein

[1] The abbreviations used are: MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; SDS, sodium dodecyl sulfate; TEA, triethanolamine.

bands were visualized with Coomassie brilliant blue R-250. Molecular weight calibration curves were obtained using β-galactosidase (M_r = 130,000), bovine serum albumin (M_r = 68,000), aldolase (M_r = 40,000), and glyceraldehyde phosphate dehydrogenase (M_r = 36,000) as standards (20).

Nondenaturating Polyacrylamide Disc Gel Electrophoresis—Electrophoresis was carried out in 7.5% polyacrylamide gels (0.4 × 8.5 cm), pH 8.6, with Tris/glycine, pH 8.6, as reservoir buffer at +4°C as described by Davis (21) with slight modifications. Prevention of hexokinase inactivation during disc electrophoresis was achieved by adding 100 mM glucose to the reservoir buffer and in the gels. After electrophoresis, the hexokinase activity was located by incubating polyacrylamide gels for 15 to 30 min in a freshly prepared solution containing 0.135 M glycylglycine (pH 8.1), 10 mM glucose, 5 mM ATP·$MgCl_2$, 1 mM $NADP^+$, 5 mM $MgCl_2$, 0.1 IU/ml of glucose-6-phosphate dehydrogenase, 75 μg/ml of phenazine methosulfate, and 200 μg/ml of MTT at 37°C.

Molecular Weight Estimation—The molecular weight of native hexokinase was determined by Ultrogel AcA 44 gel filtration using a calibrated column (2.1 × 42 cm). The column was equilibrated with 5 mM sodium potassium phosphate buffer, pH 7.5, containing 3 mM 2-mercaptoethanol, 3 mM KF, 5 mM glucose, 0.5 M NaCl, and 9% (v/v) glycerol at 4°C. The flow rate was 15 to 20 ml/h, and 1.5-ml fractions were collected. One-milliliter samples were layered on the surface of the gel. In order to detect the elution volumes of hexokinase and molecular weight standards, each fraction was assayed for enzyme activities. The void volume (V_0) was determined with blue dextran 2000. The molecular weight of hexokinase was also determined by sedimentation velocity on sucrose density gradients. The procedure of Martin and Ames (22) was used at 4°C. Linear 4.5-ml sucrose gradients from 5 to 20% sucrose (w/v) were prepared by automatic gradient former (Gilson) in 5 mM sodium potassium phosphate buffer, pH 7.5, containing 3 mM 2-mercaptoethanol, 3 mM KF, and 10 mM glucose. Purified hexokinase (100 μl containing 5 units/ml, specific activity of 145), 10 μl of aldolase (EC 4.1.2.13) (rabbit muscle, in $(NH_4)_2SO_4$), 10 μl of lactate dehydrogenase (rabbit muscle, in $(NH_4)_2SO_4$), 10 μl of uricase (EC 1.7.3.3) (porcine liver, in $(NH_4)_2SO_4$), 10 μl of phosphoglucomutase (EC 2.7.5.1) (rabbit muscle, in $(NH_4)_2SO_4$), and 10 μl of 3-phosphoglycerate kinase (EC 2.7.2.3) (yeast, in $(NH_4)_2SO_4$ and $Na_4P_2O_7$) were dialyzed for 4 h against phosphate buffer and layered onto a gradient. The gradients were developed in the SW 65 rotor of the Beckman L5-65 ultracentrifuge at 4°C for 16 h at 60,000 rpm and afterwards 3-drop fractions were collected from the bottom of the tubes and assayed for enzyme activities.

Isoelectric Focusing—Isoelectric focusing of hexokinase was carried out on LKB 8100 electrofocusing equipment in a glycerol gradient solution and in a pH gradient of 3.5 to 10 and 4 to 8 at 1% Ampholines concentration according to the instructions of the manufacturer. The sample was layered after the pH gradient was formed. The voltage was 600 V and the focusing was completed after 16 h from the introduction of the sample. Cooling temperature was 4°C, elution flow rate 50 ml/h, and fractions of 1 ml were collected and measured for the hexokinase activity and the pH value.

RESULTS

Purification of Red Blood Cell Hexokinase

All operations were performed at 4°C. All buffers were 3 mM 2-mercaptoethanol and 3 mM KF.

Step 1: Preparation of Hemolysate—Rabbit red blood cells were collected using EDTA as anticoagulant. The red cells were washed twice with isotonic sodium chloride. The buffer coat was removed by suction. After adding an equal volume of 0.4% (w/w) saponin solution, the washed cells were hemolysated for 2 h.

Step 2: Batch Treatment with DEAE-Sephadex A-50—The hemolysate was mixed with 2 volumes of DEAE-Sephadex A-50 suspension, equilibrated in 3 mM sodium potassium phosphate buffer, pH 7.3, containing 5 mM glucose, and mechanically stirred for 60 min. The suspension was rinsed in a Buchner funnel with the same buffer until the eluate was colorless. This procedure removed the bulk of the hemoglobin while the hexokinase remained bound. The enzyme was eluted with 0.5 M KCl in phosphate buffer. For a complete hexokinase recovery, the elution procedure was repeated twice.

Step 3: Ammonium Sulfate Fractionation—Solid ammonium sulfate was slowly added to the stirred enzyme solution from Step 2 to achieve 35% saturation. The suspension was gently stirred for 60 min and then centrifuged at 16,000 × *g* for 30 min. The supernatant solution was removed and brought to 65% saturation with ammonium sulfate. After 60 min, the suspension was centrifuged as before. The 35 to 65% ammonium sulfate precipitate was dissolved in 20 to 30 ml of 5 mM sodium potassium phosphate buffer, pH 7.5, containing 5 mM glucose, and then dialyzed overnight against 200 volumes of 5 mM sodium potassium phosphate buffer, pH 7.5, containing 5 mM glucose and 9% (v/v) glycerol. The dialyzed enzyme solution was then centrifuged at 16,000 × *g* for 20 min. At this stage the enzyme can be stored at −20°C over a period of weeks, with no significant loss of activity.

Steps 4 and 5: Affinity Chromatography—Before affinity chromatography, the glucose present in the enzyme solution was completely removed by enzymatic phosphorylation. By adding 7.5 mM ATP·$MgCl_2$ to the hexokinase solution (30 min at room temperature), the glucose present was phosphorylated and removed by passage through a Sephadex G-25 column (2.1 × 12 cm) equilibrated with 2.5 mM sodium potassium phosphate buffer, pH 7.5, containing 9% (v/v) glycerol.

The fractions containing hexokinase activity were applied to a Sepharose-*N*-aminohexanoyl glucosamine column (1 × 4 cm) equilibrated with the same buffer. After the sample application, the affinity column was washed with buffer phosphate until the protein absorbance at 280 nm, in the eluate, was lower than 0.1 *A*. The hexokinase was eluted by adding 5 mM glucose to the equilibrating buffer. The preparation obtained at this stage is 8,000- to 10,000-fold purified. The pooled fractions containing hexokinase activity were concentrated by ultrafiltration through a PM-30 Amicon membrane. A 150,000-fold purification was obtained by a second similar affinity chromatography step in 10 mM sodium potassium phosphate buffer, pH 7.5, containing 9% (v/v) glycerol. The enzyme was eluted by adding 10 mM glucose to the developing buffer (Fig. 1). At this stage, the enzyme can be stored at −20°C over a period of months, with no significant loss of activity.

Step 6: Preparative Polyacrylamide Gel Electrophoresis—The enzyme solution from Step 5 was electrophoresed on 7.5% preparative polyacrylamide gel (1 × 8 cm). The gels were sliced into 0.2-cm sections and separately mashed in 1 ml of

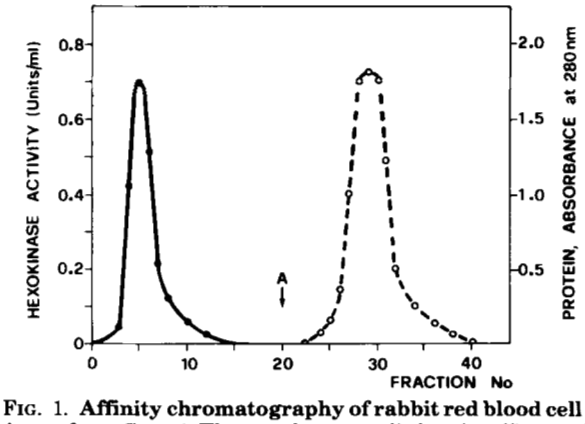

FIG. 1. **Affinity chromatography of rabbit red blood cell hexokinase from Step 4.** The sample was applied to the affinity column (1 × 4 cm) equilibrated in 10 mM sodium potassium phosphate buffer, pH 7.5, containing 9% (v/v) glycerol and operated at 15 to 20 ml/h. Fractions of 3 ml were collected. At *A*, elution was initiated by adding 10 mM glucose in the developing buffer. From *A*, protein concentration was too low to be presented on the figure. ●——●, absorbance at 280 nm; ○- - -○, hexokinase activity.

10 mM sodium potassium phosphate buffer, pH 7.5, containing 10 mM glucose and 9% (v/v) glycerol. The eluted fractions were assayed for hexokinase activity and pooled. The preparation obtained at this stage gave a single band in nondenaturating polyacrylamide disc gel electrophoresis and in the presence of sodium dodecyl sulfate, as shown in Fig. 2.

The purification procedure is summarized in Table I. The specific activity of the pure hexokinase was 145 units/mg of protein, corresponding to a 300,000-fold purification over the starting material, with an overall recovery of approximately 35%. In the final stage of purification, the presence of 9% (v/v) glycerol in the buffer for elution is essential for good recovery of hexokinase activity.

Hexokinase recoveries indicated in Table I strictly depend also upon the use of buffers 5 mM in glucose. As reported above, removal of glucose is absolutely necessary for binding hexokinase to Sepharose-*N*-aminohexanoyl glucosamine; in fact, *N*-acetylglucosamine is a competitive inhibitor (K_i = 0.7 mM) with respect to the glucose of red blood cell hexokinase (23).

Molecular Weight

The molecular weight of the pure hexokinase estimated by gel filtration on Ultrogel AcA 44 for five different preparations was about 110,000 (Fig. 3). The molecular weight was also estimated by sucrose density gradient sedimentation velocity, with five other standard enzymes, and it was found to be 112,000. The molecular weight estimated by sodium dodecyl sulfate-gel electrophoresis was 110,000, indicating that hexokinase is a monomer. In each case, a least square program was used to determine the straight line with a correlation coefficient of about 0.999.

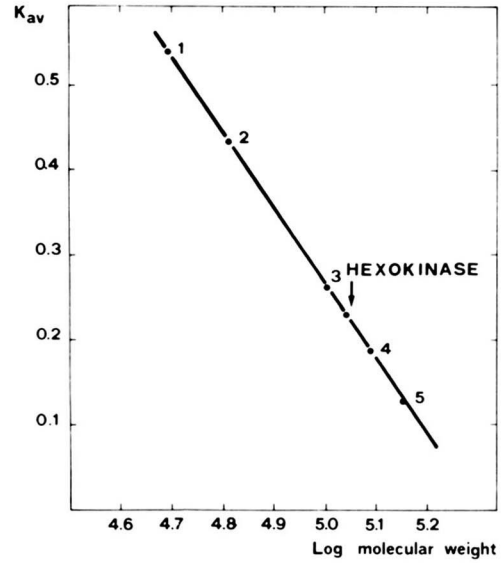

FIG. 3. **Estimation of the molecular weight of the rabbit red cell hexokinase by gel filtration on an Ultrogel AcA 44 column.** The column was calibrated with the protein standards: (*1*)3-phosphoglycerate kinase; (*2*) phosphoglucomutase; (*3*) uricase; (*4*) glutathione reductase; (*5*) lactate dehydrogenase.

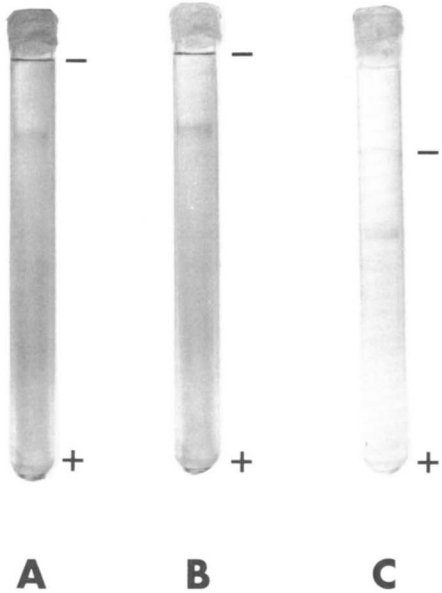

FIG. 2. **Polyacrylamide gel electrophoresis of purified red blood cell hexokinase.** *A*, stained for hexokinase activity; *B*, stained for proteins; *C*, sodium dodecyl sulfate-polyacrylamide gel.

pH Optimum

The pH profile for rabbit red blood cell hexokinase shows maximal activity between pH 7.8 and 8.5 with the optimum at pH 8 to 8.2.

Isoelectric Focusing

Isoelectric focusing of the purified hexokinase under native conditions in a glycerol gradient solution provides a single peak with a pI 6.2 to 6.3 (Fig. 4).

Kinetic Properties and Specificity

The K_m values of purified hexokinase for glucose and MgATP were estimated in 0.135 mM glycylglycine, pH 8.1, at 37°C. The double reciprocal plot, $1/V$ against $1/S$, shows a K_m value for glucose of 0.06 to 0.1 mM at MgATP concentra-

TABLE I

Purification of hexokinase from rabbit red blood cell

Step	Fraction	Volume	Protein	Activity	Specific activity	Yield	Purification
		ml	*mg/ml*	*units/ml*	*units/mg*	%	*-fold*
1	Hemolysate	900	163	0.0806	0.00049	100	
2	DEAE-Sephadex A-50 eluate	2,280	1.4	0.0318	0.0227	99.9	46
3	Ammonium sulfate precipitation, 35 to 65%	18	134	3.82	0.0285	95	58
4	First immobilized *N*-acetylglucosamine eluate	120	0.104	0.458	4.40	76	8,980
5	Second immobilized *N*-acetylglucosamine eluate	94	0.005	0.378	75.6	49	150,000
6	Preparative gel electrophoresis	5	0.0348	5.07	145.6	35	300,000

tions ranging from 0.2 to 5 mM (Fig. 5*a*). Furthermore, the K_m for MgATP was 0.56 to 1.1 mM at glucose concentrations ranging from 0.05 to 5 mM (Fig. 5*b*).

Rabbit erythrocyte hexokinase was specific for MgATP and

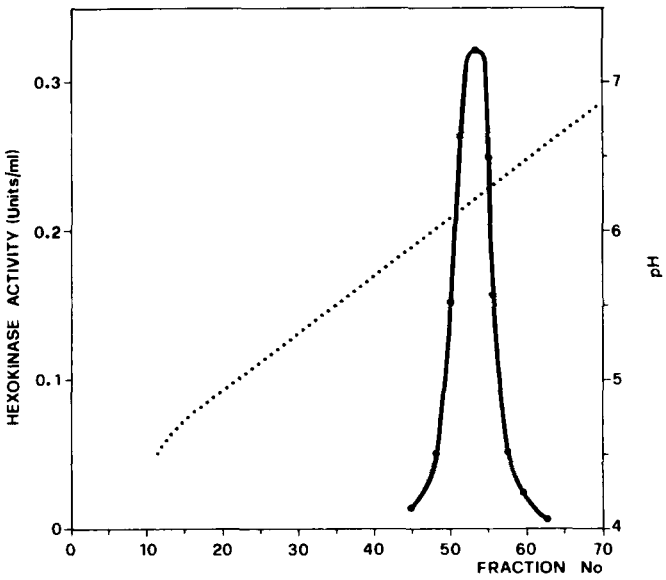

FIG. 4. **Isoelectric focusing of purified rabbit erythrocyte hexokinase.** One-milliliter sample with 4 units/ml was applied to an LKB 8100 column in a pH gradient 4 to 8.

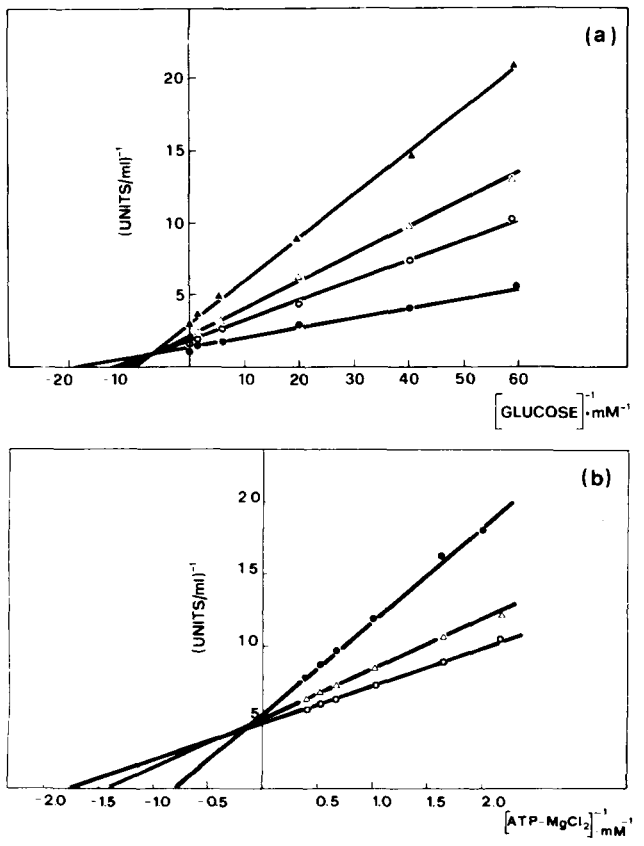

FIG. 5. **Lineweaver-Burk plots of hexokinase activity.** *a*, Lineweaver-Burk plot of hexokinase activity *versus* glucose at 5 mM (●——●), 1 mM (○——○), 0.5 mM (△——△), and 0.2 mM (▲——▲) MgATP concentrations. *b*, Lineweaver-Burk plot of hexokinase activity *versus* MgATP at 5 mM (○——○), 0.2 mM (△——△), and 0.05 mM (▲——▲) glucose concentrations. The activity determinations were carried out as described under "Experimental Procedures."

TABLE II

Specificity of purified red blood cell hexokinase

Compounds	K_m[a]	Relative velocity[b]
	mM	%
MgATP	0.6	100
MgGTP		
MgITP	1.9	36
MgUTP		
MgCTP		
D(+)-Glucose	0.06	100
D(+)-Mannose	0.71	109
D(−)-Fructose	17.8	70
2-Deoxy-D-glucose	1.33	65.7
D(+)-Glucosamine	2.0	43.4
N-Acetyl-D-Glucosamine	41.6	32.6
D(+)-Galactose		

[a] K_m values were determined using the Lineweaver-Burk plot.
[b] Maximum velocities are expressed relative to the V for glucose (100%) when the enzyme is saturated by the considered substrate.

MgITP as the nucleotide substrates. MgGTP, MgUTP, and MgCTP were not converted to their diphosphates at 20 mM concentrations.

Several hexoses can be phosphorylated by the enzyme (Table II) with different affinities and different maximum velocities. D(+)-galactose was not phosphorylated.

Enzyme Stability

Purified hexokinase can be stabilized in a specific way by hexoses such as glucose or fructose at 5 mM concentrations. Enzyme stabilization can also be achieved in a nonspecific way by high concentrations of glycerol, 9 to 15% (v/v), without significant loss of activity for 20 h at 4°C. When hexokinase was maintained in 10 mM sodium potassium phosphate buffer, pH 7.5, or 10 mM triethanolamine, pH 7.5, a rapid destabilizing effect was observed.

DISCUSSION

The rabbit red blood cell hexokinase is a protein similar to that found in a number of mammalian tissues.

The preparation of homogeneous hexokinase in sufficient quantities to study the structure and function of the enzyme has been facilitated by the use of an efficient affinity chromatography system.

Procedures for the purification of hexokinase from red blood cells have been reported by other investigators (24, 25). All previous procedures have utilized relatively laborious techniques and no one has provided a homogeneous enzyme. By our method, we have reached a final recovery of 35% with a specific activity of 145 units/mg of protein. This specific activity is also higher than that obtained for hexokinase type I from pig heart (26), from rat brain (27), and bovine brain (28) but lower than the specific activity of purified rat kidney hexokinase I (180 units/mg)(29).

The molecular weight of red blood cell hexokinase found in our experiments is slightly different from the molecular weight of hexokinase I of other tissues (26–31). These differences could depend on the starting material used for purifications. In fact, Rijksen and Staal (25) have also reported a molecular weight of 132,000 for the human red blood cell hexokinase. The isoelectric point for the pure type I hexokinase from human erythrocytes has been reported to be 4.7 (25). The value 6.2 to 6.3 found by us is in agreement with Chou and Wilson's (27) value for the rat brain enzyme. The affinity constants are in the same range as reported for hexokinase type I from erythrocytes (5, 6, 25, 32) and many other mammalian tissues (31, 33, 34).

The substrate specificity studies show that glucose is the most effective substrate, but mannose can also be used with fairly satisfactory results. This is in agreement with the conclusion reached by Beutler and Teeple (35) that in red cell the same enzyme is responsible for mannose and glucose phosphorylation. An interesting result is that rabbit hexokinase can also utilize MgITP as a phosphate donor.

The structure of the hexokinase and its precise role in the regulation of red blood cell glycolysis are currently under investigation. The availability of homogeneous hexokinase in a stable form, isolated by the purification procedure reported here, should facilitate the study of protein modifications during erythrocyte aging.

REFERENCES

1. Brewer, G. J. (1974) *The Red Blood Cell*, Vol. 1, pp. 387–433, Academic Press, New York
2. Rapoport, T. A., Heinrich, R., Jacobasch, G., and Rapoport, S. (1974) *Eur. J. Biochem.* **42,** 107–120
3. de Verdier, C. H., and Garby, L. (1965) *Scand. J. Haematol.* **2,** 305–317
4. Rose, I. A., Warms, J. V. B., and O'Connell, E. L. (1964) *Biochem. Biophys. Res. Commun.* **15,** 33–37
5. Kosow, D. P., Oski, F. A., Warms, J. V. B., and Rose, I. A. (1973) *Arch. Biochem. Biophys.* **157,** 114–124
6. Gerber, G., Preisler, H., Heinrich, R., and Rapoport, S. M. (1974) *Eur. J. Biochem.* **45,** 39–52
7. Rijksen, G., and Staal, G. E. J. (1977) *Biochim. Biophys. Acta* **485,** 75–86
8. Minakami, S., and Yoshikawa, H. (1966) *J. Biochem.* **59,** 139–144
9. Rogers, P. A., Fisher, R. A., and Harris, H. (1975) *Clin. Chim. Acta* **65,** 291–298
10. Turner, B. M., Fisher, R. A., and Harris, H. (1975) *Isozymes*, Vol. 1, pp. 781–795, Academic Press, New York
11. Chapman, R. G., and Shaumberg, L. (1967) *Br. J. Haematol.* **13,** 665–678
12. Fornaini, G., Dachà, M., Magnani, M., Fazi, A., Cuppini, C., and Bossù, M. (1978) *Enzyme* **23,** 46–51
13. Magnani, M., Dachà, M., Bossù, M., and Fornaini, G. (1978) *Comp. Biochem. Physiol.* **60B,** 323–327
14. Fornaini, G., Magnani, M., Dachà, M., Bossù, M., and Stocchi, V. (1978) *Mech. Aging Dev.* **8,** 249–256
15. Magnani, M., Stocchi, V., Bossù, M., Dachà, M., and Fornaini, G. (1979) *Mech. Aging Dev.* **11,** 209–217
16. Magnani, M., Stocchi, V., Ninfali, P., Dachà, M., Bossù, M., and Fornaini, G. (1979) *Bull. Mol. Biol. Med.* **4,** 90–99
17. Beutler, E. (1975) *Red Cell Metabolism*, 2nd Ed, p. 11, Grune & Stratton, New York
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
19. Weber, K., Pringle, J. R., and Osborn, M. (1972) *Methods Enzymol.* **26,** 3–27
20. Weber, K., and Osborn, M. (1969) *J. Biol. Chem.* **244,** 4406–4412
21. Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **121,** 404–427
22. Martin, R. G., and Ames, B. N. (1961) *J. Biol. Chem.* **236,** 1372–1379
23. Stocchi, V., Magnani, M., Ninfali, P., and Dachà, M. (1979) *J. Solid-Phase Biochem.*, in press
24. Gerber, G., Preissler, H., Heinrich, R., and Rapoport, S. M. (1974) *Eur. J. Biochem.* **45,** 39–52
25. Rijksen, G., and Staal, G. E. J. (1976) *Biochim. Biophys. Acta* **445,** 330–341
26. Easterby, J. S., and O'Brien, M. J. (1973) *Eur. J. Biochem.* **38,** 201–211
27. Chou, A. C., and Wilson, J. E. (1972) *Arch. Biochem. Biophys.* **151,** 48–55
28. Redkar, V. D., and Kenkare, U. W. (1972) *J. Biol. Chem.* **247,** 7576–7584
29. Wright, C. L., Warsy, A. S., Holroyde, M. J., and Trayer, I. P. (1978) *Biochem. J.* **175,** 125–135
30. Easterby, J. S. (1975) *Eur. J. Biochem.* **58,** 231–235
31. Grossbard, L., and Schimke, R. T. (1966) *J. Biol. Chem.* **241,** 3546–3560
32. Malone, J. I., Winegrad, A. I., Oski, F. A., and Holmes, E. W. (1968) *N. Engl. J. Med.* **279,** 1071–1077
33. Cayanis, E., and Balinsky, D. (1975) *Int. J. Biochem.* **6,** 741–749
34. Kamikashi, T., Kizaki, H., Murakami, K., and Ishibashis, S. (1974) *Biochem. J.* **137,** 133–142
35. Beutler, E., and Teeple, L. (1969) *J. Clin. Invest.* **48,** 461–466