Purification and Properties of Galactokinase from Human Red Blood Cells

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SUMMARY

1. Galactokinase from normal human red blood cells was purified 3300- to 3900-fold by ion exchange chromatography, ammonium sulfate precipitation, and dextran gel filtration with a yield of 14 to 19%. Analytical disc electrophoresis of the purified enzyme revealed a single protein band.

2. The $K_m$ of purified galactokinase for galactose is 100 to 150 $\mu$M; for ATP, 200 to 500 $\mu$M. The pH optimum for enzymic phosphorylation of galactose is in the range of pH 7.7 to 7.9. In electofocusing experiments the isoelectric point of galactokinase was found to be 5.7. The molecular weight of galactokinase was determined to be 53,000 to 57,000 by means of dextran gel chromatography and sodium dodecyl sulfate-polyacrylamide electrophoresis. The enzyme seems to have a dimeric structure consisting of monomers with a molecular weight of 25,000 to 27,000.

3. The biochemical properties of galactokinase from human red blood cells are similar to the characteristics of galactokinase from other mammalian tissues, but differ considerably from galactokinase of yeast and Escherichia coli.

Galactokinase (ATP:D-galactose-1-phosphotransferase, EC 2.7.1.6) catalyzes the first step in galactose metabolism, the phosphorylation by ATP of $\alpha$-D-galactose to $\alpha$-D-galactose-1-phosphate. Hereditary deficiency of galactokinase results in galactosemia, galactosuria, and cataracts in early childhood. Six cases of this inborn error of metabolism have been reported (1-6). In normal humans and in rats the activity of galactokinase is high at birth and decreases with age (7, 8). Kinetic alterations during maturation have also been found (8, 9). Biochemical studies with purified galactokinase from various tissues of microbiological and mammalian sources (yeast (10, 11), Escherichia coli (12, 13), and pig liver (14)) have been described.

In this paper we report a method for extensive purification of galactokinase from normal human red blood cells and its biochemical properties.

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MATERIALS AND METHODS

Human blood was collected in acid-citrate-dextrose solution (15) and stored at 4°C under sterile conditions until use. All chemicals were purchased from Sigma.

Sephadex G-75, Sephadex G-200, DEAE-Sephadex A-50, CM-Sephadex C-50, blue dextran 2000, and the columns "K9/60," "K15/90," "K15/30," and "K25/45 with flow adaptor" were obtained from Pharmacia. Concentration of the protein solutions were carried out by ultrafiltration with colodion bags from Sartorius Membran Filter GMBH, Goettingen, Germany. [1-14C]Galactose was obtained from Amersham-Searle. Electofocusing equipment, consisting of LKB 8101 column (110 ml) and ampholine, pH 3 to 10 and pH 5 to 8, was purchased from LKB-Produkter AB, Bromma-1, Sweden.

Determination of galactokinase activity was performed according to a modification (16) of the method described by Sherman (17), in which [1-14C]galactose-1-phosphate formed from purified [1-14C]galactose preparations is absorbed on DEAE-paper, followed by liquid scintillation counting. One milliunit of galactokinase was defined as the amount of activity which phosphorylates 1 nmole of galactose per min under the conditions of the assay (16). The protein content was determined by the method of Lowry et al. (18) using crystallized bovine serum albumin as a standard. Hemoglobin was determined as cyanmethemoglobin. Analytical disc electrophoresis was carried out according to the method of Davis (19). The molecular weight determinations were formed by dextran gel filtration using the method of Determann (20) and by SDS-polyacrylamide electrophoresis according to Shapiro, Vitiela, and Maizel (21). The pH optimum of the galactokinase reaction was obtained with a Tris-glycine-phosphate buffer system (22, 23) previously used for characterization of glucose 6-phosphate dehydrogenase from human red blood cells. Column electofocusing experiments were carried out according to the method of Vesterberg and Svensson (24).

RESULTS

Purification of Galactokinase

All procedures were carried out at 4°C. All centrifugations were performed for 20 min at 37,500 x g. The following buffers were used during the purification procedure. Buffer A: 10 mM KH$_2$PO$_4$, 7 mM 2-mercaptoethanol, 0.5 mM Na$_2$-EDTA, final

1 The abbreviation used is: SDS, sodium dodecyl sulfate.
Purification of erythrocyte galactokinase

Step 1. One volume of washed red cells was mixed with 4 volumes of Buffer A, frozen, and thawed twice. After centrifugation the sediment was discarded.

**Step 2.** The supernatant ("hemolysate") was applied to a DEAE-Sephadex A-50 column (2.5 × 35 cm) equilibrated with Buffer A using ascending chromatography with a flow rate of 30 ml per hour. Fractions containing galactokinase (8.4 to 26.4 ml) were pooled ("CM-Sephadex C-50 filtrate") and concentrated by ultrafiltration.

The purification procedure is summarized in Table I. The cumulative purification ranges between 3300- and 3900-fold, the yield between 14 and 19%. When stored at 4°C in 3.0 M ammonium sulfate, the preparation loses 10% of its activity within 6 days. In order to define the state of purity, the activity of the enzymes of red cell glycolysis, hexose monophosphate shunt, glucotriphosphate and galactose metabolism were determined in the purified preparations (15). Except for traces of triose phosphate isomerase activity (0.6 to 1.1 munits per mg of protein, representing less than 0.1% of the specific activity in crude hemolysates), no contaminating enzyme was detectable. By analytical disc electrophoresis in polyacrylamide a single protein band was found with the purified enzyme.

**Properties of Enzyme**

**Molecular Weight**—The molecular weight of galactokinase from purified preparations was determined by Sephadex G-75 and Sephadex G-200 filtration. Only one peak with galactokinase activity was obtained with hemolysates and purified preparations. A molecular weight of 53,000 to 57,000 was read from the calibration curve given in Fig 1. Enzyme aging in the presence of 7 mM 2-mercaptoethanol results in the appearance of two protein peaks on dextran gel chromatography. Only one peak with galactokinase activity was detectable. By analytical disc electrophoresis in polyacrylamide a single protein band was found with the purified enzyme.

**Electrophoresis**—On SDS-polyacrylamide electrophoresis of 3,600- to 3,850-fold purified preparations, two bands were observed after staining with Coomassie blue. One band was in the position of 53,000 to 57,000 molecular weight, a second band in the position of 25,000 to 27,000 molecular weight.

**Kinetics**—The Michaelis-Menten constant of purified galactokinase for galactose was 100 to 150 µM and for ATP was 200 to 500 µM.

**pH Optimum**—The pH optimum of the enzyme was found to be

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**TABLE I**

**Purification of galactokinase from human red blood cells**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Protein (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Total activity (units)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemolysate</td>
<td>500</td>
<td>47.5</td>
<td>23.8 x 10^4</td>
<td>0.021</td>
<td>501</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-Sephadex A-50 filtrate</td>
<td>66</td>
<td>1.42</td>
<td>93.7</td>
<td>4.30</td>
<td>403</td>
<td>204</td>
<td>80.5</td>
</tr>
<tr>
<td>(NH₄)₂SO₄, 35 to 60% preparation</td>
<td>1.8</td>
<td>19.2</td>
<td>34.6</td>
<td>9.29</td>
<td>321</td>
<td>440</td>
<td>64.1</td>
</tr>
<tr>
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<td>0.11</td>
<td>6.56</td>
<td>28.2</td>
<td>184</td>
<td>1530</td>
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</tr>
<tr>
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<td>1.08</td>
<td>81.2</td>
<td>87.7</td>
<td>3850</td>
<td>17.5</td>
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</tbody>
</table>

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**Fig. 1.** Determination of molecular weight of galactokinase from normal red blood cells by Sephadex G-75 chromatography (Curve A) and Sephadex G-200 chromatography (Curve B). The arrows indicate the range of five estimations of galactokinase. Curve A, Sephadex G-75, equilibration and elution with Buffer A; column size, 1.5 × 90 cm; ascending chromatography, flow rate 8.0 ml per hour. Curve B, Sephadex G-200, equilibration and elution with Buffer A; column size, 0.9 × 60 cm; ascending chromatography, flow rate 2.8 ml per hour. Whereas contaminating proteins were absorbed, galactokinase was not bound to the column exchanger. Fractions containing galactokinase (8.4 to 26.4 ml) were pooled ("CM-Sephadex C-50 filtrate") and concentrated by ultrafiltration.
Considerable differences were found between the affinity for galactose of *E. coli* (12) and yeast (10) enzyme and that of the red cell enzyme. The molecular weight of red cell galactokinase was higher than of galactokinase from *E. coli* (12, 13) and lower than of galactokinase from yeast (11). The size of the galactokinase subunits of yeast (11) was reported to be 23,000.

The kinetic properties of purified red cell galactokinase are similar to those reported earlier using crude extracts of rat tissues (8) and human red cells (9), white cells (25), and fibroblasts (25).

Chromatographic studies with Sephadex G-75 and Sephadex G-200 showed the molecular weight of galactokinase to be 53,000 to 57,000. On aging the molecule seems to be dissociated into subunits with a molecular weight of 25,000 to 27,000. This interpretation was confirmed by the findings in SDS polyacrylamide electrophoresis, where one band in position of molecular weight of 53,000 to 57,000 and a second band of molecular weight of 25,000 to 27,000 was detected. We interpret these results as indicating that the native enzyme has a molecular weight of about 55,000 and that it consists of two equal sized subunits. In SDS-polyacrylamide the dissociation into the subunits was incomplete in galactokinase samples and in some of the reference proteins. This incomplete dissociation may be caused by either the existence of very strong bonds which are only partially split by SDS in the time and conditions of treatment or to the presence of secondary bonds resistant to SDS between some subunits.

A comparison of the characteristics of galactokinase from various sources is shown in Table II. The purified enzyme obtained from pig liver (14) seems to have similar properties as galactokinase from red blood cells. Variants with enzyme deficiency may demonstrate biochemical differences. Furthermore, by using purified galactokinase as an antigen, immunological methods may reveal the abnormal nature of galactokinase in variants.

**REFERENCES**
