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Hexokinase type I multiplicity in human erythrocytes

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Hexokinase I in human erythrocytes exists in multiple molecular forms that differ in isoelectric points. By means of Western blotting and immunodetection of total glucose-phosphorylating activity by using an antibody raised in rabbit against homogeneous human placenta hexokinase I, a single protein band was detected. Identical results were also obtained by immunoaffinity chromatography of the partially purified enzyme. Separation of the three major hexokinase I subtypes (Ia, Ib and Ic) by h.p.l.c. ion-exchange chromatography and immunodetection following electrophoretic blotting confirmed that each hexokinase subtype showed the same apparent M_r of 112000, which is the value obtained for the high-M_r hexokinase I from human placenta. Purification of erythrocyte hexokinase by a combination of several procedures including dye–ligand and affinity chromatography that were previously successfully applied to the purification of other mammalian hexokinases type I produced a 35000-fold-purified enzyme that showed several contaminants after SDS/polyacrylamide-gel electrophoresis. Only one of these peptides was found to be recognized by anti-(hexokinase I) IgG, suggesting that proteolytic degradation does not occur and that hexokinases Ia, Ib and Ic have the same apparent M_r.

INTRODUCTION

Four different hexokinases (EC 2.7.1.1) catalyse the phosphorylation of glucose in mammalian tissue [1]. In human erythrocytes hexokinase type I is the predominant isoenzyme, but in recent years we [2,3] and others [4] have clearly shown that this glucose-phosphorylating activity consists of at least three different subtypes (Ia, Ib and Ic) that differ in isoelectric points and show pronounced cell-age-dependent modifications [3]. Although several pieces of evidence [5–7] seem to indicate that all hexokinase I subtypes are under the control of the same gene, the origin of these multiple forms is still an open question. Furthermore, it has been shown that hexokinase type I from human heart [8] and human placenta [9] is present in two subtypes with different M_r values in SDS/polyacrylamide-gel electrophoresis, but with identical isoelectric points. This fact raises the question whether the erythrocyte enzyme is present as two components and what relationship exists, if any, between the microheterogeneity in charge previously reported [2–4] and the differences in M_r as seen in other human hexokinases I [8,9]. This question could in principle be solved by the purification of the human erythrocyte enzyme to homogeneity, but this does not seem feasible, since all the purification procedures so far reported [10–12] have provided an enzyme protein that is not homogeneous after electrophoresis in the presence of SDS. Since the problem of enzyme microheterogeneity is of general interest, at least in the erythrocytes, where many enzymes are present in multiple molecular forms [13], we have undertaken a study of human erythrocyte hexokinase microheterogeneity to assess two specific points, namely whether or not the hexokinase in human erythrocytes is present in subtypes with different M_r values, as in heart and placenta, and what relationship there is, if any, between charge and M_r among the erythrocyte hexokinases Ia, Ib and Ic. The results reported in the present paper show that in the human erythrocyte only one component of hexokinase is present, with an M_r of 112000, and that no correlation exists between charge and M_r heterogeneity in this enzyme.

MATERIALS AND METHODS

Materials

Coenzymes, enzymes and substrates were obtained from Sigma Chemical Co. or Boehringer Biochemia. Activated carboxyhexyl- (CH-)Sepharose 4B, Protein A–Sepharose CL-4B and DEAE-Sephadex A-50 were from Pharmacia, DE-52 DEAE-cellulose was from Whatman, and Matrex Gel Red A, Matrex Gel Blue A and ultrafiltration membranes (PM 30) were from Amicon. Electrophoresis and immunoblotting equipment and reagent were from Bio-Rad Laboratories. Glutardialdehyde-activated affinity adsorbent was from Boehringer.

Assays

Hexokinase activity was measured as described in ref. [9], protein was determined by the method of Bradford [14] and haemoglobin was determined as described in ref. [5].

Purification of hexokinase

Hexokinase type I from human placenta was purified as described in ref. [9] to a specific activity of 190 ± 5 units/mg of protein and showed two protein bands (M_r 112000 and 103000) upon SDS/polyacrylamide-gel electrophoresis.

Hexokinase from human erythrocytes was partially purified up to $(NH_4)_2SO_4$ fractionation in the procedure described in ref. [3] and then by Matrex Gel Red A chromatography, affinity chromatography on immobil-

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ized glucosamine and Matrex Gel Blue A chromatography as described for the placenta enzyme [9]. The final specific activity ranged between 25 and 30 units/mg of protein in three different preparations with yields in the range 10–20%, representing a higher-than-35000-fold purified enzyme that was contaminated by several proteins as evidenced by SDS/polyacrylamide-gel electrophoresis.

Antibodies

An antiserum against homogeneous hexokinase I from human placenta was raised in rabbits. Immunization was by the injections of 100 μg of protein at 10-day intervals, with the first injection being in compete Freund's adjuvant. IgGs were prepared from serum of immunized and control rabbits by chromatography on immobilized Protein A and elution by 0.1 M-sodium citrate buffer, pH 3.5. A 50 μg portion of anti-hexokinase IgG was found to be able to inactivate 0.25 unit of hexokinase activity. No inactivation occurred with IgG from normal rabbits. Purified IgGs were coupled to the glutardialdehyde-activated affinity adsorbent according to the instructions of the manufacturers and equilibrated in 0.9% NaCl solution.

Separation of hexokinases Ia, Ib and Ic

Hexokinases Ia, Ib and Ic were obtained by h.p.l.c. ion-exchange chromatography from human erythrocyte lysate and the $(NH_4)_2SO_4$ fraction (see above). Briefly, 0.5 ml samples were applied on a Bio-Gel TSK DEAE 5PW column (75 mm × 7.5 mm internal diam.) equilibrated in 5 mM-sodium/potassium phosphate buffer, pH 7.5, containing 3 mM-2-mercaptoethanol and 5 mM-glucose, connected to a Beckman h.p.l.c. system and operated at 1 ml/min. The column was developed by a 300 ml linear gradient of 0–0.4 M-KCl in the equilibrating buffer, and 1 ml fractions were collected and assayed for hexokinase activity. The fractions containing hexokinases Ia, Ib and Ic were pooled separately, dialysed and concentrated by ultrafiltration and used for electrophoresis and electroblotting experiments as previously described [9].

RESULTS

Immunological properties of erythrocyte, hexokinases

In previous studies [7] we have shown that human erythrocyte hexokinases Ia, Ib and Ic are inactivated with the same affinity by an antibody raised in rabbit against human placenta hexokinase I. Unfortunately that antibody did not recognize the enzyme efficiently in Western blotting, so we prepared a new antiserum, The IgG used in these experiments recognized both native and denatured hexokinase I but did not recognize native or denatured hexokinase type II (results not shown) prepared from human placenta. Fig. 1(*a*) shows an immunoblot of human placenta hexokinase I and of a partially purified hexokinase fraction from human erythrocytes containing (see below) all three hexokinase subtypes. Only one band is commonly seen in the erythrocyte sample, and this corresponds to the high-M_r hexokinase found in human placenta and shows an M_r of 112000. To be sure that this protein band is not aspecifically detected by the rabbit IgG, we have performed an immunoaffinity purification of human erythrocyte hexokinase. A sample (2 activity units) of hexokinase

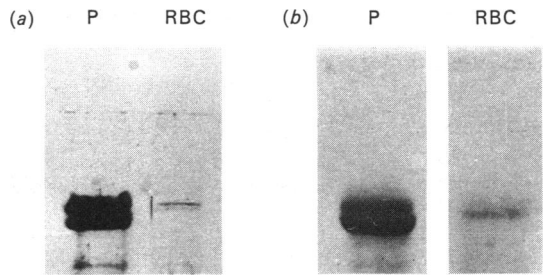

Fig. 1. Immunoblot of human hexokinase type I from placenta (P) and erythrocytes (RBC)

(*a*) Hexokinase type I purified from human placenta and that partially purified from human erythrocytes (see the text) were electrophoresed on 10% polyacrylamide slab gel in the presence of SDS, electroblotted on a nitrocellulose sheet and then incubated with Protein-A-purified immunoglobulins (1:500) from rabbits immunized with homogeneous human hexokinase type I. Immunostaining was obtained by incubation in 1:200 goat anti-(rabbit IgG) antibody–horseradish peroxidase conjugate. Colour development was obtained in 0.015% (v/v) H_2O_2 containing 0.05% (w/v) 4-chloro-1-naphthol. (*b*) As in (*a*) except that erythrocyte hexokinase was purified by immunoaffinity chromatography first on immobilized rabbit IgG (unretained) and then on rabbit anti-(human hexokinase) IgG, from which it was eluted by 0.1 M-sodium citrate buffer, pH 3.5, after extensive column washing with 3% (w/v) NaCl.

from an $(NH_4)_2SO_4$ fraction [3] of erythrocyte lysate was chromatographed first on immobilized IgG from normal rabbits and then on a 2 ml column of immobilized anti-(hexokinase I) IgG. The column was then washed with 3% (w/v) NaCl solution until protein absorbance at 280 nm was less than 0.005, and the bound enzyme was eluted with 0.1 M-sodium citrate buffer, pH 3.5. Eluted proteins were dialysed against 200 vol. of 10 mM-sodium phosphate buffer, pH 7.5, containing 3 mM-2-mercaptoethanol, concentrated in a Savant concentrator, separated by SDS/polyacrylamide-gel electrophoresis and electroblotted as described in ref. [9]. Fig. 1(*b*) shows again only one protein band with an electrophoretic mobility similar to that found for the high-M_r hexokinase of human placenta.

M_r values of hexokinases Ia, Ib and Ic

Hexokinases Ia, Ib and Ic were separated by h.p.l.c. ion-exchange chromatography from human erythrocyte lysate, but the amount of protein was too low to be detected in immunoblots. To increase the amount of hexokinase subtypes an $(NH_4)_2SO_4$ fraction of erythrocyte lysate was chromatographed (Fig. 2). The procedure required to remove the bulk of the haemoglobin (batch chromatography on DEAE-Sephadex A-50) caused some loss of hexokinase Ic, as previously shown [3]; however, this is not a serious problem, since pooled fractions of each hexokinase subtype provided enough material to show that hexokinases Ia, Ib and Ic have the same M_r of 112000 (Fig. 3).

Purification of human erythrocyte hexokinase

Attempts to have a homogeneous hexokinase preparation have been done by many authors [10–12] unsuccess-

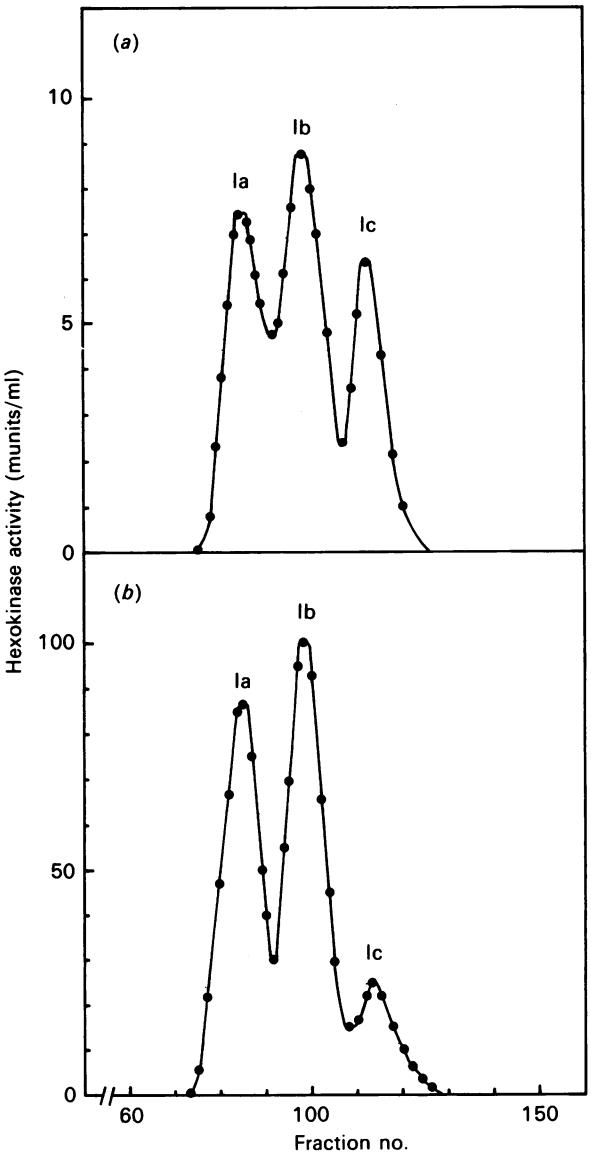

Fig. 2. H.p.l.c. ion-exchange chromatography of hexokinase from (*a*) erythrocyte lysate or (*b*) a partially purified fraction

The samples of hexokinase chromatographed were 0.7 activity unit for erythrocyte lysate or 2.8 units when an enzyme fraction after haemoglobin removal on DEAE-Sephadex A-50 and $(NH_4)_2SO_4$ fractionation as described in ref. [3] was used.

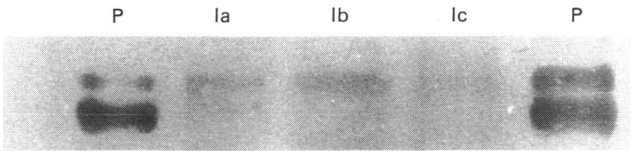

Fig. 3. Immunoblot of human placenta hexokinase type I (P) and hexokinases Ia, Ib and Ic from human erythrocytes

Erythrocyte hexokinases were obtained by h.p.l.c. ion-exchange chromatography as in Fig. 2 and immunoblot was performed as in Fig. 1.

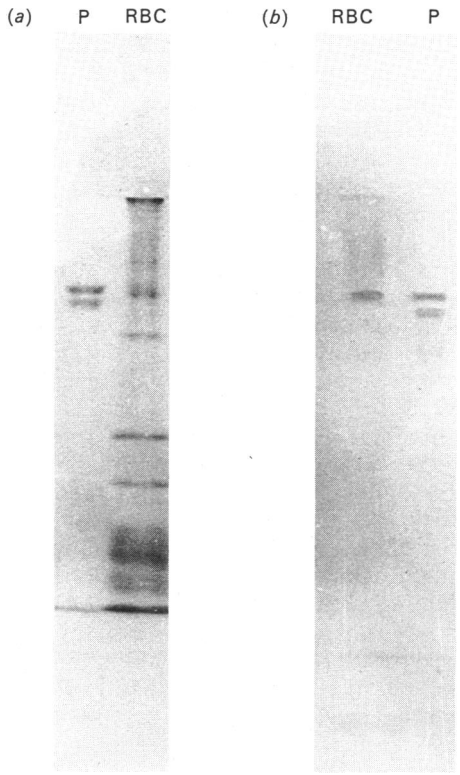

Fig. 4. (*a*) SDS/polyacrylamide-gel electrophoresis and (*b*) immunoblot of hexokinase purified from human placenta (P) and from human erythrocytes (RBC)

fully. We utilized a procedure that removed the bulk of haemoglobin by DEAE-Sephadex A-50 as described in ref. [3] and then utilized the strategy we have developed to purify hexokinase I from human placenta. A 1-litre volume of blood was commonly employed as starting material; however, we obtained enzyme preparations always contaminated by proteins as detected by SDS/polyacrylamide-gel electrophoresis. However, immunoblotting was performed on these samples and only one polypeptide was always detected (Fig. 4). Since the antibody employed was a polyclonal one able to detect denatured and proteolysed hexokinase (results not shown), we can conclude that in erythrocytes hexokinase type I consists of only one polypeptide of M_r 112000, in contrast with that previously found for placenta and heart hexokinase I [8,9].

DISCUSSION

Many reports in the last years have already shown that the occurrence of enzymes in multiple molecular forms should no longer be considered an exception, but rather a common feature of biological systems. Among the several mechanisms that could lead to the appearance of enzyme microheterogeneity, two seem to involve human hexokinase I. Independent studies in our [3] and other laboratories [4] have shown that hexokinase I in human erythrocytes, as well as in the erythrocytes of other mammalian species [15], is present in multiple molecular forms that differ in charge. More recently human heart hexokinase type I [8] and human placenta hexokinase I [9] have been isolated in homogeneous form and found to be present in two different subtypes with M_r values of

112000 and 103000 [8]. This fact prompted us to re-investigate the microheterogeneity of human erythrocyte hexokinase to see whether or not any relationship exists between charge and M_r heterogeneity in hexokinase I. The approach that we used to study this problem was based on the high sensitivity and specificity of the immunoblotting techniques. As we have reported in the present paper, this approach permits us to get round the problem of having the enzyme from erythrocytes in homogeneous form, and of having to use relatively small amounts of blood. The data reported are clear evidence that hexokinase I in human erythrocytes is present in only one component of M_r 112000, which is similar to the value previously found for the high-M_r components of hexokinase I from human placenta. It could be speculated that hexokinases Ia, Ib and Ic probably arose by mechanism(s) other than proteolysis. Several enzymes in erythrocytes undergo multiplicity by deamination [13] or oxidation. We have performed chromatographic separation of human hexokinases Ia, Ib and Ic immediately after the preparation of cell lysate or after dialysis against reducting agents (2-mercaptoethanol and dithiothreitol), but no changes in the chromatographic pattern were observed (results not shown), making the hypothesis of a partial enzyme oxidation unlikely. A definitive answer about the mechanisms leading to the erythrocyte hexokinase microheterogeneity has still not been obtained; however, it seems to be worthwhile to work on this problem, as enzyme microheterogeneity is one of the determinants of their half-life *in vivo* [16].

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