An Isozyme of Hexokinase Specific for the Human Red Blood Cell (HK$_R$)

By Koko Murakami, Francine Blei, William Tilton, Carol Seaman, and Sergio Piomelli

The hexokinase (HK) of the human red blood cell (RBC) was separated into two distinct major isozymes by fast protein liquid chromatography using a linear salt gradient on a MonoQ column. The first isozyme (HK$_1$) eluted as a sharp peak at the same position as HK of human liver. The second isozyme eluted between HK$_1$ and HK$_a$ of human white blood cells, and it appeared to be unique to the RBC (it was designated HK$_a$). From a gel filtration column, HK$_a$ eluted before HK$_a$, suggesting that it was larger than HK$_a$ by several kilodaltons. In a mitochondria-enriched fraction from human reticulocytes, no HK$_a$ was found; thus, HK$_a$ was not a mitochondrial enzyme. Despite these differences in chromatographic behavior, size, and mitochondrial binding, both forms behaved kinetically as HK$_a$. RBC from normal blood contained HK$_1$ and HK$_a$ at an equal activity, but in reticulocyte-rich RBC, HK$_a$ dominated. When RBC of increasing age was separated by buoyant density ultracentrifugation, the total HK activity decayed in a biphasic manner, with half-lives respectively of ~15 and ~51 days. When isolated by MonoQ column from each age-separated fraction, HK$_a$ was the major form in the youngest RBC, and decreased rapidly with cell age, with a t$_{1/2}$ of ~10 days, representing a negligible activity in the oldest RBC. Instead, HK$_a$ was relatively stable through the entire life span of the RBC, with a t$_{1/2}$ of ~66 days. Thus, HK$_a$ appears to be an RBC-specific isozyme that is predominant in the reticulocyte and is then rapidly degraded. During maturation of the RBC, the fast decay of HK$_a$ contributes to the early sharp decline of HK activity and the slow decay of HK$_a$ to the later gradual decline. © 1990 by The American Society of Hematology.

MATERIALS AND METHODS

Preparation of human "pure RBC" suspensions. Blood samples from healthy adult volunteers were defibrinated in a syringe with glass beads. The blood was diluted with five volumes of phosphate-buffered saline containing 10 mmol/L glucose (BSG), and filtered through two layers of Whatman #2 paper. After filtration, the cells were washed twice with BSG and packed by centrifugation. The resulting suspension was essentially free of platelets, containing less than 1% of the original leucocytes, but retained an unchanged percentage of reticulocytes. The preparation was performed in the same manner as described in the previous study. Reticulocyte-rich samples, obtained from the blood of patients with sickle cell anemia discarded at time of therapeutic exchange transfusion, were similarly filtered.

Preparation of HK from RBCs. RBC suspensions were lysed with a hypotonic buffer (5 mmol/L Tris-Cl, pH 7.4, 0.5 mmol/L diethiothreitol, 0.5 mmol/L glucose, and 1 mmol/L ethylenediamine tetra acetic acid [EDTA]). After centrifugation at 30,000 x g for 30 minutes, the supernatant was loaded on DE52 (Whatman, Clifton, NJ).
HEXOKINASE ISOZYME SPECIFIC FOR HUMAN RBC

NJ) equilibrated with column buffer (20 mmol/L Tris-Cl, pH 7.4, 0.5 mmol/L dithiothreitol, 0.5 mmol/L glucose, and 0.1 mmol/L EDTA). The hemoglobin was first washed out with column buffer, then the protein fraction containing HK was eluted with 0.5 mol/L KCl in the same buffer, and precipitated by 65% saturated ammonium sulfate. After dialysis against column buffer, the protein fraction was chromatographed on a BioGel 0.5m column (20 mmol/L Tris-Cl, pH 7.4, 0.5 mmol/L glucose, and 0.1 mmol/L EDTA). The hemoglobin was first washed out with column buffer, KCl in the same buffer, and precipitated by 65% saturated ammonium sulfate, obtained from sickle cell patients as described above, was lysed through a millipore filter (200 μm) (Millipore, Bedford, MA), and stored at −70°C.

Preparation of a mitochondria-enriched fraction from human reticulocytes. The reticulocyte-rich (16% to 25%) RBC suspension, obtained from sickle cell patients as described above, was lysed with the hypotonic buffer. The isotonicity was then rapidly restored by addition of the appropriate amount of a concentrated NaCl solution, and the lysate was centrifuged at 1,600 g for 10 minutes. From the resulting supernatant, the particulate fraction was obtained by centrifugation at 30,000 g × g for 20 minutes. This sediment was then washed twice with a mannitol buffer containing MgCl2 (0.25 mol/L mannitol, 20 mmol/L Tris-HCl, pH 7.4, 0.5 mmol/L dithiothreitol, 0.5 mmol/L glucose, 1 mmol/L MgCl2). The final pellet, enriched with mitochondria, was colorless, and thus presumably free of hemoglobin and cytoplasmic protein. Measurements of hexokinase activity showed that approximately 5% of the total lysate activity was contained in this fraction. It was then divided into two aliquots, one suspended in the mannitol buffer and the other in the mannitol buffer containing 0.2 mmol/L glucose-6-phosphate (G6P).

Preparation of platelet and white blood cell (WBC) suspensions. Blood collected in EDTA (18 mL) was centrifuged at 600 x g for 3 minutes to separate the platelet-rich plasma (PRP). From the PRP, the platelets were precipitated by centrifugation at 1,800 x g for 15 minutes; these preparations contained essentially platelets (platelet suspension). The sedimented mixture of RBC and WBC was then loaded on a dextran solution (1.25% dextran and 0.8% NaEDTA in 0.9% NaCl) and allowed to separate by gravity for 30 minutes at 37°C. The supernatant dextran layer contained 72% and 33%, respectively, of the original WBC and platelets. These were concentrated by centrifugation at 1,800 x g for 15 minutes (WBC plus platelet suspension). Contaminating RBC was removed from both preparations by lysis with the hypotonic buffer for 1 minute, followed by restoration of isotonicity with NaCl. The colorless pellet of cells was then suspended in 2 mL of hypotonic buffer, and lysed by three cycles of freezing (−70°C) and thawing. The supernatant, after centrifugation at 30,000 x g for 30 minutes, was directly applied on a MonoQ column.

Preparation of liver extracts. Human liver obtained at autopsy was homogenized in three volumes of column buffer with an Omni mixer (Dupont, Wilmington, DE), then centrifuged at 30,000 x g for 30 minutes. From the resulting supernatant, the HK fraction was obtained by the same procedure described above for RBC.

MonoQ column chromatography. A MonoQ anion exchange column (0.5 x 3 cm) was used in an FPLC system (Pharmacia). Using a GP250 gradient programmer, a linear gradient of KCl was established on two buffers (no-salt buffer: 50 mmol/L Tris-Cl, pH 7.4, 0.5 mmol/L dithiothreitol, 0.5 mmol/L glucose, and 0.05% Na Azide; high-salt buffer: 1.0 mol/L KCl added to no-salt buffer). After the injection of the enzyme-containing solution, the proteins were first eluted with 95% no-salt buffer and 5% high-salt buffer for 10 minutes, then with a linear gradient of 5% to 35% high-salt buffer (50 to 350 mmol/L KCl) over 10 to 40 minutes. The flow rate was 1.0 mL/min; 0.5 mL was collected in each fraction. The column was run at room temperature, and the eluate was collected on ice. The concentration of KCl in column fractions was verified using a CDC80 conductivity meter (Radiometer American, Westlake, OH). Repeated analyses of several preparations were reproducible without need for regenerating the MonoQ. The recovery of HK activity was 70% to 80%.

Enzyme assays. HK activity was measured at 37°C by coupling with glucose-6-phosphate dehydrogenase, and pyruvate kinase activity was measured at 25°C by coupling with lactate dehydrogenase as described previously.

Protein and hemoglobin determination. Protein concentrations were measured by the method of Lowry et al using bovine serum albumin as reference. Hemoglobin was quantitated after conversion to cyanmethemoglobin.

Buoyant density ultracentrifugation of RBC. Discontinuous density gradients (1.0973, 1.1010, 1.1048, 1.1085, 1.1122) of arabinoalactane were prepared in a cellulose nitrate tube (1.6 x 10.2 cm) as previously reported. Two milliliters of RBC suspensions diluted in BSG to a hematocrit of approximately 70% (equivalent to −4 mL blood) was placed on top of the gradient, which was then ultracentrifuged in a Spinco model (Beckman, Somerset, NJ), using the SW27 swinging bucket rotor at 60,000 g for 45 minutes. Six layers of cells were at the interfaces. They were isolated by slicing the tube, and washed with BSG. The hemoglobin content of each fraction was measured and used to establish the cumulative distribution function of each layer; from this the average cell age for each layer was estimated as previously published.

RESULTS

HK isozyme profile of human RBC and other human tissues. It has been difficult until now to reproducibly separate the HK isozymes of RBC due to their similar size and charge. This study used the MonoQ anionic exchange column in an FPLC system (Pharmacia), a technique that is particularly successful in analyzing various isozymes because of its fine and rapid resolution. The HK was adsorbed onto the MonoQ in the no-salt buffer, then eluted by a linear gradient from 50 to 350 mmol/L KCl in the same buffer. Figure 1A shows that the HK of human RBC was well-separated into two peaks, one eluting at 120 mmol/L KCl (HKα) and another at 160 mmol/L KCl (HKβ). The elution was completed within 30 minutes, at a flow rate of 1 mL/min. The resolution and rapidity of the MonoQ column exceeded by far those of conventional columns, such as DEAE-cellulose, phosphocellulose, etc. Although much of the data reported in this report result from the analysis of partially purified enzymes on DEAE-cellulose, it must be noted that a similar pattern of separation was obtained with crude lysate; thus there was no preferential loss of any isozyme during preparation.

To identify the specificity of the RBC enzyme, the HK of human platelet suspensions, of mixed WBC and platelet suspensions, and of liver extracts was similarly analyzed by MonoQ column chromatography. The platelet HK consisted only of HKα that eluted at the same salt concentration (120 mmol/L KCl) as HKα from RBC (Fig 1B). The WBC plus platelets preparation contained both HKα and HKβ, eluting at 120 and 185 mmol/L KCl, respectively (Fig 1B). Therefore, the second form (HKβ) of RBC HK, which eluted at 160 mmol/L KCl, a lower salt concentration than for HKα of WBC, cannot represent either WBC or platelet contamination. The HK from liver was separated by the MonoQ
column into two major forms: HK₁, that eluted at 120 mmol/L KCl, and HK₃₁ that eluted at 205 mmol/L KCl, later than HK₃₂ from WBC (Fig 1C), and was inhibited by glucose at a high concentration. A subtype of HK₁ was constantly observed in the liver extract, which eluted as a preceding shoulder to HK₃. Thus, the second isozyme of RBC (HK₃₂) eluting at 160 mmol/L KCl between HK₁ and HK₃₁ appeared different from any HK isozyme in other blood cell types or other tissues tested, and it most likely represents a unique isozyme of the RBC.

The kinetic parameters of HK₁ and HK₃₁ of RBC were compared. The Km (Michaelis constant) for glucose was 0.064 mmol/L (95% confidence limits, 0.04 to 0.1 mmol/L for HK₁, and 0.05 to 0.09 mmol/L for HK₃₁) for both isozymes; the Km for adenosine triphosphate was also comparable: 1.38 mmol/L (95% confidence limits, 0.95 to 2.3 mmol/L) for HK₁ and 1.27 mmol/L (95% confidence limits, 0.9 to 3.3 mmol/L) for HK₃₁. Thus, the RBC-specific isozyme behaved kinetically as HK₁.

HK from rabbit RBC similarly prepared was also analyzed on the MonoQ column. Figures 1A and 1D compare the isozyme profiles of human and rabbit RBC. In both cases there were two distinct peaks; however, while both first peaks eluted at 120 mmol/L KCl, the second peak eluted at 160 mmol/L KCl in the case of human RBC, while in the case of rabbit RBC it eluted later at 180 mmol/L KCl. Thus, although there were two isozymes in both species, the second one was much more clearly separated from the first in the case of the rabbit RBC than in the case of human RBC.

The RBC-specific isozyme of human HK (HK₃₁) is larger than HK₁. When the hemolysate of human RBC was first directly applied on DEAE-cellulose to wash out hemoglobin and later chromatographed on a BioGel column, the HK eluted in a sharp peak in fractions 38 to 41 (Fig 2, inset). The fractions were not pooled but were instead individually analyzed on MonoQ columns. In the first of the BioGel fractions (no. 38), HK₃ represented most of the activity with a small percentage of the activity due to HK₁ (Fig 2). On the other hand, HK₁ dominated in the last BioGel fraction (no. 41). Thus, since HK₃₁ clearly migrated faster than HK₁ on BioGel, HK₁ appeared to be larger than HK₁ by several kilodaltons.

HK₁, but not HK₃₁, is eluted by G6P from the particulate fraction of human reticulocytes. The particulate-bound HK in a mitochondria-enriched fraction from human reticulocytes, which contained approximately 5% of the total hexokinase activity in the original lysate, was solubilized with 0.2 mmol/L G6P for 10 minutes at 30°C. The supernatant from a 1-mL suspension (pellet:buffer ratio = 1:1)
contained 10.2 mU of HK in the G6P-containing preparation and 2.4 mU in the untreated one. Thus, the bound form of RBC HK eluted specifically with G6P, as shown for bovine brain HK by Rose and Warms. The G6P-solubilized HK was analyzed on a MonoQ column; no HK$_r$ was found; thus, also in the human RBC, HK$_r$ is not a mitochondria-bound isozyme. The G6P-solubilized HK eluted in a closely overlapping peak with HK$_r$. We could not clearly identify an additional mitochondrial-bound form of HK that was separable from HK$_r$ by ionic exchange high performance liquid chromatography (HPLC) as Stocchi et al recently described in rabbit RBC.

HK$_r$ is predominant in the reticulocyte. The HK isozyme patterns of normal and reticulocyte-rich RBC suspensions were compared (Fig 3A). RBC from normal blood contained approximately equal activities of HK$_r$ and HK$_r$. On the other hand, HK$_r$ was predominant in reticulocyte-rich RBC suspensions, representing 75% to 80% of the total HK activity.

RBC of increasing mean cell age can be separated by buoyant density ultracentrifugation on a discontinuous gradient of arabinogalactane. Six layers of cells were isolated, and their respective mean age was estimated. From each layer the cells were lysed and centrifuged, and the resulting supernatants were divided into two aliquots. One was directly applied on the MonoQ column. The hemoglobin content in the void volumes of each eluate was measured and the HK activities were expressed as a specific activity against the hemoglobin content. In the other aliquot the total activity of HK was measured.

Figure 3B shows the HK activity profiles from a MonoQ column of each layer in a typical experiment. In the lightest cells (L1, mean age = 22 days), HK$_r$ predominated, constituting 82% of total activity. HK$_r$ then decreased dramatically in the successive layers, in inverse proportion to the age of the cells. In the heaviest cells (L6, mean age = 79 days), HK$_r$ was negligible. Thus it was clear that HK$_r$ was relatively stable and that HK$_r$ rapidly decayed during aging of the cells. The specific activities of each HK isozyme were then plotted against the mean age of the cells in each layer. As shown in the inset, HK$_r$ activity decreased exponentially from 50 to 10 mU/μmole hemoglobin from L1 to L3, with an apparent t$_{1/2}$ of ~10 days (95% confidence limits, 8 to 12 days). For this estimate, only the specific activities of HK$_r$ in L1, L2, and L3 were used since the contribution of HK$_r$ into the HK$_r$ peak became overwhelming in L4, L5, and L6. The apparent t$_{1/2}$ of HK$_r$ activity could be precisely estimated in all layers using only the left limb of the HK$_r$ peak, which did not include any contribution by HK$_r$. The decay rate of HK$_r$ was much slower, with an estimated t$_{1/2}$ of ~66 days (95% confidence limits, 60 to 84 days).

The above results indicating a biphasic rate of decay were in agreement with the results of Rogers et al, but in conflict with our previous report of a linear decline of the total HK activity of the RBC. Therefore we re-examined several separations recently performed in our laboratory to obtain a topmost layer of only 2% to 3% of the RBC population. (This was in contrast to our earlier study in which the topmost layer usually contained more than 10% of the RBC population to provide sufficient yield to measure many enzymes simultaneously.) When the HK activity of each layer was plotted against its cumulative distribution function, it was apparent that the rate of decay was clearly biphasic with two half-lives of ~15 (95% confidence limits, 8 to 18 days) and ~51 days (95% confidence limits, 46 to 57 days), respectively. In the same layers, the activity of pyruvate kinase decayed very linearly, with a single slope and a t$_{1/2}$ of ~30 days (95% confidence limits, 28 to 32 days) (Fig 4).

**DISCUSSION**

Conflicting reports are present in the literature with regard to the HK isozymes of human RBC. Stocchi et al reported the separation of three closely overlapping peaks...
using a very narrow DEAE cellulose column. Gahr,\textsuperscript{13} using the same column with a finer gradient, could show only two peaks. In our laboratory, using the same DEAE columns as Stocchi et al, we also obtained two peaks, as did Gahr. To clarify this issue, we used an FPLC system with a MonoQ column that offered superior resolution. With this technique we could clearly separate the human RBC HK into two peaks. These two forms were also distinguished from each other by molecular size on a gel filtration column and by affinity for the mitochondrial fraction. Thus, it can be concluded that human RBC contains two forms of HK isozymes.

The HK of rabbit RBC had been separated into two isozymes by DEAE-cellulose column chromatography.\textsuperscript{10,14} When the HK isozyme profile from a MonoQ column of human and rabbit RBC were compared, the two isozymes of human RBC eluted at 120 mmol/L and 160 mmol/L KCl, whereas those of rabbit RBC eluted at 120 mmol/L and 180 mmol/L KCl, respectively. Thus, the two rabbit RBC isozymes were even further apart from each other than those of human RBC. Previous studies that used DEAE-cellulose chromatography could not resolve the isozymes as efficiently as the MonoQ column. While the two forms of rabbit RBC HK could be easily separated on DEAE-cellulose, this technique could not adequately resolve the much closer two peaks of human RBC HK, and hence yielded conflicting results.

Our study demonstrates that HK\textsubscript{R}, the second form of human RBC HK, is eluted in a position different from any other HK isozyme present in other tissues. Therefore, the HK\textsubscript{R} isozyme appears to be unique to the RBC.

In normal RBC, the activities of HK\textsubscript{R} and HK\textsubscript{K} were approximately equal. In reticulocyte-rich RBC suspensions, HK\textsubscript{R} was predominant. When RBC were separated on buoyant density gradients into age-dependent layers, HK\textsubscript{R} was shown to be predominant in the youngest cell population, and to be negligible in the oldest RBC. Thus, HK\textsubscript{R}, the RBC-specific isozyme, appears to be present at a high level in the reticulocyte and to degrade rapidly. From the relative specific activity in the layers, the t\textsubscript{1/2} of both isozymes could be estimated. While HK\textsubscript{R} decreased very rapidly, with a t\textsubscript{1/2} of \(-10\) days, HK\textsubscript{K} was much more stable with a t\textsubscript{1/2} of \(-66\) days. Therefore it appears that the rapid in vivo decay of HK, observed by us and others, reflects the rapid decay of HK\textsubscript{R}. Our findings for human RBC closely parallel the findings by Magnani et al\textsuperscript{14} for rabbit RBC. In that species as well the second RBC isozyme decreased during cell aging. Therefore, it appears likely that the presence of an isozyme of HK in RBC precursors, which rapidly decays on entry into the circulation, is a general phenomenon. It has been recently suggested by Butler\textsuperscript{23} that buoyant density separation of RBC only results in a redistribution of reticulocytes and therefore estimates of half-life with these techniques could be artifactual if the change in enzyme activity takes place only at the reticulocyte-mature RBC transition. However, this view is not shared by others.\textsuperscript{24-26}

It has been shown by others that HK decays in a biphasic manner during aging of RBC.\textsuperscript{27} On reanalysis of several separations performed recently in our laboratory, in which 2\% to 3\% of the youngest RBC were isolated in the topmost layer, a biphasic decay was similarly apparent. Our previous study that had erroneously suggested a single slope of decline was based on separations that did not contain many fractions with predominantly young RBC.\textsuperscript{28} The present results suggest that, during maturation and aging of the RBC, the initial rapid decay of HK reflects the rapid decay of HK\textsubscript{R}, and the later slow decrease reflects the slow decay of HK\textsubscript{K}.

Both isozymes of human RBC HK\textsuperscript{10,12,27} appear to behave kinetically as HK\textsubscript{R}, as do also the two isozymes of rabbit RBC. Magnani et al\textsuperscript{27} indicated that all HK isozymes of human RBC were cross-reactive with a polyclonal antibody against HK\textsubscript{R}. In studies of rabbit RBC, they showed that only HK\textsubscript{R} bound to the mitochondria and speculated that this binding stabilized this isozyme, while the unbound one rapidly decayed.\textsuperscript{14} They also suggested that the subtypes of RBC HK resulted from posttranslational modifications of HK\textsubscript{R}. Our study has shown that (1) HK\textsubscript{R} is an isozyme specific to the RBC, and is not found in any other tissues and cells; (2) HK\textsubscript{R}, but not HK\textsubscript{K}, binds to the mitochondrial fraction; (3) HK\textsubscript{R} is clearly larger by several kilodaltons than HK\textsubscript{K}; and (4) the two forms independently decay at different rates.

Thus, our data suggest that HK\textsubscript{R} and HK\textsubscript{K} are separate gene products that are independently regulated, and that the HK\textsubscript{R} gene is expressed only in the RBC. Although HK\textsubscript{R} is smaller than HK\textsubscript{K}, it does not appear to be a degradative intermediate of HK\textsubscript{R}. In fact, as HK\textsubscript{R} decreases, HK\textsubscript{K} does not.
not increase and the two forms decay at the independent rates. Altay et al. showed in their electrophoretic studies that the fast-moving band (presumably our HK2) was increased in hemolytic anemia and deficient in a patient with HK deficiency. These results are consistent with our conclusion that HK1 and HK2 are products of two separate genes. Studies of the molecular mechanisms for the regulation of the HK2 gene and of the molecular structure of both HK1 and HK2 may clarify the process of aging of the RBC.

ACKNOWLEDGMENT

We thank Drs E. Beutler and L. Corash for their thoughtful review of the manuscript and helpful suggestions.

REFERENCES