ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 239, No. 2, June, pp. 352-358, 1985

Regulatory Properties of Human Erythrocyte Hexokinase During Cell Ageing[1]

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Received October 31, 1984

Human red blood cell hexokinase exists in multiple molecular forms with different isoelectric points but similar kinetic and regulatory properties. All three major isoenzymes (HK Ia, Ib, and Ic) are inhibited competitively with respect to Mg·ATP by glucose 6-phosphate (K_i = 15 μM), glucose 1,6-diphosphate (K_i = 22 μM), 2,3-diphosphoglycerate (K_i = 4 mM), ATP (K_i = 1.5 mM), and reduced glutathione (K_i = 3 mM). All these compounds are present in the human erythrocyte at concentrations able to modify the hexokinase reaction velocity. However, the oxygenation state of hemoglobin significantly modifies their free concentrations and the formation of the Mg complexes. The calculated rate of glucose phosphorylation, in the presence of the mentioned compounds, is practically identical to the measured rate of glucose utilization by intact erythrocytes (1.43 ± 0.15 μmol h^{-1} ml red blood $cells^{-1}$). Hexokinase in young red blood cells is fivefold higher when compared with the old ones, but the concentration of many inhibitors of the enzyme is also cell age-dependent. Glucose 6-phosphate, glucose 1,6-diphosphate, 2,3-diphosphoglycerate, ATP, and Mg all decay during cell ageing but at different rates. The free concentrations and the hemoglobin and Mg complexes of both ATP and 2,3-diphosphoglycerate with hemoglobin in the oxy and deoxy forms have been calculated. This information was utilized in the calculation of glucose phosphorylation rate during cell ageing. The results obtained agree with the measured glycolytic rates and suggest that the decay of hexokinase during cell ageing could play a critical role in the process of cell senescence and destruction.

It is well known that living functions of the mature red cell are entirely dependent upon the utilization of glucose. In humans, this limited metabolic ability permits erythrocyte survival for approximately 120 days, after which it is sequestered and phagocytosed in the reticuloendothelial system. The selective nature of this removal of aged cells is mediated by the binding of autologous IgG[3] on the surface of senescing erythrocytes (1, 2). Subsequently the Fc region of the autoantibody is recognized by a macrophage, which phagocytoses the erythrocyte (3). ATP was found to be an important factor in determining IgG binding (4, 5); therefore, the metabolic impairment, which occurs during cell ageing (6), could be considered the primary and fundamental step responsible for ATP decay and the red cell sequestration and destruction.

The hexokinase reaction (EC 2.7.1.1; HK) is the first step in red blood cell glycolysis (the only ATP-producing pathway of this cell) and is also considered a crucial rate-limiting enzyme (7-9). In fact, compared with the other glycolytic en-

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[3] Abbreviations used: IgG, immunoglobulin G; HK, hexokinase; RBC, red blood cells.

0003-9861/85 $3.00

zymes, it shows the lowest activity as it is suppressed by its product, glucose 6-phosphate (10–14) and many other glycolytic intermediates (13, 15–18), and cellular concentrations of Mg·ATP (one of its substrates) are in the range of the respective K_ms (6, 19). Furthermore, among all the enzymes of the glycolytic pathway that show an age-dependent decay of activity, HK seems to be the most critical, with a half-life of 33 days (20, 21). In view of the apparent importance of HK in red cell metabolism, we have investigated the regulatory role of this enzyme during cell ageing. The information derived from measurement of HK inhibitors and substrate concentrations and from determination of the respective K_i is utilized to calculate the reaction velocity under conditions close to those *in vivo*. The values obtained are compared with the glycolytic flux measured in cells of different ages and found to be very similar.

MATERIALS AND METHODS

Materials. Coenzymes, enzymes, substrates, and buffers were obtained from Sigma (St. Louis, Mo.). Ficoll was purchased from Pharmacia (Uppsala, Sweden); Triosil was from Nyegaard (Oslo, Norway). All other chemicals were from standard supply houses, and were of the highest grade available.

Enzyme purification. Human erythrocyte hexokinases Ia, Ib, and Ic were prepared as reported earlier (22). Immediately before use, the enzyme solution was dialyzed against 3 mM Tris-HCl, pH 7.2, 0.25 mM glucose, 3 mM mercaptoethanol.

Enzyme assay. The enzyme activity was determined spectrophotometrically at 37°C in a system coupled with glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and by a radioisotopic assay as described in Refs. (23, 24).

Kinetic studies. The kinetic properties of purified HK Ia, Ib, and Ic were determined spectrophotometrically at 30°C in the coupled glucose-6-phosphate dehydrogenase assay in the presence of 80 mM Tris-HCl, pH 7.2.

Determinations. Red blood cell glucose 6-phosphate, ATP, ADP, and reduced glutathione were determined by the methods of Beutler (26). 2,3-Diphosphoglycerate was determined by an ultraviolet test (Boehringer, Mannheim, FRG), and glucose 1,6-diphosphate was assayed as previously described (27). Red blood cell Mg was determined by atomic absorption at 285.2 nm, subtracting the level in plasma from the level found in whole blood after evaluation of the hematocrit.

Glycosylated hemoglobin was determined by column chromatography utilizing the Helena Glyco Hb test kit (Helena Laboratories, Texas).

Glucose utilization. Glucose utilization by intact cells was determined as previously described (23).

Red blood cell fractionation. Red blood cells were separated into fractions of different mean age by ultracentrifugation through a density gradient of Ficoll-Triosil as described in Ref. (28). After centrifugation the red cells were separated into six fractions, numbered from 1 to 6, and represented erythrocytes of increasing density (age).

RESULTS

Hexokinase isozymic pattern in red blood cells of different ages. Hexokinase in human red blood cells exists in multiple molecular forms which can be separated by DEAE column chromatography (29). Of the three major forms (hexokinase Ia, Ib, and Ic on the basis of their order of elution from the DE-52 column) only HK Ia corresponds to HK type I from human liver, while all the other isoenzymes were eluted at intermediate positions between HK type I and type II (22). In young red blood cells, obtained by density gradient ultracentrifugation, HK Ib is the predominant form but becomes one of the minor components in the old erythrocyte (22).

Kinetic and regulatory properties. HK Ia, Ib, and Ic prepared from the whole red blood cell population show the same K_m for glucose (47 ± 3 μM) and the same K_m for $Mg \cdot ATP^{2-}$ (0.6 ± 0.03 mM) at pH 7.2, 30°C. Human erythrocyte HK Ia, Ib, and Ic are also inhibited by glucose 6-phosphate, glucose 1,6-diphosphate, 2,3-diphosphoglycerate, ATP^{4-}, and reduced glutathione competitively compared with $Mg \cdot ATP^{2-}$. The secondary plot of slope of the Lineweaver-Burk plots against inhibitor (not shown) are linear over the range of concentrations used, and the determined K_i values are reported in Table I. HK Ia, Ib, and Ic prepared from young or old red blood cells, obtained by density gradient ultracentrifugation, show kinetic and regulatory properties similar to those reported in Table I for the isoenzymes of the total red cell population.

Intracellular concentration of some erythrocyte constituents. The total concentration of the compounds found to be

TABLE I

TOTAL CONCENTRATIONS AND K_i OF SOME INHIBITORS OF HEXOKINASE

Compound	K_i (μM)	Total concentration (μM)
Glucose 6-phosphate	15	28.0 ± 7.0
Glucose 1,6-diphosphate	22	75.6 ± 12.0
2,3-diphosphoglycerate	4000	4800 ± 200
ATP	1500	1250 ± 150
Glutathione (reduced)	3000	2000 ± 300

Note. The K_m for glucose was estimated to be 47 μM and that of Mg·ATP, 0.6 mM, at pH 7.20 and 37°C. Similar K_i values have been obtained on all three hexokinase isozymes (Ia, Ib, and Ic). The K_i of ATP refers to the uncomplexed form (ATP^{4-}).

inhibitors of erythrocyte HK are reported in Table I. However, because of the binding of many phosphorylated compounds to hemoglobin and the formation of Mg complexes, the intracellular concentration of each species must be calculated to evaluate the reaction velocity at conditions close to cellular. In this sense, the actual substrate of HK is known to be the complex $Mg \cdot ATP^{2-}$, but this species accounts for only a part of the total ATP. On the other hand, only the free form of 2,3-diphosphoglycerate is able to influence the HK reaction (30). In Table II the intracellular distribution of Mg and hemoglobin complexes of various phosphorylated compounds is reported. These data have been obtained by measuring the total amount of ATP, 2,3-diphosphoglycerate, hemoglobin, Mg, and ADP, and from the dissociation constants of the various complexes derived from the literature (37). Further details can be found in Ref. (30).

Age-dependent decay of some erythrocyte constituents. In order to evaluate the HK reation velocity as a function of cell age we have determined the age-dependent decay of compounds known to be HK modulators. In Fig. 1 the decays of glucose 6-phosphate, 2,3-diphosphoglycerate, ATP, glucose 1,6-diphosphate, and Mg are shown. Since to evaluate their intracellular distribution it is also important to know the total concentration of hemoglobin and because glycosylated hemoglobin does not bind phosphorylated compounds (31, 32), we have measured the glycosylated hemoglobin as a function of cell age (Fig. 1). The values obtained are then utilized in the calculation of intracellular distribution of each free and bound species considered as a function of cell age.

Hexokinase activity and cell age. As occurs with many other enzymes, HK decays as a function of cell age (Fig. 2). However, the values obtained from the conventional spectrophotometric assay do not represent the true HK activity because of the presence of endogenous 6-phosphogluconate dehydrogenase. In fact, this last enzyme produces additional NADPH by oxidation of part of the 6-phosphogluconate formed in the gluocse-6-phosphate dehydrogenase reaction (24, 26). To avoid

TABLE II

INTRACELLULAR DISTRIBUTION OF Mg AND HEMOGLOBIN COMPLEXES OF VARIOUS PHOSPHORYLATED COMPOUNDS AT pH 7.20, 37°C, AND μ = 0.14

	K_D (mM)	Intracellular concentration (mM)	
		$+O_2$	$-O_2$
Mg·ATP	0.038	0.714	0.624
Mg·DPG	1.5	0.704	0.328
Mg·ADP	0.430	0.042	0.061
Hb·ATP	0.127	0.057	0.156
Hb·DPG	0.103	1.603	3.875
Hb·ADP	0.830	0.032	0.025
Hb·MgATP	1.150	0.404	0.372
HbO_2·ATP	3.430	—	—
HbO_2·DPG	4.750	—	—
HbO_2·ADP	4.000	—	—
HbO_2MgATP	5.400	—	—
Mg	—	0.424	0.848
ATP	—	0.064	0.029
DPG	—	2.491	0.581
ADP	—	0.043	0.031
Hb	—	3.057	0.687

Note. The dissociation constants reported were from Ref. (38). Intracellular concentrations of free and complexed species were calculated in the presence or absence of O_2 from the conservation equations and the definitions of the various dissociation constants. Further details are reported in Ref. (30).

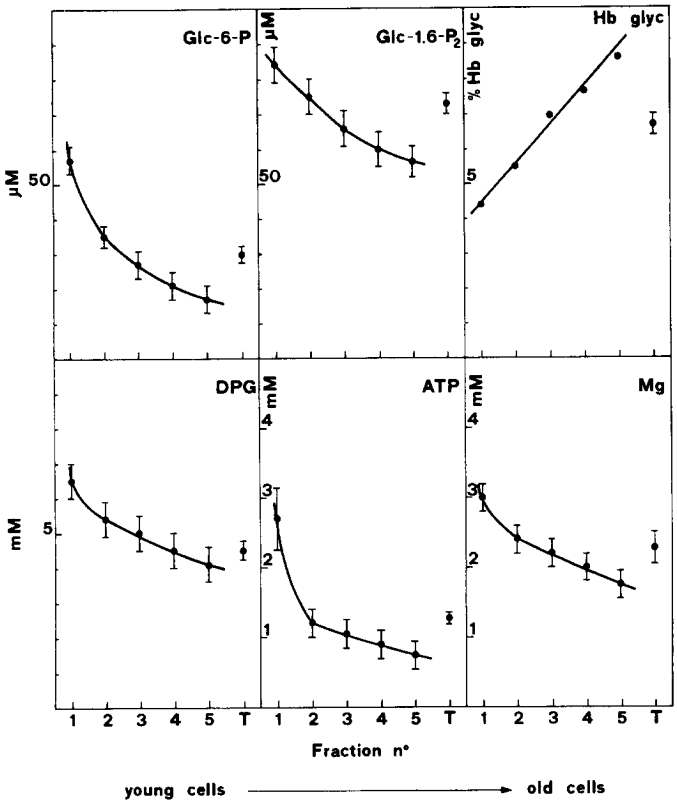

FIG. 1. Red blood cell age-dependence of some cellular compounds. Human erythrocytes were fractionated into six fractions of different mean ages by density gradient ultracentrifugation. Fractions from 1 to 5 represent cells of increasing density (age); T is the whole red cell population. Not enough cells were available in fraction 6 to be utilized for biochemical determinations. All values are means ± SD of five different samples. Glc-6-P, glucose 6-phosphate; Glc-1,6-P_2, glucose 1,6-diphosphate; Hb glyc, glycosylated hemoglobin; DPG, 2,3-diphosphoglycerate.

this problem we have utilized a radioisotopic assay by direct measurement of glucose 6-phosphate produced (24). The results obtained are 25–28% lower than the data provided by the spectrophotometric assay and they probably show the true hexokinase activity of the erythrocyte (Fig. 2).

Regulation of hexokinase under conditions close to cellular in erythrocytes of different ages. As reported above, human erythrocyte HK exists in multiple molecular forms with different decay rates during cell ageing. As we have shown that all three major isoenzymes of human erythrocyte HK possess similar kinetic and regulatory properties, to calculate the reaction velocity under conditions close to cellular we consider them as a single enzyme whose activity is the sum of activities of each isozymic form. Furthermore, glucose is present at a saturating level in all red blood cell fractions (6), the enzyme exhibits Michaelis-Menten kinetics (22), and all the inhibitors considered are competitive versus $Mg \cdot ATP^{2-}$. Taking into account these facts the rate equation can be written as

$$v = \frac{V_{max}}{1 + \frac{K_m(Mg \cdot ATP)}{[Mg \cdot ATP^{2-}]}\left(1 + \sum_{n=1}^{i} \frac{[I]}{K_i}\right)},$$

where I and K_i, respectively, refer to the concentration and dissociation constant of each compound considered. The values of K_m for $Mg \cdot ATP^{2-}$ and K_i do not change during cell ageing, but significant differences occur for the concentrations of ATP and that of inhibitors considered (Fig. 1). Consequently the intracellular distribu-

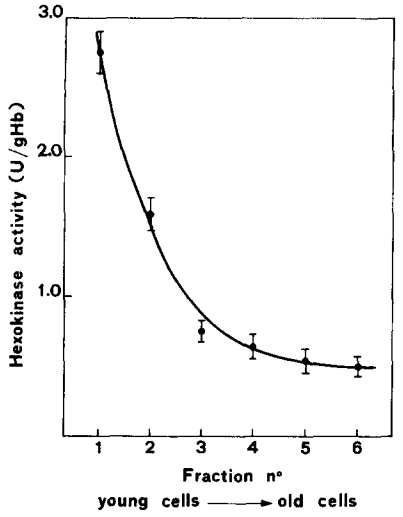

FIG. 2. Red blood cell age-dependence of hexokinase in human erythrocytes. The hexokinase activity was assayed by the radioisotopic method reported under Materials and Methods; means ± SD of five determinations.

tion of free and complexed species of ATP and 2,3-diphosphoglycerate is also modified during cell ageing (Fig. 3). Calculation of the HK reaction velocity according to the rate equation reported above provides values of 6 μmol h^{-1} ml RBC^{-1} in young cells and 0.7 μmol h^{-1} ml RBC^{-1} in the old ones. A comparison of these values with those of the glycolytic flux of intact erythrocytes is reported in Fig. 4 and provides a good example for the control role of hexokinase in the regulation of glucose metabolism during cell ageing.

DISCUSSION

The mechanisms by which senescent erythrocytes are recognized and removed from circulation have been recently clarified (1, 2). The main problem, which is still open, is the nature of the event responsible for the appearance of an antigen on the surface of old RBC recognized

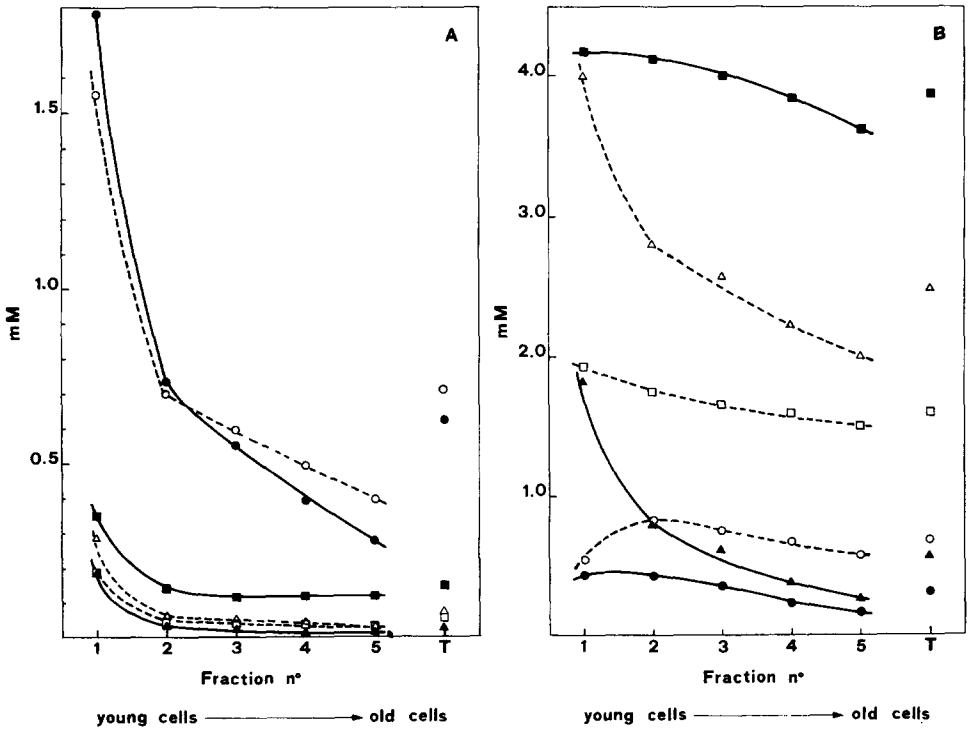

FIG. 3. Intracellular distribution of free and complexed ATP (A), and 2,3-diphosphoglycerate (B), in human erythrocytes of different ages. The values reported were calculated from the K_D reported in Table II and the total concentrations shown in Fig. 1. Details of the calculation procedure as in Ref. (30) (○, ●), $Mg \cdot ATP^{2-}$ or 2,3-diphosphoglycerate Mg; (△, ▲), ATP^{4-} or 2,3-diphosphoglycerate; (□, ■), hemoglobin · ATP or hemoglobin · 2,3-diphosphoglycerate complexes. Open symbol, values in the presence of O_2; filled symbols, values in the absence of O_2.

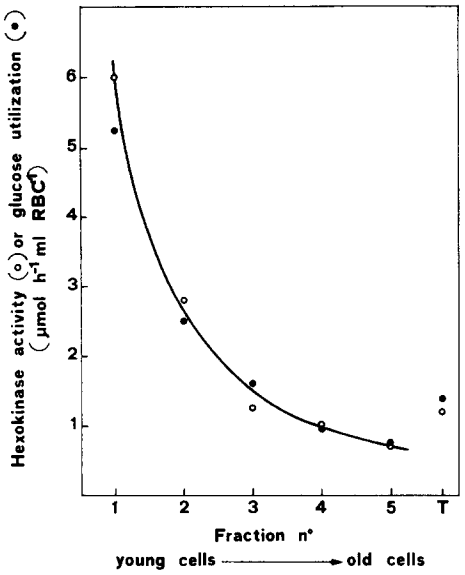

FIG. 4. Comparison between the calculated hexokinase reaction velocity (O), and the measured glucose utilization by intact erythrocytes (●), as a function of cell age. The hexokinase reaction velocity was calculated by the equation reported under Results.

by autologous IgG. Some reports (4, 5) have shown that a correlation exists between the appearance of the senescent antigen and the cellular level of ATP.

ATP in the erythrocyte is produced only in the glycolytic pathway and this, as we have previously reported (6), shows a pronounced age-dependent decay which results in a reduced production of ATP in the old cell. Among glycolytic enzymes, the hexokinase reaction has the lowest turnover (7) and shows the faster decay rate during cell ageing, with a 33-day half-life (20, 21). Because of these properties a rate-limiting role for HK in red cell glycolysis (and, consequently, ATP production) has been proposed by several authors (8, 9, 23). Recent results from our laboratory have provided further evidence for this idea. In fact, we have been able to show that erythrocytes of individuals carrying a *de novo* trisomy 10p have an increased HK activity and a proportional high glycolytic rate (28), while individuals heterozygous for HK deficiency, hematologically normal, have a reduced rate of glycolysis (Magnani *et al.*, submitted for publication). The results reported in this paper further explain the key role of HK in red cell glycolysis in erythrocytes of different ages. Attempts to study the regulation of HK activity related to the cell conditions have been reported by several authors. However, in many cases the experiments were performed on crude hemolysate (10, 33, 34) or at pH values too far away from the physiological values (14, 35). Moreover, the intracellular concentration of some effectors has been misinterpreted, or not considered at all as in the case of orthophosphate, which has received great emphasis because of its ability *in vitro* to remove the HK inhibition brought about by many phosphorylated compounds (10, 13). Unfortunately, orthophosphate is present in the human erythrocyte at only 210 μM concentration (36), a value too low to have any practical significance (22). On the other hand, little attention has been devoted to the age-dependent variation of glucose 1,6-bisphosphate, a powerful HK inhibitor, which has been extensively studied in our laboratory (27, 37). All these problems have been considered in this paper in an attempt to evaluate the reaction velocity of HK under conditions close to cellular. The results obtained show the importance of this approach especially when the study is performed as a function of cell age. In fact, the total intracellular concentration of 2,3-diphosphoglycerate in young erythrocytes in 6.5 mM, but 64.6% is bound to deoxyhemoglobin, 6.8% is bound to Mg, and only 28.6% is, in the free form, able to inhibit the HK reaction. This figure is reduced to only 8.4% in the old erythrocytes (Fig. 3). A further critical parameter in the study of glycolysis control is the real amount of HK present in the erythrocyte. The conventional spectrophotometric assay does not provide information on the true level of HK because of the presence of endogenous 6-phosphogluconate dehydrogenase (24). By a new radioisotopic method we have found HK values which are about 25% lower compared with the spectrophotometric analyses (24). Taking into account the hexokinase activity measured in this way and the effect of many intracellular inhibitors of the enzyme, the HK reaction velocity becomes

practically similar to the glycolytic flux. In other words, the decay of HK activity as a function of cell age could probably explain the decline in the erythrocyte glycolytic rate. This phenomenon, in turn, could be responsible for the decay of cellular ATP. These data cannot be considered conclusive proof that HK is responsible for the cellular loss of ATP during cell ageing. In fact, we could show that the rate of ATP production decreases as the cell becomes senescent but we still do not have information on the ATP-requiring processes of erythrocytes as a function of age. When these data are available, an ATP balance will be possible and the role of HK in cell senescence will be completely understood.

ACKNOWLEDGMENTS

This work was supported by the Cassa di Risparmio di Pesaro and the Ministero della Pubblica Istruzione.

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