Human erythrocyte hexokinase deficiency. Characterization of a mutant enzyme with abnormal regulatory properties.

G Rijksen, G E Staal

J Clin Invest. 1978;62(2):294-301. https://doi.org/10.1172/JCI109129.

Research Article

In the erythrocytes of a patient with hereditary nonspherocytic hemolytic anemia, a homozygous expression of hexokinase deficiency was detected. The mutant enzyme was characterized by normal kinetic parameters with respect to its substrates, glucose and MgATP2-, normal pH optimum, normal heat stability at 40 degrees C, but abnormal behavior with respect to its regulation by glucose-1,6-diphosphate and inorganic phosphate, and an altered electrophoretic pattern. Interpretation of the results revealed the presence of two different hexokinases type I in normal human erythrocytes: one enzyme with a high affinity for glucose-1,6-diphosphate, the inhibition of which is regulated by inorganic phosphate; and another enzyme with a lower affinity for the inhibitor, not regulated by inorganic phosphate. The former enzyme was not detectable in the erythrocytes of the patient, whereas the presence of the latter enzyme could be demonstrated.

Human Erythrocyte Hexokinase Deficiency

CHARACTERIZATION OF A MUTANT ENZYME WITH ABNORMAL REGULATORY PROPERTIES

GERT RIJKSEN and GERARD E. J. STAAL, *Unit of Medical Enzymology, Haematological Department, State University Hospital, Utrecht, The Netherlands*

ABSTRACT In the erythrocytes of a patient with hereditary nonspherocytic hemolytic anemia, a homozygous expression of hexokinase deficiency was detected. The mutant enzyme was characterized by normal kinetic parameters with respect to its substrates, glucose and $MgATP^{2-}$, normal pH optimum, normal heat stability at 40°C, but abnormal behavior with respect to its regulation by glucose-1,6-diphosphate and inorganic phosphate, and an altered electrophoretic pattern. Interpretation of the results revealed the presence of two different hexokinases type I in normal human erythrocytes: one enzyme with a high affinity for glucose-1,6-diphosphate, the inhibition of which is regulated by inorganic phosphate; and another enzyme with a lower affinity for the inhibitor, not regulated by inorganic phosphate. The former enzyme was not detectable in the erythrocytes of the patient, whereas the presence of the latter enzyme could be demonstrated.

INTRODUCTION

Hexokinase deficiency was first described in 1965 by Löhr et al. (1) in the blood cells of three patients with a panmyelopathy (type Fanconi) with multiple malformations. The residual hexokinase activity in the erythrocyte was 25–60% of normal and the enzyme showed decreased affinities for its substrates glucose and $MgATP^{2-}$.

However, the metabolic defects in these patients are acquired rather than truly genetic. Hexokinase deficiency of human erythrocytes was first related to hereditary nonspherocytic hemolytic anemia in 1967 by Valentine et al. (2). The hexokinase activity of the patient's erythrocytes was only slightly decreased, but was markedly lowered when the degree of reticulocytosis was taken into account. The kinetic (2) and electrophoretic (3) behavior of the enzyme showed no abnormalities. Since the report of Valentine et al. (2), some other variants of hexokinase deficiency related to hereditary nonspherocytic hemolytic anemia have been reported (4–7). Most variants showed increased Michaelis-Menten constants for the substrates glucose and $MgATP^{2-}$ (5–7), and in one case a decreased heat stability was found (5). The hexokinase deficiency described by Necheles et al. (4) was kinetically and electrophoretically characterized by the absence of the "low-K_m" hexokinase type III, which is normally present in low concentrations, whereas hexokinase type I, which is the predominant form of hexokinase in human erythrocytes (8), was present in a normal amount. Hexokinase deficiency appears to be inherited as an autosomal recessive disorder (1, 2).

Recently (9, 10), we described the regulation of purified erythrocyte hexokinase type I by hexosephosphate and diphosphate and inorganic phosphate (P_i).[1] Hexokinase is strongly inhibited by glucose-6-phosphate (glc-6-P) and glucose-1,6-diphosphate (glc-1,6-P_2), whereas P_i is able at least partly to overcome this inhibition.

To explain the results, two alternative models were suggested: (*a*) Hexokinase exists in equilibrium between a free and a phosphate-associated form, the latter having a reduced affinity for glc-1,6-P_2 with respect to the first. (*b*) There are two different hexokinases: one enzyme with a high affinity for glc-1,6-P_2, the inhibition of which is competitively influenced by P_i; the other enzyme with a lower affinity for the inhibitor and insensitive to regulation by P_i.

In this paper we present a patient with nonspherocytic hemolytic anemia associated with a homozygous hexokinase deficiency. The defective enzyme could be characterized by a different electrophoretic pattern

Received for publication 9 September 1977 and in revised form 3 April 1978.

[1] *Abbreviations used in this paper:* glc-1,6-P_2, glucose-1,6-diphosphate; glc-6-P, glucose-6-phosphate; P_i, inorganic phosphate.

 J. Clin. Invest. © *The American Society for Clinical Investigation, Inc., 0021-9738/78/0801-294 $1.00*

and altered regulatory properties. The results are used to discriminate between the models proposed for the regulation of the normal erythrocyte enzyme.

Case Report

The patient is a 30-yr-old woman suffering from chronic hemolysis since birth. During the first years of life many blood transfusions were necessary. At the age of 2 yr, splenectomy was performed, after which the need for blood transfusions decreased.

The parents of the patient are second cousins. They both had a normal blood picture except for a slightly increased packed cell volume and hemoglobin content in the mother's blood. These increases were consistent on two different occasions; the cause of these deviations remains unclear. The patient is their only child.

In 1967 a hexokinase deficiency of the erythrocytes was found as the possible cause of the hemolytic anemia (11). The hexokinase activity of the erythrocytes was only slightly lowered with respect to normal, but was markedly decreased when the degree of reticulocytosis was taken into account. The most recent hematological data, obtained at the time of the present extensive enzyme studies in the patient, are shown in Table I. There was a slight anemia and pronounced reticulocytosis, the latter accounting for the markedly increased mean corpuscular volume and mean corpuscular hemoglobin. The blood smear showed 60 erythroblasts per 100 white cells, marked anisocytosis, macrocytosis, polychromasia, and basophilic stippling of the erythrocytes. Many Howell-Jolly bodies and a few target cells and spherocytes were present.

METHODS

Substrates, coenzymes, and auxiliary enzymes for determination of glycolytic enzymes and intermediates, except for the determination of 2,3-diphosphoglycerate, were obtained from Boehringer (Boehringer & Soehne, Mannheim, Germany). Sulfoethyl-cellulose SE-23 was supplied by Serva (Heidelberg, Germany) and Sephadex G-25 (coarse) and DEAE-Sephadex A-50 by Pharmacia Fine Chemicals (Piscataway, N. J.). Acrylamide and bisacrylamide were obtained from Ega-chemie (Albuch, Germany) and phenazine methosulfate and MTT-tetrazolium (bimethyl-thiozolyl diphenyltetrazolium bromide) from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals were of analytical grade purity. Venous blood was collected with heparin (30 U/ml) from the patient and her parents, from a normal healthy donor, and from two control patients with reticulocytosis. The first control (reticulocytosis control I) had a glucosephosphate isomerase deficiency of the erythrocytes with a very high reticulocyte count (72%) and was splenectomized (12). The second control (reticulocytosis control II) was suffering from a hemolytic anemia probably of viral origin. No metabolic abnormalities were found. This patient was not splenectomized and had a reticulocyte count of 20%.

Immediately after collection of the blood, a part of it was deproteinized for the determination of glycolytic intermediates. Deproteinization and determination of all glycolytic intermediates except 2,3-diphosphoglycerate were performed according to the methods of Minakami et al. (13). The 2,3-diphosphoglycerate content of the acid extract was determined with a Sigma-testkit (Sigma Technical Bulletin 35-UV, December 1974). All determinations were performed within 5 h after extract preparation.

The leucocytes and platelets of the remaining blood cells were removed according to the method of Nakao et al. (14) by filtrating through a mixture of sulfoethyl-cellulose and Sephadex G-25. However, instead of phosphate buffer we used 0.154 M NaCl which does not affect the results of the separation (15).

The erythrocyte glycolytic enzymes were determined by the methods of Beutler (16). Erythrocyte hexokinase was partly purified by batchwise treatment with DEAE-Sephadex A-50 and by ammonium sulfate precipitation as previously described (17). The ammonium sulfate precipitate was dissolved in 0.2 M Tris-HCl (pH 8.0) and 3 mM β-mercaptoethanol. Before enzyme characterization the sample was desalted by gel filtration on Sephadex G-25 equilibrated with the same buffer. The procedure resulted in a 30- to 50-fold purification with an overall yield of approximately 50%. Hexokinase activity of the partially purified preparations was determined at 37°C in a glc-6-P dehydrogenase-coupled assay as previously described (17).

$MgCl_2$ was added in concentrations necessary to maintain an excess of 5 mM Mg^{2+} over ATP. Routinely, the concentration of glucose was 10 mM. The pH of the assay buffer was 7.15 at 37°C. The reaction was started by adding 0.03 U hexokinase. 1 U of enzyme activity is defined as the amount of

TABLE I
Some Routine Hematological Data of the Patient and Her Parents

	Patient (♀)	Father	Mother	Normal ♀	Normal ♂
Erythrocytes, × 10^{12}/*liter*	2.39	4.91	5.23	3.7–5.0	4.1–5.6
Hemoglobin, *g/dl*	11.3	15.3	16.6	11.9–15.5	13.8–17.6
Packed cell volume, *ml/100 ml*	37	48	52	36–46	41–51
Mean corpuscular volume, *fl*	156	98	100	84–104	
Mean corpuscular hemoglobin, *pg*	47.8	31.9	32.5	28.2–35.7	
Mean corpuscular hemoglobin concentration, *g/100 ml erythrocyte*	30.6	32.5	32.9	32.3–37	
Reticulocytes, %	39	1.6	1.2	0.4–1.8	

enzyme that catalyzes the formation of 1 μmol glc-6-P per min at 37°C.

The heat stability of the partly purified enzyme preparations was determined by measuring the decrease of activity vs. the time of incubation at 40°C.

Disk-electrophoresis on poly-acrylamide gels was performed by method II of Davis (18) at pH 8.8. No sample gel was used and some modifications were made. The buffer contained 3 mM β-mercaptoethanol to protect the enzyme from oxidation. Glucose was incorporated in the gels at the same concentration as that used in the staining mixture to stabilize the enzyme. The staining mixture contained in a final volume of 80 ml: 0.1 M Tris/HCl (pH 7.2), 7.5 mM $MgCl_2$, 0.2 mM $NADP^+$, 35 IU glc-6-P dehydrogenase, 12 IU phosphogluconate dehydrogenase, 2 mM ATP, 1.25 mM EDTA, 2 mM KCN, 6 mg phenazine methosulfate, 16 mg MTT, and glucose at the concentrations as indicated in the results.

The staining was stopped with 2.5% acetic acid and the gels were scanned at 625 nm on a Zeiss spectrophotometer model PMQ 3 provided with a ZK 5 Disc Attachment (Carl Zeiss, Inc., New York).

RESULTS

Erythrocyte metabolism. Hexokinase activity of the erythrocytes of the patient was only slightly decreased (Table II). The enzyme activity was lowered to about 15–20% of normal, however, when the degree of reticulocytosis was taken into account. Due to the very high percentage of reticulocytes, the enzymes pyruvate kinase, 6-phosphofructokinase, glc-6-P dehydrogenase and aldolase showed a high activity and were comparable to the values found for the "reticulocytosis controls." The activities of the other enzymes were in the normal range.

Hexokinase activity in the erythrocytes of both parents was decreased to 50–80% of normal, indicating that both parents are heterozygous for the defect.

The contents of glycolytic intermediates of the patient's erythrocytes are shown in Table III. The data of reticulocytosis control I are of no use here, because of the metabolic nature of the defect in this patient. The 2,3-diphosphoglycerate level in the patient's erythrocytes was strongly decreased, whereas the content of fructose-1,6-diphosphate was increased. The decreased 2,3-diphosphoglycerate content had caused a shift in the oxygen dissociation curve to the left. The P_{50} (mm Hg of oxygen at 50% saturation) was shifted to 20.4 mm Hg (normal range 26–28 mm Hg), which implied that the release of oxygen to the tissues was impaired.

Enzyme characterization. At two opportunities erythrocyte hexokinase was purified from the erythrocytes of the patient and the reticulocytosis controls. The results of the enzyme characterization on both occasions were comparable. The parents' erythrocyte hexokinase was investigated only once.

Kinetics. The partially purified hexokinase preparation of the patient's erythrocytes showed normal Michaelis-Menten kinetics. In agreement with other reports on human erythrocyte hexokinase (17, 19), the results were consistent with a rapid equilibrium random reaction mechanism. K_m for the substrate glucose was 0.050 mM, SD = 0.027, $n = 3$ (normal: 0.064 mM, SD = 0.016, $n = 30$), and for the other substrate, $MgATP^{2-}$, 1.19 mM, SD = 0.20, $n = 5$. The latter value was slightly increased with respect to normal (0.72 mM, SD = 0.19, $n = 30$); however, the significance of this increase is doubtful.

The kinetic parameters obtained for the enzymes from the parents and the reticulocytosis controls fell in the normal range (Table IV).

TABLE II
Erythrocyte Enzyme Activities

	Patient	Reticulocytosis controls I	Reticulocytosis controls II	Father	Mother	Normal controls, mean±SD ($n = 50$)
Hexokinase	0.70	6.7	2.7	0.77	0.81	1.31±0.23
Phosphoglucose isomerase	24.2	8.0	30.4	21.8	23.2	26.2±4.7
6-Phosphofructokinase	15.0	16.2	16.2	7.8	9.5	10.7±1.5
Aldolase	3.0	5.0	4.8	1.8	1.8	2.54±0.57
Triosephosphate isomerase	2,659	2,596	1,850	2,217	1,955	1,950±700
Glyceraldehyde dehydrogenase	79.4	38.1	91	25	39.3	79±56
Phosphoglycerate kinase	150	244	223	133	140	179±59
Biphosphoglyceromutase	34.6	40.8	30.7	25.8	25.6	25.0±5.2
Enolase	13.8	14.4	10.1	6.5	7.0	10.0±4.3
Pyruvate kinase	13.2	22.6	9.5	5.4	5.1	6.0±1.2
Glucose-6-P dehydrogenase	19.6	26.5	17.8	9.4	10.0	10.6±2.0
Gluconate dehydrogenase	12.4	ND*	13.4	6.1	6.1	8.5±1.7
Gluthathione reductase	7.0	ND*	8.3	6.7	6.6	5.7±1.6
Reticulocytes, %	39	72	20	1.6	1.2	0.4–1.8

Enzyme activities are expressed as units per gram hemoglobin.
* Not determined.

TABLE III
Erythrocyte Glycolytic Intermediates

	Patient	Reticulocytosis control II	Normal controls, mean±SD (n = 20)
Glc-6-P	34.5	62	35.7±6.6
Fructose-6-phosphate	17.3	28.3	13.6±2.5
Fructose-1,6-diphosphate	35.5	27.7	10.2±3.4
Dihydroxyacetone-phosphate	10.0	21.9	12.1±5.1
Glyceraldehyde-phosphate	5.0	6.5	4.8±1.6
2,3-Diphospho-glycerate	3,000	5,100	5,250±400
3-Phospho-glycerate	69.8	ND*	55±35
2-Phospho-glycerate	10.0	ND*	9.1±5.3
2-Phosphoenol-pyruvate	22.1	22.2	13.4±8.0
Pyruvate	78.2	63.9	125±75
Lactate	1,500	ND*	990±340
ATP	1,700	2,300	1,300±250

Concentrations are expressed as nanomoles per milliliter erythrocytes except for pyruvate and lactate (in nanomoles per milliliter of blood).
* Not determined.

Influence of glc-1,6-P_2 and inorganic phosphate. Human erythrocyte hexokinase activity is strongly regulated by glc-6-P (9, 19–21) and glc-1,6-P_2 (10, 19, 21). The inhibition by both ligands is regulated by inorganic phosphate in the same manner (9, 10). Hexokinase activity in the presence of glc-6-P cannot be measured in the glc-6-P dehydrogenase-coupled assay, but is determined in the pyruvate kinase-lactate dehydrogenase-coupled assay (9). However, because of the interference of contaminating enzymes in crude hexokinase preparations with the latter assay, we investigated only the influence of glc-1,6-P_2. The inhibition of the partially purified mutant enzyme by glc-1,6-P_2 is shown in Fig. 1A. The inhibition was competitive with respect to $MgATP^{2-}$. The secondary plot of the slope of the Lineweaver-Burk plot vs. the inhibitor concentration was linear, and a K_i (glc-1,6-P_2) of 115 μM could be calculated. This value was increased with respect to the reticulocyte control enzyme (Fig. 1B). The secondary plot of Fig. 1B is deviating from linearity at higher concentrations of inhibitor. From the linear part a K_i (glc-1,6-P_2) of 40 μM was calculated.

About the same results were obtained for the partially purified enzymes from the parents and normal controls. The values obtained (Table IV) are the same as for the pure erythrocyte enzyme at pH = 7.15 (10). The inhibition of pure human erythrocyte hexokinase by glc-1,6-P_2 was partly counteracted by P_i (10). This influence of P_i was found to be competitive with respect to glc-1,6-P_2. To evaluate the influence of inorganic phosphate, a function, f (P_i), was derived describing the influence of P_i on the inhibition constant of the enzyme for any inhibitor as a function of phosphate concentration (9).

The influence of P_i on the inhibition by glc-1,6-P_2 of partially purified hexokinase from patient, parents, and controls was evaluated by calculating f (P_i) (Fig. 2). For the parents and controls f(P_i) was linear up to concentrations of about 2 mM P_i, at higher concentrations of P_i a maximum was reached. This behavior reflects the inability of P_i to overcome completely the inhibition by glc-1,6-P_2.

These results were comparable to those obtained for the pure enzyme (10) although the maxima of the functions vary. The function f(P_i) of the patient's enzyme behaved entirely differently: f(P_i) did not increase

TABLE IV
Properties of Partially Purified Hexokinase

				Reticulocytosis controls		
	Patient	Father	Mother	I	II	Normal controls
K_m $MgATP^{2-}$, *mM*	1.19 SD = 0.20 (n = 5)	0.75	0.89	0.61	0.60	0.72 SD = 0.19 (n = 30)
K_m glucose, *mM*	0.050 SD = 0.027 (n = 3)	0.035	0.079	0.066	0.053	0.064 SD = 0.016 (n = 30)
K_i glc-1,6-P_2, *mM*	0.115	0.063–0.029 (n = 2)	0.043	0.025–0.040 (n = 2)	0.038	0.039 SD = 0.006 (n = 4)
pH Optimum	Normal	Normal	Normal			
Heat stability	Normal	Normal	Normal			
Electrophoresis	Altered	Normal	Normal			

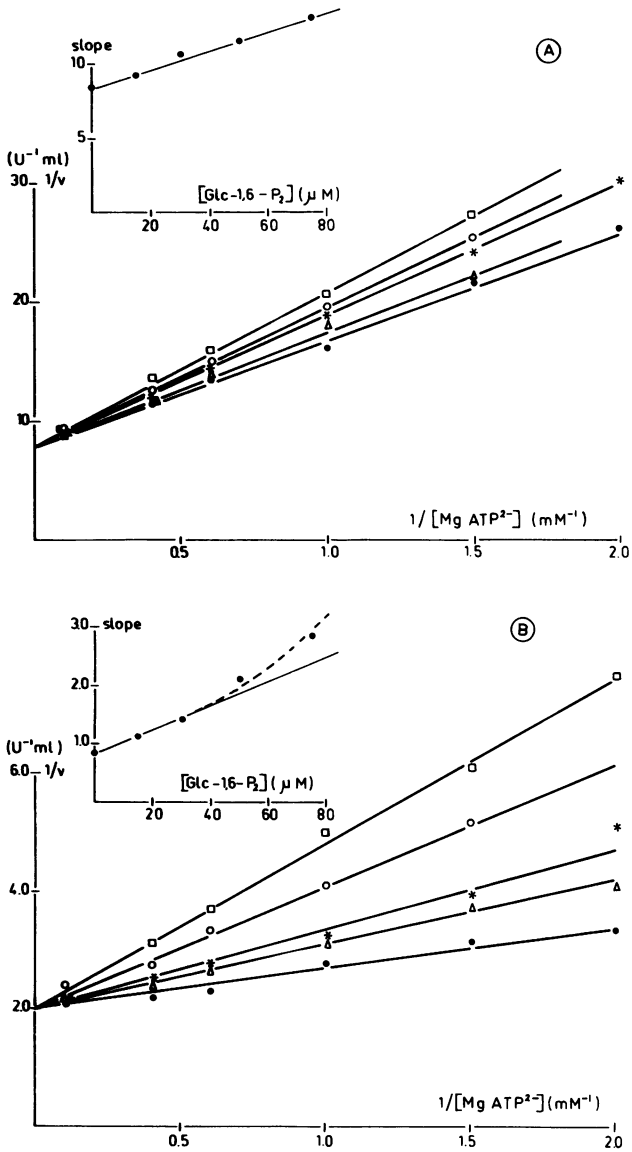

FIGURE 1 Lineweaver-Burk plots of hexokinase activity from the patient's erythrocytes (A) and from reticulocytosis control I (B) vs. $MgATP^{2-}$ at concentrations of glc-1,6-P_2 of 0 (●), 15 (▲), 30 (*), 50 (○), and 75 μM (□).

and remained 1, indicating that P_i has no influence at all on the inhibition by glc-1,6-P_2.

pH-Optimum. The partially purified enzyme preparations from patient, parents, and controls all had the same broad pH optimum ranging from pH 6.9 to 8.2 (results not shown), which is supposed to be normal (17).

Heat stability. The heat stability of the partially purified hexokinase preparations from patient, parents, and controls was not significantly different. 50% of total activity was lost after an incubation period of 4.5–5 min at 40°C.

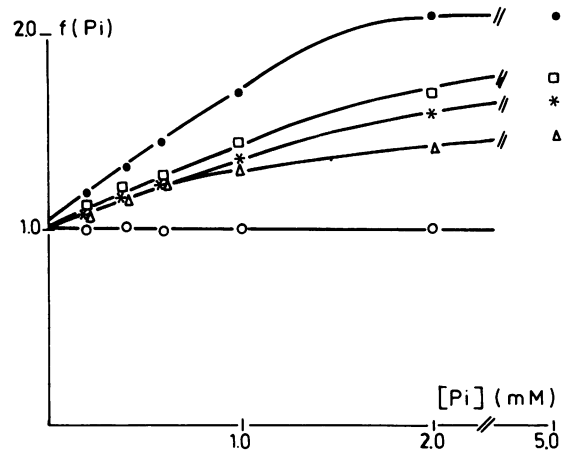

FIGURE 2 Influence of inorganic phosphate on the inhibition constant for the enzyme from patient (○), father (*), mother (△), normal control (□), and reticulocytosis control I (●) at a concentration of 0.5 mM $MgATP^{2-}$ and 0.1 mM glc-1,6-P_2. $f(P_i)$ is determined as defined in the text.

Electrophoresis. The electrophoretic pattern of semipurified human erythrocyte hexokinase normally shows a predominant triple hexokinase type I band (8) and a more anodal faint hexokinase type III band (8, 22, 23). The ratio of hexokinase activity in the second (band I_b) and third band (band I_c) from the cathode in the type I region is dependent on the cell age. Band I_b is predominant in reticulocytes and its activity is shifted to band I_c with increasing cell age (8). The electrophoretic pattern of the mutant enzyme showed a relatively increased hexokinase activity in the type III band (Fig. 3), indicating that the hexokinase deficiency was located in the type I region. In Fig. 4 the electrophoretic patterns of semipurified hexokinase from the patient, parents, and controls are shown. The relative intensity of the bands was evaluated by scanning the gels at 625 nm.

The samples used in these experiments had been kept frozen at −70°C, resulting in a loss of activity in the hexokinase type III band. No alterations in the type I region were found after storage. It appeared that in the type I region of the patient's electrophoretic pattern only two bands were present. The second band (band I_b) from the cathode was absent, although this band normally is predominant in reticulocytes (Fig. 4). Band I_c seemed to be more pronounced than usual in reticulocytes. Hexokinase preparations from the parents' erythrocytes showed essentially normal electrophoretic patterns.

DISCUSSION

The nonspherocytic hemolytic anemia in our patient was found to be associated with a markedly decreased hexokinase activity in the erythrocyte.

The hexokinase content was about 15–20% of normal when the degree of reticulocytosis was taken into account. Clinical, metabolic, and enzymological investigations of both parents revealed almost no abnormalities, except that the hexokinase activity of their erythrocytes was about 50–80% of normal, indicating heterozygosity for the defect. Because hexokinase deficiency is a very rare disease with autosomal recessive inheritance (1, 2) and because the parents are consanguineous, it is very likely that both parents are heterozygous for the same variant of hexokinase and that the patient is consequently homozygous for the mutant enzyme.

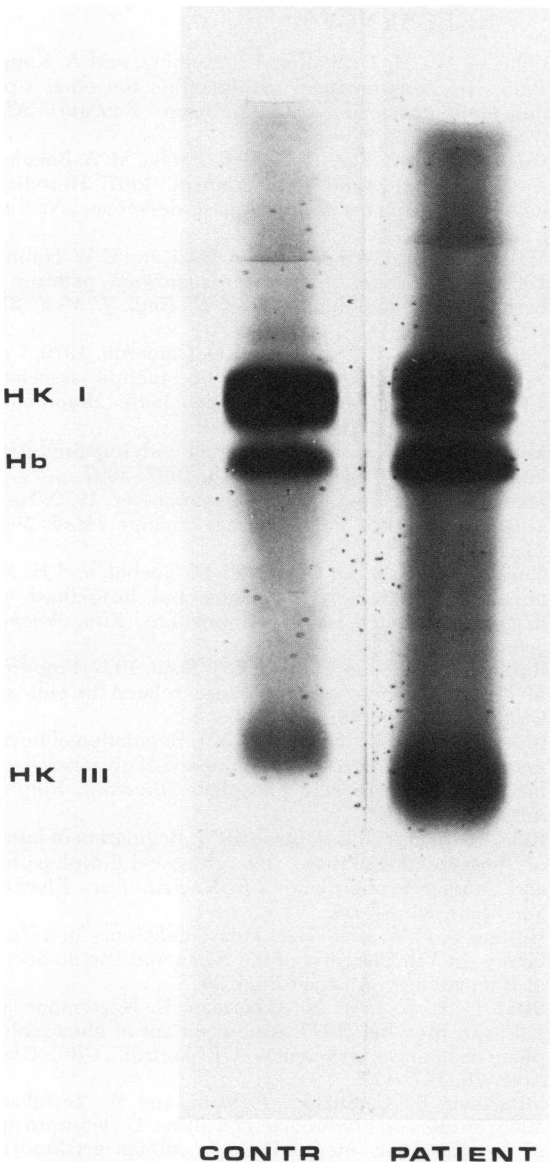

FIGURE 3 Polyacrylamide-gel electrophoresis of partially purified hexokinase from patient and from a normal control (Contr). The gels are stained at a concentration of 10 mM glucose.

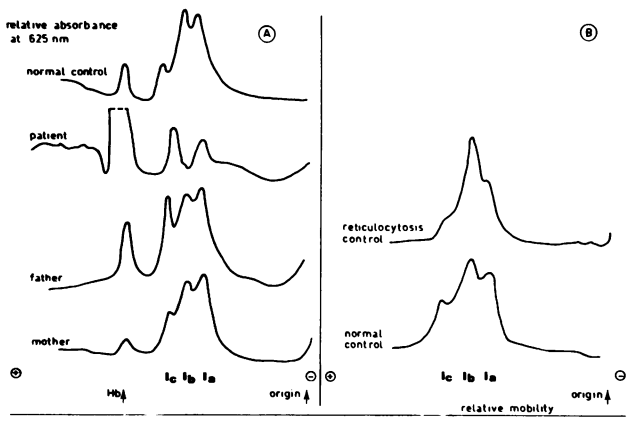

FIGURE 4 Graphic representation of the electrophoretic patterns of partially purified hexokinase preparations from patient, parents, and controls. The relative intensities of the bands are given as their absorbances of 625 nm. The gels are stained at a concentration of 0.2 mM glucose. A and B represent two separated experiments.

Interpretation of the data on the contents of glycolytic intermediates is rather complicated because of the high reticulocytosis. In normal reticulocytes and young erythrocytes, the contents of glc-6-P, fructose-6-phosphate, and fructose-1,6-diphosphate are increased because of the high activity of hexokinase (24, 25). Furthermore, the levels of ATP and 2,3-diphosphoglycerate are elevated in reticulocytes (24, 25). With this in mind we conclude that with respect to the reticulocytosis, the levels of glc-6-P, fructose-6-phosphate, ATP, and 2,3-diphosphoglycerate are decreased in our patient, reflecting a block at the hexokinase level with consequent impairment of glycolysis. The results of the enzyme characterization are summarized in Table IV. The mutant enzyme had normal kinetic parameters, with respect to its substrates, normal heat stability, and normal pH optimum. The K_i(glc-1,6-P_2), however, was significantly increased, and no influence of P_i on this parameter could be detected.

Furthermore, the electrophoretic pattern was altered. Recently (10), we described the regulation of purified human erythrocyte hexokinase type I by glc-1,6-P_2 and P_i. Hexokinase is strongly inhibited by glc-1,6-P_2, whereas P_i is able to at least partly overcome this inhibition. To explain the results, two alternative models were suggested: (*a*) Hexokinase exists in equilibrium between two forms of the enzyme, which differ in their affinity for hexose-phosphate inhibitor. The position of the equilibrium is influenced by P_i, which induces a shift in the equilibrium to the enzyme with the lower affinity. (*b*) There are two different hexokinases: one enzyme with a high affinity for glc-1,6-P_2, the inhibition of which is influenced by P_i; the other

enzyme with a lower affinity for the inhibitor and insensitive to regulation by P_i.

The patient's enzyme shows a decreased affinity for glc-1,6-P_2 and insensitivity to regulation by P_i. In terms of the first model this should mean that regardless of the presence of P_i the equilibrium between the two enzyme forms in the hexokinase-deficient erythrocytes is shifted entirely to the enzyme with the lower affinity for hexose-phosphate inhibitor. This implies that the equilibrium is affected by another factor not present during the in vitro experiments which means that the supposed equilibrium is static rather than dynamic and that model 2 is preferred to model 1. The enzyme with the higher affinity for glc-1,6-P_2, being regulated by P_i, is deficient in the patient's erythrocytes, whereas the other enzyme is still present. The electrophoretic pattern of the patient's enzyme is in agreement with this hypothesis. The second band from the cathode in the type I region (band I_b), which is normally predominant in reticulocytes (8), is absent, whereas the third band (I_c) is increased. From these observations we conclude that band I_b is the phosphate-regulated form of the enzyme, whereas band I_c represents the phosphate-insensitive form. In agreement with this hypothesis is the observation that the maximum in the $f(P_i)$ plot (Fig. 2) of the reticulocytosis control is increased with respect to normal, indicating that there is an increased quantity of phosphate-regulated hexokinase present in reticulocytes. The possibility that the phosphate-insensitive form of hexokinase could be identical with hexokinase type III was considered and, as this form is relatively increased in the patient's erythrocytes, the results of the inhibition experiments might have been masked. However, there are some arguments against this explanation: (*a*) The inhibition experiments are performed at a concentration of 10 mM glucose, which is strongly inhibitory for type III hexokinase (26). (*b*) The presence of two differently regulated hexokinases is suggested from experiments with pure erythrocyte hexokinase (9, 10). This preparation contained only type I hexokinase (17). During the purification procedure, type III is lost, probably as a result of its greater instability.

Therefore, we conclude that the phosphate-insensitive form of hexokinase is not identical with type III hexokinase, but is located in the hexokinase type I region. The proportion of hexokinase activity in bands I_b and I_c is shifted in favor of band I_c during cell senescence accompanied by a loss of overall activity (20).

Therefore, we now postulate that the phosphate-regulated form of the enzyme is modified during senescence to the phosphate-insensitive form. In this light we can regard the patient's erythrocyte hexokinase as being "old before its time."

As hexokinase is considered to be an important factor in erythrocyte senescence, it means that the patient's erythrocytes have to be considered as old cells with consequently increased destruction.

ACKNOWLEDGMENTS

We thank Miss A. M. C. Vlug, Miss M. van der Vlist, and Miss M. Hamelink for technical assistance, and Mrs. E. L. Huisman-Backer Dirks for secretarial help. Miss C. C. Bos is acknowledged for examination of the blood smear, and Prof. J. J. Sixma and Dr. K. Punt are thanked for helpful discussion of the manuscript.

This work was supported by the Netherlands Foundation for Fundamental Medical Research (FUNGO, grant 13-39-12).

REFERENCES

1. Löhr, G. W., H. D. Waller, F. Anschütz, and A. Knopp. 1965. Hexokinasemangel in blutzellen bei einer sippe mit familiärer panmyelopathie (typ. Fanconi). *Klin. Wochenschr.* **43**: 870–875.
2. Valentine, W. N., F. A. Oski, D. E. Paglia, M. A. Baughan, A. S. Schneider, and J. L. Naiman. 1967. Hereditary hemolytic anemia with hexokinase deficiency. *N. Engl. J. Med.* **276**: 1–11.
3. Malone, J. I., A. I. Winegrad, F. A. Oski, and E. W. Holmes. 1968. Erythrocyte hexokinase isoenzyme patterns in hereditary hemoglobinopathies. *N. Engl. J. Med.* **279**: 1071–1077.
4. Necheles, T. F., U. S. Rai, and D. Cameron. 1970. Congenital nonspherocytic hemolytic anemia associated with an unusual erythrocyte hexokinase abnormality. *J. Lab. Clin. Med.* **76**: 593–602.
5. Keitt, A. S. 1969. Hemolytic anemia with impaired hexokinase activity. *J. Clin. Invest.* **48**: 1997–2007.
6. Moser, K., M. Ciresa, and J. Schwarzmeier. 1970. Hexokinasemangel bei hämolytischer anämie. *Med. Welt.* **21**: 1977–1981.
7. Goebel, K. M., W. D. Gassel, F. D. Goebel, and H. Kaffarnik. 1972. Hemolytic anemia and hexokinase deficiency associated with malformations. *Klin. Wochenschr.* **50**: 849–851.
8. Rijksen, G., I. Schoop, and G. E. J. Staal. 1977. Properties of human erythrocyte hexokinase related to cell age. *Clin. Chim. Acta.* **80**: 193–202.
9. Rijksen, G., and G. E. J. Staal. 1977. Regulation of human erythrocyte hexokinase. The influence of glycolytic intermediates and inorganic phosphate. *Biochim. Biophys. Acta.* **485**: 75–86.
10. Rijksen, G., and G. E. J. Staal. 1977. Regulation of human erythrocyte hexokinase by glucose-1,6-diphosphate and inorganic phosphate. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **80**: 61–65.
11. Helleman, P. W. 1969. Hexokinase deficiency in a Dutch family. *In* Vth Congress of the Asian and Pacific Society of Haematology, Abstract Book 24.
12. Staal, G. E. J., J. W. N. Akkerman, E. Eggermont, and J. P. van Biervliet. 1977. A new variant of glucosephosphate isomerase deficiency: GPI-kortrijk. *Clin. Chim. Acta.* **78**: 121–127.
13. Minakami, S., C. Suzuki, T. Saito, and W. Yoshikawa. 1965. Studies on erythrocyte glycolysis. I. Determination of the glycolytic intermediates in human erythrocytes. *J. Biochem. (Tokyo).* **58**: 543–550.
14. Nakao, M., T. Nakayama, and T. Kankura. 1973. A new method for separation of human blood components. *Nat. New Biol.* **256**: 94.
15. Beutler, E., C. West, and K. G. Blume. 1976. The removal

of leucocytes and platelets from whole blood. *J. Lab. Clin. Invest.* **88:** 328–333.
16. Beutler, E. 1975. Red cell metabolism: a manual of biochemical methods. Grune & Stratton Inc., New York. 2nd edition. 38–70.
17. Rijksen, G., and G. E. J. Staal. 1976. Purification and some properties of human erythrocytes hexokinase. *Biochim. Biophys. Acta.* **445:** 330–341.
18. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* **121:** 404–427.
19. Gerber, G., H. Preissler, R. Heinrich, and S. M. Rapoport. 1974. Hexokinase of human erythrocytes. Purification, kinetic model and its application to the conditions in the cell. *Eur. J. Biochem.* **45:** 39–52.
20. Kosow, D. P., F. A. Oski, J. V. B. Warms, and I. A. Rose. 1973. Regulation of mammalian hexokinase: Regulatory differences between isoenzyme I and II. *Arch. Biochem. Biophys.* **157:** 114–124.
21. Rose, I. A., J. V. B. Warms, and D. P. Kosow. 1974. Specificity for the glucose-6-P inhibition site of hexokinase. *Arch. Biochem. Biophys.* **164:** 729–735.
22. Kaplan, J. C., and E. Beutler. 1968. Hexokinase isoenzymes in human erythrocytes. *Science (Wash. D. C.).* **159:** 215–216.
23. Rogers, P. A., R. A. Fisher, and H. Harris. 1975. An examination of the age-related patterns of decay of the hexokinases of human red cells. *Clin. Chim. Acta.* **65:** 291–298.
24. Buc, H. A., J. P. Leroux, H. Garreau, J. C. Marchand, and P. Cartier. 1974. Metabolic regulation in enzyme-deficient red cells. *Enzyme (Basel).* **18:** 19–36.
25. Oelshlegel, F. J., G. J. Brewer, and C. F. Sing. 1977. Red cell glycolytic intermediates in sickle cell anemia. I. Values and first analyses. *J. Mol. Med.* **2:** 51–60.
26. Katzen, H. M., and R. T. Schimke. 1965. Multiple forms of hexokinase in the rat: tissue distribution, age dependency and properties. *Proc. Natl. Acad. Soc. U. S. A.* **54:** 1218–1225.