Use of cultured lymphoblastoid cells for the study of abnormal enzymes: Molecular abnormality of a phosphoglycerate kinase variant associated with hemolytic anemia

(genetic disease/mutant enzyme/amino acid substitution/cell culture/phosphoglycerate kinase-Tokyo)

HISAICHI FUJII*, SHI-HAN CHEN†, JUNICHI AKATSUKA‡, SHIRO MIWA§, AND AKIRA YOSHIDA*¶

*Department of Biochemical Genetics, City of Hope Research Institute, Duarte, California 91010; †Department of Pediatrics, University of Washington School of Medicine, Seattle, Washington 98105; ‡Department of Pediatrics, Jikei Medical College, Tokyo, Japan; and §Department of Internal Medicine, Institute of Medical Science, University of Tokyo, Tokyo, Japan

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ABSTRACT A phosphoglycerate kinase (PGKase; ATP:3-phosphoglycerate 1-phosphotransferase, EC 2.7.2.3; X chromosome-linked) variant, PGKase-Tokyo, is associated with enzyme deficiency, nonspherocytic hemolytic anemia, and neurological disturbances. Because a sufficient amount of the patient's erythrocytes was not available, the variant enzyme was purified to homogeneity from the cultured lymphoblastoid cells of the patient. The enzyme activity of the variant lymphoblastoid cells was about 16% of that of the normal lymphoblastoid cells. PGKase-Tokyo, compared to the normal enzyme, had a lower specific activity (31% of normal in the backward reaction and 15% of normal in the forward reaction), higher than normal Michaelis constants for ATP and 3-phosphoglycerate, a more acidic pH optimum, and increased thermal instability. Microscale peptide mapping analysis revealed that the structural abnormality of PGKase-Tokyo is a single amino acid substitution from valine to methionine at position 266. Thus, the use of the cultured lymphoblastoid cells is proven to be useful for the study of structural and functional abnormalities of mutant enzymes.

Except for hemoglobin and a few other enzymes that are abundant in blood and tissues, it is difficult and often impossible to obtain a sufficient amount of sample for the study of functional and structural abnormalities of mutant enzymes associated with genetic disorders in man. The use of cultured tissues of patients, particularly virus-transformed lymphoblastoid cells that grow vigorously, is an alternative approach for obtaining sufficient amounts of variant enzymes. In the present work, we isolated a variant enzyme from cultured lymphoblastoid cells of a patient with phosphoglycerate kinase (PGKase; ATP:3-phosphoglycerate 1-phosphotransferase, EC 2.7.2.3; X chromosome-linked) deficiency and nonspherocytic hemolytic anemia (1). This variant enzyme is designated "PGKase-Tokyo." Human phosphoglycerate kinase is a single polypeptide chain consisting of 417 amino acid residues. The complete amino acid sequence of normal human PGKase has been determined (2, 3). This paper reports functional and structural abnormalities of PGKase-Tokyo, which were determined using cultured tissues of the patient.

MATERIALS AND METHODS

Blood Sample. A heparinized blood sample (about 18 ml) from the patient, a hemizygote man with a PGKase abnormality (PGKase-Tokyo), was shipped from Japan to the University of Washington. The culture was started within 24 hr after venesection.

Establishment of a Transformed Lymphoblastoid Cell Line.

Lymphocytes were prepared from normal blood and the patient's blood by Ficoll/Hypaque gradient centrifugation (4). The lymphoblastoid cell line was established from the lymphocytes by the previous method (5). Cells ($10\text{--}14 \times 10^6$) were suspended in 2 ml of an Epstein-Barr virus suspension for 30 min at 37°C. Cells were then pelleted, and fresh RPMI 1640 medium (GIBCO) with 20% (vol/vol) fetal calf serum (GIBCO) and antibiotics (100 units of penicillin and 50 μg of streptomycin per ml) were added. The culture was grown at 37°C in 5% CO_2 /95% air and was examined daily for blastoid formation. The culture was kept in the flask for further maintenance and characterization.

Expansion and Harvesting of the Cells. A starting culture of 10×10^6 cells was transferred to a tissue culture flask (600 ml). The fresh media was then added to obtain a starting cell density of about 0.5×10^6 cells per ml. Hemocytometric counts were made daily, and fresh media was added as needed to ensure a logarithmic phase growth. The maximum cell density was about 2×10^6 cells per ml. When the desired number of cells was obtained, cultures were pooled and centrifuged at $300 \times g$. Cell pellets were resuspended and washed with saline/phosphate buffer, pH 7.4. The final cell pellets were immediately frozen at -70°C . Karyotype of the transformed lymphoblastoid cells examined in metaphase was found to be normal (i.e., 46 autosomal chromosomes with X and Y).

Purification of the Normal and Variant Enzymes. The cell pellets were suspended in 10 ml of 50 mM Tris-HCl, pH 8.0/5 mM MgCl_2 /1 mM EDTA/1 mM 2-mercaptoethanol and were lysed by freezing and thawing three times. This suspension was centrifuged at $20,000 \times g$ for 30 min. The extraction was repeated once. The total extracts were used for purification by affinity chromatography as described (6). The normal enzyme was also purified from the normal lymphoblastoid cells and from the normal erythrocytes.

Assay of Enzyme Activity. The forward (1,3-diphosphoglycerate + ADP $\rightarrow$ 3-phosphoglycerate + ATP) and backward (3-phosphoglycerate + ATP $\rightarrow$ 1,3-diphosphoglycerate + ADP) reactions of the enzyme were measured as described (7, 8).

Peptide Mapping. S-Carboxymethylation and tryptic digestion of the normal and variant enzymes were carried out as described (9). Peptide mapping of the tryptic digests on microscale thin-layer cellulose was carried out as described (9).

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Abbreviation: PGKase, phosphoglycerate kinase (ATP:3-phosphoglycerate 1-phosphotransferase, EC 2.7.2.3; X chromosome-linked).

¶ To whom reprint requests should be addressed.

Table 1. Purification of PGKase from normal and variant lymphoblastoid cells

Step	Normal PGKase*				PGKase-Tokyo†			
	Total vol, ml	Total protein, mg	Total activity, units	Specific activity, units/mg	Total vol, ml	Total protein, mg	Total activity, units	Specific activity, units/mg
Crude extract	20.0	43	547	12.7	24.5	164	311	1.89
Affinity chromatography	43.0	1.51	420	270	55.0	2.70	248	92
Sephadex gel filtration	29.0	0.43	241	560	50.5	1.03	154	150
CM-Sephadex chromatography					27.0	0.67	116	173

Enzyme activity (unit) is expressed as μmol of 1,3-diphosphoglycerate produced per min in the backward reaction under the assay conditions (16).

* Normal PGKase was purified from 1.2×10^9 cells.

† PGKase-Tokyo was purified from 4.4×10^9 cells. In another preparation, 0.54 mg of the variant enzyme was obtained from 3.6×10^9 cells.

Polyacrylamide Gel Electrophoresis. Electrophoresis was performed in the absence and presence of NaDodSO_4 as established (10, 11). Starch gel electrophoresis was carried out with a Tris/citrate buffer, pH 7.5, and the enzyme activity was detected as described by Beutler (12). Protein was assayed by Lowry's method with bovine serum albumin as a standard (13). The peptides Met-Lys and Ile-Met-Lys were synthesized chemically by the modified Merrifield procedure (14). The peptides Ile-Lys, Val-Lys, and Ile-Val-Lys were previously obtained from tryptic digests of the normal enzyme (2). The source of chemicals has been described (8, 15).

RESULTS

Purification of Normal and Variant Enzymes. The enzyme activity of the variant lymphoblastoid cells was about 16% of that of the normal lymphoblastoid cells (i.e., 71 units per 10^9 variant cells and 456 units per 10^9 normal cells). Two preparations of PGKase-Tokyo were purified separately from the same lymphoblastoid cell line. In the first preparation, 0.67 mg of the variant enzyme was obtained from 4.4×10^9 cells, and in the second preparation, 0.54 mg of the enzyme was obtained from 3.6×10^9 cells. The results of purification are summarized in Table 1. The purified normal and variant enzyme preparations showed a single protein band in polyacrylamide gel electrophoresis in the presence and absence of NaDodSO_4 . The two enzymes cannot be distinguished by polyacrylamide gel electrophoresis, but they can be clearly distinguished in starch gel electrophoresis (Fig. 1). The normal enzyme obtained from erythrocytes was identical to that obtained from lymphoblastoid

cells in both polyacrylamide gel electrophoresis and starch gel electrophoresis.

Kinetic Properties. The specific activity (i.e., V_{max} per mg of enzyme) of PGKase-Tokyo is about 31% of that of the normal enzyme in the backward reaction and 15% in the forward reaction (Table 2). The Michaelis constants (K_m) for ATP and 3-phosphoglycerate were higher with PGKase-Tokyo than with the normal enzyme, but K_m values for ADP and 1,3-diphosphoglycerate of the variant enzyme were normal (Table 2). The pH optimum of the variant enzyme was markedly shifted towards the acidic side of the normal enzyme curve (Fig. 2). The thermal stabilities of the normal and variant enzymes were determined in 80 mM Tris-HCl, pH 7.5/8 mM MgCl_2 /0.1% bovine serum albumin at 45°C. The variant enzyme was unstable compared to the normal enzyme (Fig. 3). The normal enzyme from erythrocytes and that from lymphoblastoid cells are identical with respect to their specific activities, K_m values, pH optima, and thermal stabilities.

Amino Acid Substitution. The peptide maps of the tryptic digests are shown in Fig. 4. The pattern of PGKase-Tokyo obtained from cultured lymphoblastoid cells is identical to that of the normal enzyme from erythrocytes except for the marked spots. The normal enzyme always has three clearly separated spots (Fig. 4A, I, II, and III), whereas PGKase-Tokyo has only two spots (Fig. 4B, IV and V) in the same area. As shown before (18), three, not two, peptide spots were always observed in this peptide map area of normal PGKase and other PGKase variants (16, 18). Therefore, the structural abnormality of PGKase-Tokyo must reside in the peptides of this particular area. The amino acids found in peptides I, II, III, IV, and V after acid hydrolysis are shown in Table 3. By comparing the amino acid

FIG. 1. Starch gel electrophoresis patterns of the normal and variant PGKase. Lanes: 1, PGKase-Tokyo; 2, normal enzyme from erythrocytes; 3, normal enzyme from lymphoblastoid cells; 4, PGKase-Tokyo plus normal enzyme from lymphoblastoid cells; 5, PGKase-Tokyo.

Table 2. Enzymatic properties of normal PGKase and PGKase-Tokyo

Enzymatic property	Normal PGKase from erythrocytes	Normal PGKase from lymphoblastoid cells	PGKase-Tokyo from lymphoblastoid cells
V_{max} , units/mg			
Forward reaction	1635	1714	263
Backward reaction	562	560	173
K_m , μM			
ADP	125	111	143
1,3-Diphosphoglycerate	2.0	2.7	1.9
ATP	560	570	870
3-Phosphoglycerate	375	345	667

The kinetic properties of the normal and variant enzymes were determined as described (17).

FIG. 2. Effect of pH on enzyme activity of the normal and variant PGKase. Buffer solutions used were acetate (pH 4–6), imidazole/saline buffer (pH 6–8), Tris-HCl buffer (pH 7–9), or sodium carbonate/sodium bicarbonate buffer (pH 9–10.5). □, Normal range; ●—●, PGKase-Tokyo.

composition of these peptides and the known amino acid sequence of the normal enzyme (2, 3), peptide I, peptide II, and peptide III should be Ile-Lys at positions 40 and 41, Val-Lys at positions 140 and 141, and Ile-Val-Lys at positions 265–267, respectively. The variant peptide IV must be identical to peptide I, as judged from their position in the peptide maps and their amino acid composition. The variant peptide V must be a superimposed spot of a peptide at positions 140 and 141 and a peptide at positions 265–267. Because of its amino acid composition, peptide V should be either a mixture of Val-Lys and Ile-Met-Lys or a mixture of Met-Lys and Ile-Val-Lys.

To distinguish the two possibilities, electrophoretic and chro-

FIG. 3. Thermal stability test at 45°C. ●—●, Normal enzyme from erythrocytes; ○—○, normal enzyme from lymphoblastoid cells; Δ—Δ, PGKase-Tokyo.

matographic mobilities of these authentic peptides were compared under the same conditions of peptide mapping. The cathodal electrophoretic mobility of Met-Lys was distinctively faster than that of Ile-Lys. However, Ile-Met-Lys and Val-Lys had identical electrophoretic and chromatographic mobility, and the two peptides were superimposed under the peptide mapping conditions used. It can be concluded that peptide V is a mixture of Val-Lys and Ile-Met-Lys. Consequently, the amino acid substitution of PGKase-Tokyo should be Val to Met at position 266 of the enzyme.

DISCUSSION

Cultured cells, particularly vigorously growing transformed lymphoblastoid cells, would be an attractive source for obtaining sufficient amounts of variant enzymes associated with genetic diseases in man. Despite the recent development of micro methods for the structural study of proteins, it is often impos-

FIG. 4. Microscale peptide maps of tryptic digests of reduced, s-carboxymethylated PGKase on thin-layer cellulose. (A) Normal enzyme. (B) PGKase-Tokyo.

Table 3. Amino acid composition of tryptic peptides

Amino acid	Normal PGKase			PGKase-Tokyo	
	P. I	P. II	P. III	P. IV	P. V
Valine	—	0.467 (1)	0.266 (1)	0.093	0.453 (1)
Methionine	—	—	—	—	0.161 (1) [†]
Isoleucine	0.254 (1)	0.227*	0.254 (1)	0.432 (1)	0.497 (1)
Lysine	0.319 (1)	0.536 (1)	0.318 (1)	0.536 (1)	0.971 (1)

The values are amino acids (nmol) from acid hydrolysates of the tryptic peptides extracted from a thin-layer plate. The numbers in parentheses are probable molar ratios of amino acids. P., Peptide.

* Presumed contamination from nearby peptides I and III.

[†] During acid hydrolysis, part of the methionine was oxidized to methionine sulfoxide, which was not included in this table.

sible to obtain a sufficient amount of the patient's tissues and cells for the detailed study of functional and structural abnormalities of the mutant enzymes.

Cultured cells and virus-transformed cells might produce quantitatively and qualitatively altered enzymes and proteins (19, 20). If this is the case, the enzyme obtained from cultured cells would not be adequate for the study. However, the present study indicates that the normal PGKase obtained from the normal lymphoblastoid cells is functionally and structurally identical to the enzyme obtained from erythrocytes. PGKase is an enzyme controlled by a single gene locus located on the X-chromosome (21), and the *PGK* gene action could be particularly well preserved during transformation and cultivation of the cells. It is likely that most, if not all, enzymes and proteins might not be changed by transformation and cultivation, and other enzymes from the cultured cells also could be used for the structural study. In fact, a complete change in primary structure of any protein has never been found in cultured cells.

Inherited deficiency of PGKase is associated with chronic nonspherocytic hemolytic anemia and mental disorders. Thus far, structural and functional abnormalities of three human PGKase variants have been elucidated. These are PGKase-II (Thr → Asn at 352), which is an electrophoretic variant not associated with enzyme deficiency (16), PGKase-München (Asp → Asn at 268), which is associated with enzyme deficiency and heat instability but not with hemolytic anemia (17); and PGKase-Uppsala (Arg → Pro at 206), which is associated with enzyme deficiency, lower specific activity, chronic hemolytic anemia, and mental disorders (18). Based on the primary structure of the normal human PGKase and the three dimensional model deduced from horse PGKase, correlations between the structural and functional abnormalities of these PGK variants have been discussed (18).

The present variant, PGKase-Tokyo, is associated with erythrocyte enzyme deficiency (about 10% of normal activity in the backward reaction), nonspherocytic hemolytic anemia, and mental retardation (1). The patient's lymphoblastoid cells also had lower enzyme activity (about 16% of normal in the backward reaction).

The variant enzyme had lower specific activity, lower affinity for the substrates, thermal instability, and an abnormal pH/activity profile. It should be pointed out that the forward reaction (i.e., ATP production) was more severely retarded than the backward reaction in the variant enzyme. Therefore, the

actual PGKase activity in the glycolytic pathway is expected to be more severely diminished than is the activity in the backward reaction measured *in vitro*. This could be the reason for the severe hemolytic anemia found in the patient. E. Beutler (personal communication) has suggested that the change in catalytic activity of variant enzymes is not necessarily parallel in the forward and backward reactions. PGKase-Tokyo is indeed such an abnormal enzyme.

The structural abnormality of PGKase-Tokyo was found to be a single amino acid substitution from Val to Met at position 266, which is proximal to the substrate binding site of the enzyme (18, 22). The insertion of a large hydrophobic residue (i.e., methionyl residue) at this position is expected to induce a conformational change of the enzyme, resulting in the instability and kinetic abnormalities of PGKase-Tokyo.

The substitution from Val to Met is compatible with a single-step base transition in the *PGK* gene. Although another amino acid substitution, which cannot be detected by the peptide mapping system used, might exist, this possibility is highly unlikely on genetic grounds. The substitution from Val to Met at position 266 can explain the functional abnormalities of PGKase-Tokyo, as described above.

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1. Akatsuka, J., Saito, F., Okabe, N., Maekawa, K., Kitani, N., Nishina, T. & Hashimoto, F. (1973) *Clin. Hematol.* (in Japanese) 14, 1189–1197.
2. Huang, I-Y., Rubinfiel, E. & Yoshida, A. (1980) *J. Biol. Chem.* 255, 6408–6411.
3. Huang, I-Y., Welch, C. D. & Yoshida, A. (1980) *J. Biol. Chem.* 255, 6412–6420.
4. Boyum, A. (1974) *Tissue Antigens* 4, 269–274.
5. Nilsson, K. (1976) in *In Vitro Methods in Cell Mediated and Tumor Immunity*, eds. Bloom, E. R. & Davis, J. R. (Academic, New York), pp. 713–721.
6. Kuntz, G. W. K., Eber, S., Kessler, W., Krietsch, H. & Krietsch, W. K. G. (1978) *Eur. J. Biochem.* 85, 493–501.
7. Bücher, T. (1955) *Methods Enzymol.* 1, 415–422.
8. Yoshida, A. (1975) *Methods Enzymol.* 42, 144–148.
9. Watanabe, S. & Yoshida, A. (1971) *Biochem. Genet.* 5, 541–547.
10. Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* 121, 404–427.
11. Laemmli, U. K. (1970) *Nature (London)* 227, 680–685.
12. Beutler, E. (1969) *Biochem. Genet.* 3, 189–195.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275.
14. Corley, L., Sachs, D. H. & Anfinsen, C. B. (1972) *Biochem. Biophys. Res. Commun.* 47, 1353–1359.
15. Yoshida, A. & Watanabe, A. (1972) *J. Biol. Chem.* 247, 440–445.
16. Yoshida, A. & Watanabe, S. (1972) *J. Biol. Chem.* 247, 446–449.
17. Fujii, H., Krietsch, W. K. G. & Yoshida, A. (1980) *J. Biol. Chem.* 255, 6421–6423.
18. Fujii, H. & Yoshida, A. (1980) *Proc. Natl. Acad. Sci. USA* 77, 5461–5465.
19. O'Farrell, P. H. (1978) *Cell* 14, 545–557.
20. Parker, J., Pollard, J. W., Friesen, J. D. & Stanners, C. P. (1978) *Proc. Natl. Acad. Sci. USA* 75, 1091–1095.
21. Chen, S.-H., Malcolm, L. A., Yoshida, A. & Giblett, E. R. (1971) *Am. J. Hum. Genet.* 23, 87–91.
22. Banks, R. D., Blake, C. C. F., Evans, P. R., Haser, R., Rice, D. W., Hardy, G. W., Merrett, M. & Phillips, A. W. (1979) *Nature (London)* 279, 773–777.