Biochimica et Biophysica Acta, 522 (1978) 104—112

BBA 68316

PLATELET PYRUVATE KINASE

TWO INTERCONVERTIBLE FORMS OF THE ENZYME

* JUNJI NISHIMURA, NAOTAKA HAMASAKI and SHIGEKI MINAKAMI

Department of Biochemistry, Kyushu University School of Medicine, Fukuoka 812 (Japan)

(Received May 25th, 1977)

Summary

1. Electrophoretic mobility and kinetic properties of the pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) of human and pig platelets indicate that the enzyme belongs to the M_2-type isozyme.

2. The enzyme extracted from platelets showed a two-phasic curve in respect to phospho*enol*pyruvate concentration. It was converted to an allosteric type with low affinity for phospho*enol*pyruvate (A-type) when treated with EDTA and was converted to a Michaelis-Menten type with high affinity for phospho*enol*pyruvate (B-type) when treated with fructose 1,6-bisphosphate. The enzyme from actively glycolyzing platelets was essentially that of B-type, whereas that from the platelets in acid/citrate/dextrose solution was partially changed to the A-type. The conversion of the A-type to B-type was so slow and so dependent on the enzyme concentration, that the addition of fructose 1,6-bisphosphate into an assay mixture scarcely affected the catalytic rate.

3. The enzyme was purified from pig platelet extract by fractionations with ammonium sulfate and ethanol, CM-Sephadex chromatography and Sephadex G-200 gel filtration. About 300-fold purification was achieved (3.1 kat/kg protein at 25°C). The molecular weights of the A-type and B-type enzyme as determined by a gel filtration were approx. 120 000 and 240 000, respectively, corresponding to a dimer and a tetramer. Kinetic properties of these two forms of the enzyme were essentially in agreement with those of enzyme type M_2 reported for various tissues of other animal sources.

* Present address: Third Department of Internal Medicine, Kyushu University School of Medicine, Fukuoka 812 (Japan)

Introduction

In mammalian tissues, multiple species of pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) have been found with different kinetic and physicochemical behaviors [1—3]: M_1, M_2 and L-types according to the nomenclature of Imamura and Tanaka [1]. The enzyme M_2 is most widely distributed and considered to be the primitive species of the enzyme (which has also been called III, A or K type by different authors [4—6]). Pogson [5] has shown that there are two interconvertible forms of the enzyme type M_2 in rat adipose tissue: one allosteric type designated as pyruvate kinase A and another Michaelis-Menten type designated as pyruvate kinase B.

In platelets, glycolysis plays an important role for energy supply and high activities of glycolytic enzymes have been reported [7,8], but there has been no precise report on characterization of platelet pyruvate kinase which is involved in control enzyme for glycolytic metabolism. Moreover, platelets are available in homogeneous state from human sources other than red cells. The enzyme extracted from human platelets, as well as that from pig platelets, belongs to the M_2 type and exists as two interconvertible forms. In the present paper, some kinetic and physicochemical properties of these two forms of platelet pyruvate kinase are presented. An involvement of dimer-tetramer changes in the interconversion of the A type to B type of the purified pig platelet enzyme was shown by gel filtration. The interconversion of these two forms is discussed in connection with metabolic states of platelets.

Methods

Preparation of human platelet extracts

One-day-old platelet-rich plasma concentrate in acid/citrate/dextrose solution was kindly provided by Fukuoka Red Cross Blood Center. After the removal of contaminating red cells and white cells by centrifugation at 120 × *g* for 10 min, platelets were collected by centrifugation at 1000 × *g* for 10 min and washed with an isotonic saline solution. They were suspended in a cold, isotonic KCl solution containing 20 mM Tris · HCl buffer, pH 7.4 to approx. $1.5 \cdot 10^{10}$ cells/ml and were lyzed by the addition of four volumes of cold, distilled water. The extract was obtained by centrifugation at 9200 × *g* for 15 min at 4° C and contained less than 0.2 μM fructose 1,6-bisphosphate.

Purification of pyruvate kinase from pig platelets

Platelets obtained as described previously [7] were suspended in isotonic KCl buffered with 20 mM Tris · HCl, pH 7.4 and stored at —70° C until use. Usually, a batch of approx. 200 g (wet weight) of the platelets obtained from 100 l blood was used for the purification.

The frozen platelets were thawed at 4° C and the extract was obtained as described for human platelets. All the purification procedures were carried out at 4° C unless otherwise stated. The extract was buffered with 20 mM Tris · HCl (pH 7.4) containing 5 mM $MgCl_2$/0.1 mM dithiothreitol/0.02 mM fructose 1,6-bisphosphate. All the solutions used in the purification procedure contained the same concentrations of $MgCl_2$, dithiothreitol and fructose 1,6-bisphosphate

to protect the enzyme from inactivation. The extract was fractionated by the addition of solid $(NH_4)_2SO_4$. The material precipitating between 45—60% saturation of $(NH_4)_2SO_4$ was collected and suspended in imidazole · HCl buffer (pH 7.0) and dialyzed against the same solution overnight.

Ethanol, chilled to −20°C, was slowly added with constant stirring to the dialyzed solution kept in an ethanol/water bath at −5°C. The precipitate between concentrations of 10—35% ethanol (v/v) was collected by centrifugation at −10°C. The precipitate, dissolved in a 20 mM imidazole · HCl buffer, pH 6.5, was applied at 26 ml/h to a CM-Sephadex C-50 column (2.5 × 8 cm) equilibrated with the buffer and washed with 170 ml of it. Enzyme was eluted by applying a linear gradient (600 ml) of 0—0.24 M KCl in the same buffer. Fractions with specific activity of more than 0.6 kat/kg protein were collected and concentrated in a collodion bag (Sartorius SM 13201).

The concentrated CM-Sephadex eluate (1.5 ml) was applied to a column of Sephadex G-200 (2.5 × 63 cm) equilibrated with 20 mM imidazole · HCl buffer (pH 7.0)/50 mM KCl and eluted with the same buffer at 11 ml/h. Fractions with specific activity of more than 2.9 kat/kg protein were collected. The enzyme was precipitated with $(NH_4)_2SO_4$ (80% saturation) and suspended in a minimum volume of the $(NH_4)_2SO_4$ solution/50 mM KCl/5 mM $MgCl_2$/0.1 mM dithiothreitol/0.1 mM fructose 1,6-bisphosphate. The enzyme was stable for more than 3 months when kept at 4°C. Before use, the enzyme was sedimented by centrifugation and dissolved in 50 mM triethanolamine · HCl buffer (pH 7.4)1 mg/ml bovine serum albumin/50 mM KCl/5 mM $MgCl_2$/0.1 mM dithiothreitol and with or without 0.1 mM fructose 1,6-bisphosphate. A typical result of the purification is given in Table I. The enzyme showed negligible adenylate kinase, ATPase and lactate dehydrogenase activities, but two contaminating minor bands were observed on SDS polyacrylamide gel electrophoresis. Isoelectric point of the purified enzyme was found to be approx. 8.0 by isoelectric focusing.

Analytical procedures

Pyruvate kinase activity was assayed at 25°C by coupling with lactate dehydrogenase [9]. The reaction mixture contained (unless otherwise indicated) 50 mM triethanolamine · HCl (pH 7.5)/100 mM KCl/8 mM $MgCl_2$/0.12 mM NADH/2 mM ADP/1 mM phospho*enol*pyruvate and 5 units of lactate dehydro-

TABLE I

PURIFICATION OF PYRUVATE KINASE FROM PIG PLATELETS

Step	Volume (ml)	Total protein (mg)	Total activity * (μkat)	Specific activity (kat/kg protein)	Recovery (%)
Crude extract	2040	13400	139	0.010	100
$(NH_4)_2SO_4$ (45—60%)	98	2130	124	0.058	89
Ethanol (10—35%)	84	545	94.8	0.173	68
CM-Spehadex C-50	72	42.3	67.3	1.58	48
Sephadex G-200	27	18.2	56.2	3.08	40

* These values were obtained by the measurement for the samples kept with 0.1 mM fructose 1,6-biphosphate more than 1 h at 4°C.

genase in a final volume of 1.25 ml. The reaction was started by the addition of 5 µl enzyme. The oxidation of NADH was measured on a Hitachi 124 spectrophotometer equipped with a recorder. The activity and specific activity are expressed in katals and katal/kg protein, respectively.

Plate polyacrylamide gel electrophoresis was performed according to the procedure of Imamura and Tanaka [1]. Electrophoresis was carried out in 10 mM Tris · HCl buffer (pH 8.2)/5 mM $MgCl_2$/0.5 mM dithiothreitol/0.5 mM fructose 1,6-bisphosphate at constant voltage of 30 V/cm for 4 h at 4°C and gel was stained by coupling reaction with lactate dehydrogenase. SDS polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn [10]. Isoelectric focusing was carried out with an apparatus and reagents obtained from LKB instruments, Inc. Fructose 1,6-bisphosphate was assayed enzymatically [11]. Protein was estimated by the method of Lowry et al. [12] using bovine serum albumin as a standard.

Reagents used

Enzymes and coenzymes were obtained from Boehringer u. Söhne, Mannheim. CM-Sephadex C-50 and Sephadex G-200 were purchased from Pharmacia Fine Chemicals. All other reagents were of analytical grade.

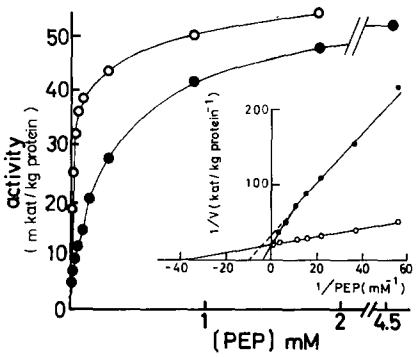
Results

General properties of platelet pyruvate kinase

The electrophoretic pattern of the pyruvate kinase of human pig platelet extract was different to that of red cells or L-type (major component in liver) but similar to that of M_2-type (leukocyte or minor component in liver): on plate polyacrylamide gel, pyruvate kinase of platelet migrated toward the anode much slower than that of red cells or the major component in liver (not shown). This observation, together with the inhibition of the platelet pyruvate kinase activity by alanine (described below), indicate that the enzyme belongs to the class of M_2, as shown with the leukocyte enzyme [13]. The activity was about 2.5 mkat/kg wet weight of human platelets, which is about 100 times higher than that of red cells [14].

The pyruvate kinase activity of human platelet extract

Fig. 1 shows the dependence of the pyruvate kinase activity of the platelet extract on the phospho*enol*pyruvate concentration. The curve is apparently two-phasic, suggesting the existence of two enzyme activities with different affinity for phospho*enol*pyruvate, one with a high $K_{0.5s}$ (>0.3 mM) and the other with a low $K_{0.5s}$ (<0.1 mM). When the extract was preincubated with 0.1 mM fructose 1,6-bisphosphate for more than 1 h at 4°C, the curve became hyperbolic with an apparent K_m of approx. 0.03 mM. This activation was so dependent on time and the concentration of the enzyme as will be shown below, that the addition of the fructose 1,6-bisphosphate 0.1 mM to the assay mixture caused only slight activation at the low substrate portion of the curve. The enzyme extracted from actively glycolyzing platelets, which were incubated in Ca^{2+}-free Krebs-Ringer phosphate buffer, pH 7.4 with 10 mM glucose at 37°C for 30 min, was essentially that of B-type (not shown).



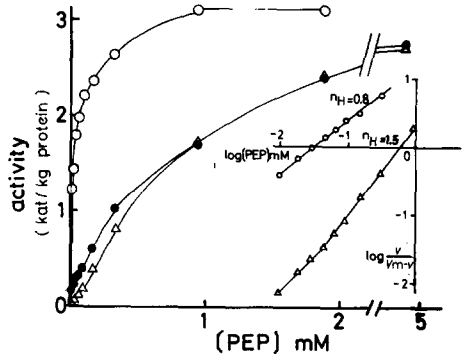


Fig. 1, Activity of pyruvate kinase in human platelet extract at various concentrations of phospho*enol*-pyruvate (PEP). The insert is a Lineweaver-Burk plot of the same result. The extract was preincubated at 4°C for 1 h with (○) or without (●) 0.1 mM fructose 1,6-bisphosphate. Assay conditions are given in Methods.

Fig. 2. Activity of purified pyruvate kinase from pig platelets at various concentrations of phospho*enol*-pyruvate (PEP). The insert is a Hill plot of the same result. The enzyme was dissolved in a fructose 1,6-bisphosphate-free buffer (●), in a buffer containing 0.1 mM fructose 1,6-bisphosphate (○) or in a Mg^{2+}-free buffer containing 2 mM EDTA (△) and was preincubated at 4°C for 1 h. Assay conditions are given in Methods.

Activation of purified pyruvate kinase by fructose 1,6-bisphosphate

To avoid complexities arising from other components in the extract, we purified the enzyme from pig platelets, which can be obtained in large quantities. The procedure consisted of $(NH_4)_2SO_4$ fractionation, ethanol fractionation, CM-Sephadex chromatography and Sephadex G-200 gel filtration (see Methods and Table I). Electrophoretic as well as kinetic properties of this enzyme were essentially the same as those of the human platelet extract.

Fig. 2 shows the effect of phospho*enol*pyruvate concentration on the pyruvate activity of the enzyme. When the enzyme protein in the stock suspension was dissolved in the fructose 1,6-bisphosphate-free buffer, the kinetic curve was two-phasic as was the pyruvate kinase activity of the platelet extract. The curve became sigmoidal when the enzyme was dissolved in a Mg^{2+}-free buffer containing 2 mM EDTA, with a Hill coefficient (n_H) of about 1.5 and a $K_{0.5s}$ of about 0.7 mM, whereas the curve for the enzyme dissolved in a buffer containing 0.1 mM fructose 1,6-bisphosphate (fructose 1,6-bisphosphate buffer) was hyperbolic with an apparent K_m of about 0.02 mM. These two forms were interconvertible: when the enzyme was diluted with the fructose 1,6-bisphosphate free buffer, the activity was promptly decreased and reached a constant level of about one third of the original activity when measured at the phospho*enol*-pyruvate concentration of 0.1 mM. This was reversed by preincubation in the fructose 1,6-bisphosphate buffer. The former process (inactivation) was too fast to follow, but the activation was dependent on the concentration of the enzyme protein and the higher the concentration the faster the activation rate, as shown in Fig. 3.

The observation suggests the possible existence of two convertible forms of the enzyme, the conversion of which may be related to the quaternary structure of the enzyme. Accordingly, the molecular weight of the enzyme was

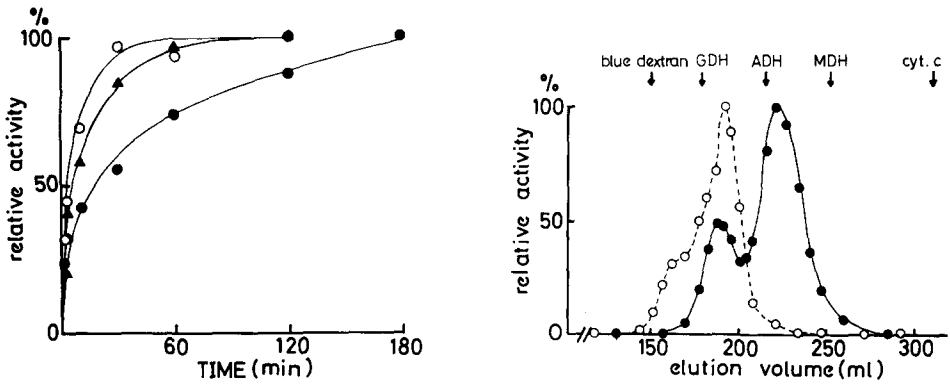


Fig. 3. Time course of the activation by fructose 1,6-bisphosphate of purified pyruvate kinase from pig platelets at various concentration of the enzyme. The 0 and 100% values indicate the initial activity after dilution in fructose 1,6-bisphosphate-free buffer and maximum activity after incubation with 0.1 mM fructose 1,6-bisphosphate at 4°C, respectively. ○, 40 μg protein/ml; ▲, 13 μg protein/ml; ●, 4 μg protein/ml.

Fig. 4. Gel filtration of purified pyruvate kinase from pig platelets. The enzyme (2 mg protein) was applied to a column of Sephadex G-200 (2.5 × 67 cm) equilibrated with 20 mM imidazole · HCl buffer (pH 7.0)/50 mM KCl/5 mM $MgCl_2$/0.5 mM dithiothreitol with or without 0.02 mM fructose 1,6-bisphosphate and eluted with the same buffer at 11 ml/h, 4°C. Glutamate dehydrogenase (GDH) (beef liver, mol. wt, 350 000), alcohol dehydrogenase (ADH) (yeast, mol. wt. 148 000), malate dehydrogenase (MDH) (pig heart, mol. wt. 70 000) and cytochrome *c* (cyt. c) (horse heart, mol. wt. 13 500) were used as reference compounds. The void volume was determined with Blue Dextran 200 separately because the dye combines with pyruvate kinase. 100% indicates the value in fraction with maximal pyruvate kinase activity. ○- - - - -○, the enzyme dissolved in a fructose 1,6-bisphosphate buffer; ●———●, dissolved in a fructose 1,6-bisphosphate-free buffer.

determined by the use of Sephadex G-200 gel filtration (Fig. 4). When the enzyme was dissolved in the fructose 1,6-bisphosphate-free buffer and applied to a Sephadex column equilibrated with the fructose 1,6-bisphosphate-free buffer, it gave a major peak at approx. 120 000 daltons and a minor peak at approx. 250 000. The enzyme dissolved in the fructose 1,6-bisphosphate buffer and applied to the column equilibrated with the fructose 1,6-bisphosphate buffer gave a major peak at approx. 230 000 daltons and a shoulder at approx. 500 000 daltons. Since a subunit of this enzyme had a molecular weight of approx. 60 000 as determined by SDS-polyacrylamide gel electrophoresis, it may be assumed that there are at least two interconvertible forms of the enzyme: the allosteric form with a dimeric structure and the Michaelis-Menten form with a tetrameric structure.

Effect of amino acids on the pyruvate kinase activity

The pyruvate kinase activity of the human platelet extract, as well as that of the pig platelet enzyme, were strongly inhibited by the addition of some amino acids, such as alanine and phenylalanine to an assay medium. The apparent Ki for the alanine inhibition was about 0.03 mM. The inhibition was essentially complete when the phospho*enol*pyruvate was 0.05 mM whereas it was only partial when the phospho*enol*pyruvate concentration was 2 mM (Fig. 5). The inhibition at low concentration of phospho*enol*pyruvate was relieved by the subsequent addition of fructose 1,6-bisphosphate in the assay system, but a

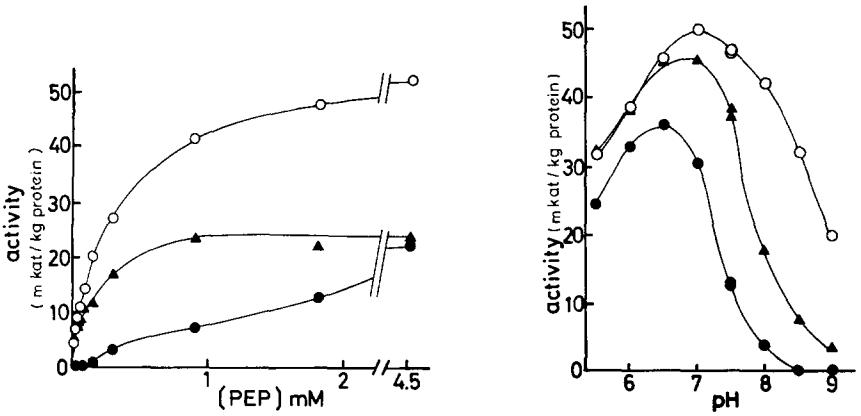


Fig. 5. Effect of alanine on pyruvate kinase of human platelet extract at various concentrations of phospho*enol*pyruvate (PEP). Assay conditions are as given in Methods. ○, control; ●, with 1 mM alanine; ▲, with 1 mM alanine + 0.1 mM fructose 1,6-bisphosphate.

Fig. 6. pH dependence curve of pyruvate kinase activity in human platelet extract. The buffers used in assay mixtures are 50 mM imidazole · HCl (pH 5.5—7.5) and 50 mM triethanolamine · HCl (pH 7.5—9.0). Other conditions are as given in Methods. ○, control; ●, with 1 mM alanine; ▲, with 1 mM alanine + 0.1 mM fructose 1,6-bisphosphate.

complete recovery of the inhibition was not observed at high phospho*enol*-pyruvate, even with higher fructose 1,6-bisphosphate concentration (1 mM). The inhibition was strongly dependent on pH and the extent of the inhibition decreased as the pH of the assay medium decreased, so that the apparent optimum pH shifted from pH 7.0 to 6.5 when amino acid was added to the assay medium (Fig. 6).

Some other properties of platelet pyruvate kinase

The ADP-dependence curve of the pyruvate kinase activity was hyperbolic irrespective of the source of the enzyme with a K_m of about 0.4 mM. The relative rates with other nucleotides, at 2 mM concentration, were 10% (UDP), 4% (GDP) and 3% (CDP), relative to that of ADP at a fixed phospho*enol*pyruvate concentration of 1 mM. The activity was moderately inhibited by ATP at 10 mM concentration but fructose 1,6-bisphosphate could not reverse the inhibition. The enzyme was susceptible to SH-inhibitors.

Discussion

The M_2-type of pyruvate kinase has been found in most tissues: adipose tissue, small intestine, kidney, spleen, lung and many other tissues [1,2,5,15.16]. It is found in adult liver as a minor component but is present as a major component in dividing cells such as fetal or regenerating liver, cultured liver cells or hepatoma cells [1,4,17]. It is also present in non-dividing specialized cells such as leukocytes [13]. The present report shows that platelets have M_2-type pyruvate kinase similar to leukocytes and in contrast to red cells [18], which originate from common bone marrow stem cells, but have an isozyme similar to that of the liver. Crude extract and purified enzyme from platelets showed a

biphasic curve for phospho*enol*pyruvate concentration. This seems to correspond to a mixture of the two interconvertible forms of the enzyme rather than negative and positive co-operativity for the substrate according to Engel and Ferdinand [19], as indicated by the incubation with fructose 1,6-bisphosphate and EDTA. The presence of convertible forms of the enzyme, similar to these described here, was observed in adipose tissue, cultured liver cells, tumor cells, kidney and small intestine [3—5,15—17].

Pogson [5] observed by a partition-cell ultracentrifugation, values of 5.3—5.6 S and 7.2—7.3 S for the A and B types, respectively, and concluded that the differing sedimentation constants of the two forms may reflect large conformational changes rather than alterations in molecular weight. On the other hand, Imamura et al. [3] observed no differences between two types of tumor pyruvate kinase in their ultracentrifugal patterns. More recently, Sparmann et al. [20] observed that two forms had sedimentation coefficients of 5.3 and 10 S, which correspond to molecular weights of approx. 100 000 and 220 000 assuming globular structure. Our present observations by gel filtration show that the molecular weights are approx. 120 000 and 240 000 for the A and B types, corresponding to a dimer and a tetramer, respectively.

An important property of the association reaction is its relative slowness in respect to the catalytic reaction and, hence, it may be classified as a hysteretic response according to Frieden [21]. The rate was strongly dependent on the enzyme protein concentration, with a half-time for the activation with fructose 1,6-bisphosphate of about 4 min at the protein concentration of 40 μg protein/ml and a half-time of 20 min at 4 μg protein/ml. This protein concentration-dependent slow activation of the A-type enzyme by fructose 1,6-bisphosphate may explain the negligible effect of fructose 1,6-bisphosphate when added in a reaction medium. A similar protein concentration-dependent slow change was reported by Schulz et al. [22] for tumor pyruvate kinase with respect to alanine-mediated, temperature-dependent, reversible inactivation of the enzyme. It should be pointed out, however, that the inactivation by the amino acid is in several respects different from the interconversion described here: (1) alanine depressed not only the affinity for phospho*enol*pyruvate, but also depresses the maximal velocity; (2) the addition to assay medium of low concentration of fructose 1,6-bisphosphate reverses the affinity but has little effect on the maximal velocity.

Finally, it is necessary to discuss the physiological significance of these two interconvertible forms. Walker and Potter [4] have shown that the extraction from cultured liver cells (which have been maintained without glucose in the medium) yields the A-type enzyme, whereas the enzyme extracted from cells maintained in the presence of glucose up to the time of the harvest is the B type. This is in accord with our observation that the enzyme extracted from the platelets suspended in acid/citrate/dextrose solution was partially converted to that of the A type, whereas the enzyme extracted from the platelets preincubated with glucose at 37° C was essentially that of the B type. The intracellular concentrations of fructose 1,6-bisphosphate under these conditions were about 50 and more than 400 μM, respectively [7,23]. These observations suggest that the interconversion between the A and B types are actually taking place in cells, although the possibility remains that the conversion takes place during

extraction processes, owing to the release of the fructose 1,6-bisphosphate in the medium. Further studies are necessary to examine the intracellular forms of the enzyme; and platelets may be a suitable preparation for this kind of study.

References

1 Imamura, K. and Tanaka, T. (1972) J. Biochem. 71, 1043—1051
2 Carbonell, J., Felíu, J.E., Marco, R. and Sols, A. (1973) Eur. J. Biochem. 37, 148—156
3 Imamura, K., Taniuchi, K. and Tanaka, T. (1972) J. Biochem. 72, 1001—1015
4 Walker, P.R. and Potter, V.R. (1973) J. Biol. Chem. 248, 4610—4616
5 Pogson, C.I. (1968) Biochem. J. 110, 67—77
6 Ibsen, K.H. and Krueger, E. (1973) Arch. Biochem. Biophys. 157, 509—513
7 Nishimura, J. and Minakami, S. (1975) Biochim. Biophys. Acta 396, 1—10
8 Doery, J.C.G., Hirsh, J. and de Gruchy, G.C. (1970) Brit. J. Haematol. 19, 145—157
9 Bücher, T. and Pfleiderer, G. (1955) in Methods in Enzymology Vol. I (Colowick, S.P. and Kaplan, N.O., eds.), pp. 435—440, Academic Press, New York
10 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
11 Michal, G. and Beutler, H.O. (1974) in Methods in Enzymatic Analysis Vol. III (Bergmeyer, H.U., ed.), pp. 1314—1319, Academic Press, New York
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
13 Van Berkel, Th.J.C. and Koster, J.F. (1973) Biochim. Biophys. Acta 293, 134—139
14 Beutler, E. (1971) Red Cell Metabolism. A manual of biological methods, pp. 56—58, Grune and Stratton, New York
15 Van Berkel, Th.J.C., de Jonge, H.R., Koster, J.F. and Hülsmann, W.C. (1974) Biochem. Biophys. Res. Commun. 60, 398—405
16 Ibsen, K.H. and Trippet, P. (1972) Biochemistry 11, 4442—4450
17 Felíu, J.E. (1975) FEBS Lett. 50, 334—338
18 Nakashima, K., Miwa, S., Oda, S., Tanaka, T., Imamura, K. and Nishina, T. (1974) Blood 43, 537—548
19 Engel, P.C. and Ferdinand, W. (1973) Biochem. J. 131, 97—105
20 Sparmann, G., Schulz, J. and Hofmann, E. (1973) FEBS Lett. 36, 305—308
21 Frieden, C. (1970) J. Biol. Chem. 245, 5788—5799
22 Schulz, J., Sparmann, G. and Hofmann, E. (1975) FEBS Lett. 50, 346—350
23 Kim, B.K. and Baldini, M.G. (1972) Haematologia 6, 437—447