Tokai J Exp Clin Med., Vol. 7, Supplement, pp.113—119, 1982

Effects of Inorganic and Organic Anions on the Transport of Phosphoenol-Pyruvate Across the Erythrocyte Membrane

Naotaka HAMASAKI, Hiroyuki MATSUYAMA, Chiyo HIROTA-CHIGITA and Hiroki NANRI*

Department of Biochemistry, Fukuoka University School of Medicine, Fukuoka, Japan
**Department of Biochemistry, Kyushu University School of Medicine, Fukuoka, Japan*

(Received June 15, 1982)

Phosphoenolpyruvate is transported across the erythrocyte membrane by a carrier-mediated transport system. The transport of phosphoenolpyruvate was competitively inhibited by inorganic phosphate (Ki = 24 mM) and pyridoxal 5-P (Ki = 0.2 mM), whereas the transport was non-competitively inhibited by L-(+)-lactate (Ki = 37 mM). Specific inhibitors for the inorganic anion transport system such as 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid strongly inhibited the phosphoenolpyruvate transport. Inhibitors for the monocarboxylate transport system, including *p*-chlormercuribenzoic acid and α-cyano-4-hydroxycinnamate, had less inhibitory effects on the phosphoenolpyruvate transport as high as 100 μM. The transport was inhibited irreversibly by treating erythrocytes with pryridoxal 5-P/$NaBH_4$. Transport activities of phosphoenolopyruvate and inorganic phosphate in the treated cells were similarly inhibited depending on the pyridoxal 5-P concentration.

The major integral membrane protein, Band 3, was preferentially labelled by treating erythrocytes with pyridoxal 5-P/[^{3}H]-$NaBH_4$. These results suggest that Band 3 mediates the transport of phosphenolopyrvate as well as inorganic phosphate.

(Key Words: Phosphoenolopyruvate transport, Erythrocyte membrane, Band 3, Anion transport, Pyridoxal 5-P)

INTRODUCTION

Organic phophate compound have been generally regarded as impermeable to the erythrocyte membrane. We have previously shown, however, that phosphoenolpyruvate (PEP)† is transported across the erythrocyte membrane by a carrier-mediated transport system (5). The transport appears to be specific to PEP among the phosphorylated glycolytic intermediates. 2-Phosphoglycerate, whose molecular weight and p*K* values are quite similar to those of PEP, is impermeable to the cell membrane. The lower rate of PEP transport is observed in erythrocytes from patients with hereditary spherocytosis, suggesting that the lower rate may be a reflection of some abnormalities of the affected cell membrane (11). The conditions for the transport of PEP are similar to the conditions for blood storage, and PEP transported into the cells is metabolized rapidly to

Naotaka HAMASAKI, Department of Biochemistry, Fukuoka University School of Medicine, Fukuoka 814—01, Japan

†Abbreviation: PEP, phosphoenolpyruvate; SITS, 4-acetamido-4′-isothiocyanostilben-2,2′-disulfonic acid; PCMB, *p*-chloromercuribenzoic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophorosis; IEF, isoelectric focusing.

2,3-bisphosphoglycerate and ATP. Thus, an acid/citrate/dextrose solution containing PEP has been developed as a new preservative for blood storage (6—8, 15).

The transport of PEP is dependent on pH, osmolarity and the concentration of sucrose or citrate in the incubation media, and is inhibited by SITS or pyridoxal 5—P/$NaBH_4$ treatment (5,9). The present report deals with a further characterization of the PEP transport, suggesting that the inorganic anion transport system in the erythrocyte membrane mediates the PEP transport.

EFFECTS OF INORGANIC AND ORGANIC ANIONS ON THE TRANSPORT OF PEP.

PEP was transproted across the erythrocyte membrane in a 0.1M citrate buffer containing 10mM NaF, pH 6.00 at 37°C. When a double reciprocal plot was made of the uptake velocity versus PEP concentration, it was a straight line with Km = 62mM and Vmax = 1.54 μmol/ml of cells per min (Fig. 1). Inorganic phosphate and pyridoxal 5—P inhibited the PEP transport in the manner of competitive inhibition, whereas pyridoxal had no effect on the PEP transport up to the concentration of 1mM (Fig. 1). Their Ki values were 24mM of inorganic phosphate and 0.2mM of pyridoxal 5—P. L-(+)-lactate inhibited the PEP transport noncompetitively and its Ki value was 37mM (data not shown). The PEP transport was completely

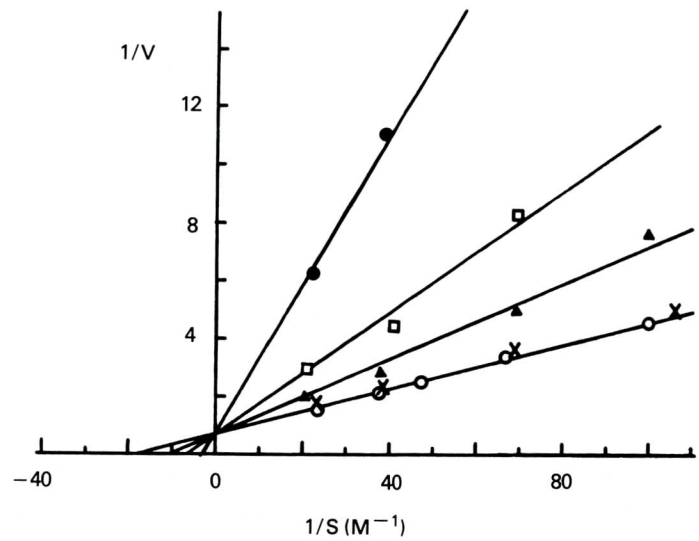

Fig. 1 Effects of inorganic and organic anions on the transport of PEP. Washed erythrocytes were preincubated with a 0.1M citrate buffer containing 10mM NaF, pH 6.00 at 37°C for 5—10 min (hematocrit about 10%). Effectors were added to cell suspensins at the end of preincubation. The incubation was started by the addition of 0.4—0.8M PEP which was dissolved in the citrate buffer and adjusted to pH 6.00. The pH of erythrocyte suspension was 6.0 at the begining of the incubation and the pH was constant during the incubation for 15 min. ○——○, control; □——□, 0.5mM pyridoxal 5 —P; ●——●, 1mM pyridoxal 5 —P; ▲——▲, 10mM inorganic phosphate; X——X, 1mM pyridoxal.

inhibited by 3μM of DIDS but inhibitor for the monocarboxylate transport system such as PCMB and α-cyano-4-hydroxycinnamate (3, 4) had less inhibitory effect on the PEP transport as high as 100μM (Fig. 2).

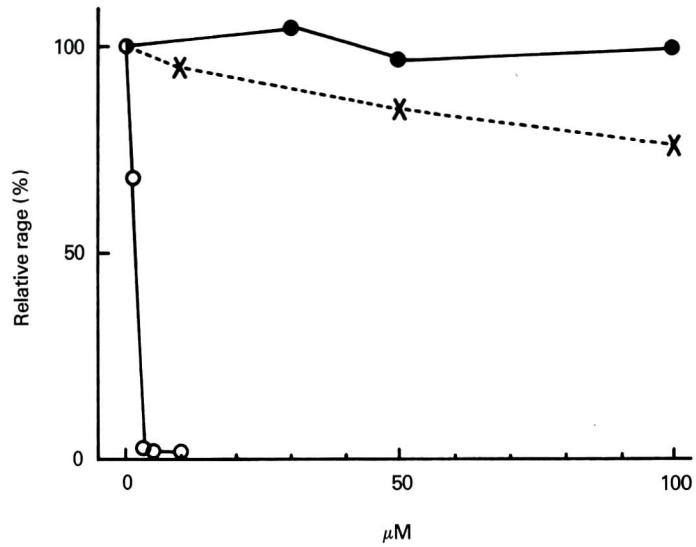

Fig. 2 Effects of inhibitors for anion transport on the transport of PEP. Incubation conditions are shown in Fig. 1. ○———○, DIDS; ●———●, PCMB; X--------X, α-cyano-4-hydroxycinnamate.

LOSS OF THE PEP TRANSPORT AND INORGANIC PHOSPHATE TRANSPORT ACTIVITIES IN THE CELLS TREATED WITH PYRIDOXAL 5—P AND SODIUM BOROHYDRIDE.

Pyridoxal 5—P forms a Schiff's base with a reactive lysine residue and has been used to identify active sites of enzymes (13). Pyridoxal 5—P inhibited competitively the PEP transport (Fig. 1) and pyridoxal 5—P itself is also transported across the erythrocyte membrane by a carrier-mediated transport system, which appears to be the inorganic anion transport system (14). Therefore, the tansport activities of PEP and inorganic phosphate were investigated in erythrocytes pretreated with pyridoxal 5—P and $NaBH_4$. Both the PEP transport and inorganic phosphate transport activities were inhibited in the cells treated with pyridoxal 5—P/$NaBH_4$ (Fig. 3). Both the transport activities of PEP and inorganic phsphate in the treated cells were inhibited proportionally to the pyridoxal 5—P concentrations used.

AFFINITY LABELLING THE MEDIATOR OF PEP TRANSPORT BY PYRIDOXAL 5—P.

As mentioned above, it is natural to assume that PEP and pyridoxal 5—P share a common transport system which seems to be the inorganic anion transport system in the cell membrane. Thus, we have tried to identify the possible mediater protein by labelling the cell membrane with pyridoxal

5—P. Erythrocytes were incubated with pyridoxal 5—P at 37°C for 5 min in a citrate buffer (pH 6.00). The cell fraction was separated from supernatant and washed with 10 volumes of the ice cold citrate buffer to remove pyridoxal 5—P which bound nonspecifically to the cell surface. Pyridoxal 5—P trapped in the transport system was fixed covalently to the transport system by reducing with [^{3}H]-$NaBH_4$. The major integral membrane protein, Band 3, was preferentially labelled by the treatment (Fig. 4). The radioactivity incorporated into Band 3 was reduced by pretreating the cells with DIDS, which is a specific inhibitor for the inorganic anion transport system (data not shown). The radioactive incorporation into Band 3 was confirmed by a two-dimensional gel electrophoresis combining isoelectric focusing in the first dimension and SDS—PAGE in the second dimension, which separate glycophorins completely from Band 3 (1, 10). Fig. 5 shows

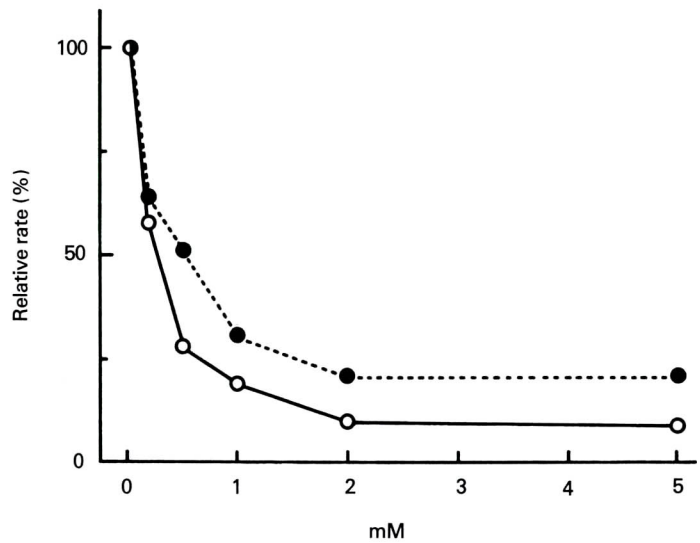

Fig. 3 Loss of the PEP transport and inorganic phosphate transport activities in the cells treated with pyridoxal 5 —P/$NaBH_4$. Washed erythrocytes were suspended in a 0.1 M citrate buffer containing 10 mM NaF, pH 6.00 at 37°C (hematocrit about 10%). The suspension was preincubated at 37°C for 5 min and erythrocytes were incubated with pyridoxal 5—P at 37°C for 8 min and were then chilled on ice. The supernatant was separated by centrifugation. The incubated cells were washed with 10 volumes of the ice cold citrate buffer to remove the weakly bound pyridoxal 5 —P from the cells surface. The washed cells were resuspended in the citrate buffer at hematocrit about 10% and sodium borohydride (freshly prepared in 0.01 M NaOH) was added to the suspension at the final concentration of 10 mM. The reduction with sodium borohydride was done in an ice bath for 30 min with continuous stirring. The reduction was terminated by washing the cells with 20 volumes of phosphate buffered saline for times. The treated cells were used for the transport assay of PEP and inorganic phosphate. ○———○, transport activity of PEP; ●-------●, transport activity of inorganic phosphate.

a fluorograph of the two-dimensional gel. Only Band 3 was lebelled with pyridxal 5—P/[^{3}H]-$NaBH_4$. Band 3 labelled with pyridoxal 5—P was also segregated into three major bands as in the case of nontreated erythrocyte membranes (12). No other radioactive spot was observed especially in the position where the glycophorins should be mobilized.

The results shown above suggest that the PEP transport is mediated by the inorganic anion transport system which may consist of Band 3. The transport is strongly dependent on pH of media and in fact no PEP is transported across the cell membrane at pH 7.4(5). It means that any conformational change of Band 3 induced by pH facilitates the transport of PEP.

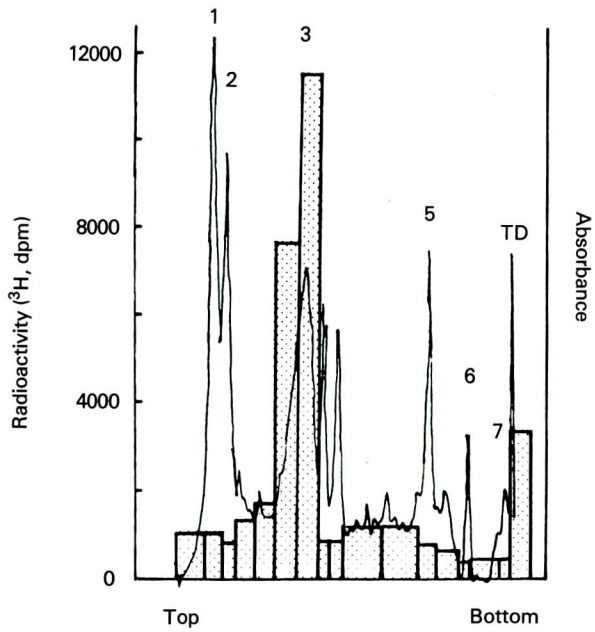

Fig. 4 Labelling the mediator of the PEP transport by pyridoxial 5—P. Washed erythrocytes were treated with 10mM pyridoxal 5—P and were washed as mentioned in Fig. 3. [^{3}H]-$NaBH_4$ was then added directly to the packed cells. The final concentration of sodium borohydride was 3.6mM. The cells were incubated in ice for 30 min with occasionally mixing. The labelled cells were washed extensively with phosphate buffered saline. The cell membrane(ghosts) were prepared from the labelled cells. The ghosts were analyzed by SDS—PAGE. The solid line indicates the densitometer scan of Coomassie blue-stained gel and the shaded bars represent the radioactivities [(^{3}H), dpm] of the slice gel. The protein peaks are numbered according to Yu *et al,* (16). TD = tracking dye.

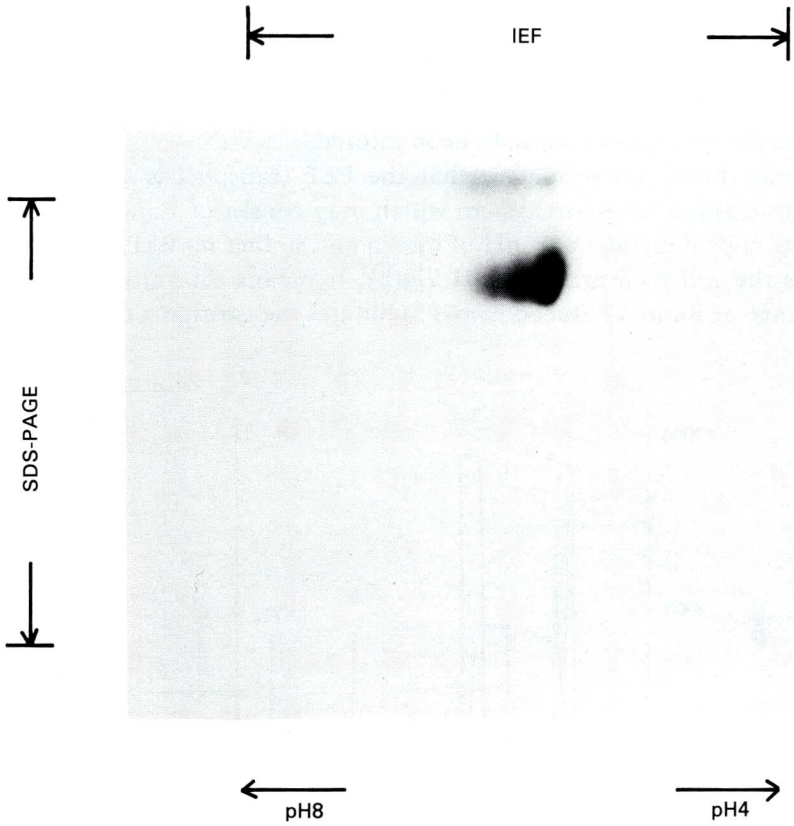

Fig. 5 A fluorograph of a two-dimensional electrophoretic analysis of membranes treated with pyridoxal 5—P/[^{3}H]-$NaBH_4$. Erythrocytes were treated as mentioned in Fig. 4. The treated cell membranes were separated in the first dimension by isoelectric focusing and in the second dimension by SDS—PAGE as described previously (12). Fluorography of the two-dimensional gel was performed according to Bonner and Laskey (2).

REFERENCES

1) Bhakdi S, Bjerrum OJ and Knüfermann H: The major intrinsic membrane protein of human erythrocytes; Preparative isolation and immunoelectrophoretic analysis. Biochim Biophys Acta 446: 419—431, 1976.
2) Bonner WM and Laskey RA: A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur J Biochem 46:83—88, 1974.
3) Deuticke B, Beyer E and Forst B: Discrimination of three paralles pathways of lactate transport in the human erythrocyte membrane by inhibitors and kinetic properties. Biochim Biophys Acta 684: 96—110, 1982.
4) Halestrap AP: Transport of pyruvate and lactate into human erythrocytes; Evidence for the involvement of the chloride carrier and a chloride-independent carrier. Biochem J 156: 193—207, 1976.
5) Hamasaki N, Hardjono IS and Minakami S: Transport of phosphoenolpyruvate through the erythrocyte membrane. Biochem J 170: 39—46, 1978.
6) Hamasaki N, Hirota C, Ideguchi H and Ikehara Y: Regeneration of 2,3-DPG and ATP of stored erythrocytes by phosphoenolpyryvate. In The Red Cell: Fifth Ann Arbor Conference (ed. Brewer GJ) Alan R. Liss, Inc., New York, pp. 577—592, 1981.

7) Hamasaki N, Ideguchi H and Ikehara Y: Regeneration of 2,3-bisphosphoglycerate and ATP in stored erythrocytes by phosphenolpyruvate; A new preservative for blood storage. Transfusion 21: 391—396, 1981.
8) Hamasaki N and Hirota-Chigita C: Acid-citrate-dextrose-phosphoenolpyruvate medium as a potent rejuvenant for blood storage. Transfusion 23: 1—7, 1983.
9) Hardjono IS, Hamasaki N, Minakami S and Fujino T: Effects of osmolarity and echinocytogenic drugs on the phosphoenolpyruvate through the red cell membrane. Cell Struc Func 5:21—25, 1980.
10) Harell D and Morrison M: Two-dimensional separation of erythrocyte membrane proteins. Arch Biochem Biophys 193: 158—168, 1979.
11) Ideguchi H, Hamasaki N and Ikehara Y: Abnormal phosphoenolpyruvate transport in erythrocytes of hereditary spherocytosis. Blood 58: 426—430, 1981.
12) Ideguchi H, Matsuyama H and Hamasaki N: Heterogeneity of human erythrocyte Band 3 analyzed by two-dimensinal gel electrophorsis. Eur J Biochem 125: 665—671, 1982.
13) Means GE and Feeney RE: in Chemical modification of proteins (eds. Means GE and Feeney RE) Holden-Day Inc., San Francisico, pp. 132—138, 1971.
14) Nanri H, Minakami S and Hamasaki N: Affinty labelling of Erythrocyte Band 3 protein with pyridoxal 5-phosphate: Involvement of 35,000-Dalton Fragment in Anion Transport. J Biol Cem 258: (1983) (in press).
15) Sohmer PR and Scott RL: Rejuvenation of CPD stored blood with phosphoenolpyruvate. Clin Res 29:42A, 1981.
16) Yu J, Fischman DA and Steck TL: Selective Solubilization of proteins and phospholipids from red blood cell membranes by nonionic detergents. J Supramlec Struct 1: 233—247, 1973.