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Modification of Human Erythrocyte Pyruvate Kinase by an Active Site-directed Reagent: Bromopyruvate

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Human erythrocyte pyruvate kinase was modified with bromopyruvate and the kinetic behavior of the modified enzyme was investigated. When the enzyme was modified with bromopyruvate in the absence of adenosine-5′-diphosphate, phosphoenolpyruvate or fructose-1,6-diphosphate the inactivation followed a pseudo first-order kinetics. The inactivation rate constant, k_s, was $1.84 \pm 0.15\,min^{-1}$. K_d of the bromopyruvate–enzyme complex was $0.14 \pm 0.03\,mM$.

The presence of adenosine-5′-diphosphate, phosphoenolpyruvate or fructose-1,6-diphosphate in the modification medium or the presence of fructose-1,6-diphosphate in the assay medium resulted in deviation of the inactivation kinetics from pseudo first-order. Phosphoenolpyruvate was better than adenosine-5′-diphosphate for protection against bromopyruvate modification whereas fructose-1,6-diphosphate was ineffective. The modified enzyme showed negative cooperativity in the presence of fructose-1,6-diphosphate whereas in the absence of it no activity was detected.

Keywords: Pyruvate kinase; Cysteine residues; Modification; Bromopyruvate; Human erythrocytes

INTRODUCTION

Pyruvate kinase (PK, E.C. 2.7.1.40) catalyzes the transfer of phosphate groups from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP) to generate adenosine triphosphate (ATP) and pyruvate. The enzyme requires K^+ as monovalent and Mg^{2+} or Mn^{2+} as divalent cations.[1] Human erythrocyte PK (R isoenzyme) is an allosteric enzyme, composed of four identical subunits, shows sigmoidal kinetics with respect to PEP and subject to activation by fructose-1,6-diphosphate (FDP) and inhibition by ATP.[2–4] Monosaccharide diphosphates other than FDP also convert the sigmoidal kinetics to hyperbolic, but at non-physiological concentrations.[2]

For elucidation of the role of functional groups located at the active or allosteric sites of the enzyme, several chemical modification studies have been performed.[5,6] An arginine residue was found at the PEP binding site of pig heart muscle and rabbit skeletal muscle PKs.[7,8] At the PEP binding site of rabbit muscle PK, in addition to

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arginine, a histidine residue and at the ADP binding site a lysine residue were found.[8–10] Human erythrocyte PK contains an arginine at the ADP binding site and two histidine residues, one at the region between the PEP and ADP binding sites and the other at a site other than the active site, responsible for the active conformation of the enzyme.[11,12]

Two cysteinyl residues in the PEP binding site of rabbit muscle PK are essential for catalysis. Conversion of these thiols to disulfide results in total inactivation of the enzyme.[13] In yeast PK, sulfhydryl groups are classified into three types according to their reactivities with 5,5′-dithio-bis-2,2′-dinitrobenzoic acid (DTNB) and it was shown that none of these sulfhydryls seems directly involved in catalysis.[14] The prevention of the modification of four sulfhydryl groups following the addition of substrate, PEP, led the researchers to consider that these groups might be in the vicinity of the active site.[4,15] Human erythrocyte PK is also sensitive to sulfhydryl reacting agents.[16–18] A sulfhydryl group was found at the PEP binding site.[18] Modification of the cysteine groups in PK results in inactivation and/or increase in the K_s for PEP.[16–18]

Bromopyruvate (BrPyr), although not a specific S-alkylation reagent as DTNB, has been successfully used as an active site-directed modifier for several enzymes.[19–26] The common features of these enzymes are that one of their substrates or products are pyruvate or a pyruvate analog.

PK deficiency causes nonspherocytic anaemia.[27] In order to understand the molecular basis of PK deficiency, the structural and functional properties of the normal enzyme need to be elucidated in detail. It is reported that a PK variant shows kinetic abnormalities that are closely mimicked by sulfhydryl modification in the normal enzyme.[28] This research aims to investigate the effect of BrPyr modification on the kinetic behavior of human erythrocyte PK so that it can be compared with the kinetic behavior of the mutant enzyme obtained from patients associated with nonspherocytic haemolytic anemia.

MATERIALS AND METHODS

Materials

Sephadex G-75, Sephadex G-25, Sepharose 4B, and Blue Dextran 2000 were from Pharmacia, Sweden. Rabbit muscle lactate dehydrogenase and nicotinamide adenine dinucleotide (reduced form, disodium salt) were from Boehringer Manheim, *N*-[2-Hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), was from Serva and ammonium sulfate was from Merck, Germany. Cyanogen bromide, monopotassium salts of ADP and PEP, FDP disodium salt, dithiotreitol (DTT) and 3-bromopyruvic acid were from Sigma, USA.

All other chemicals were standard products of Sigma or Aldrich (USA).

Enzyme Purification

Human erythrocyte PK was purified from outdated human blood obtained from Hacettepe University Hospitals, Ankara, Turkey. The details of the purification procedures have been described elsewhere.[29,30]

Enzyme Activity Determination

In kinetic and modification experiments PK activity was measured using the spectrophotometric method of Tietz and Ochoa.[31] The reaction medium contained 50 mM HEPES-KOH, pH 7.4, 0.18 mM NADH, 2 mM ADP, 2 mM PEP, 0.5 mM FDP, 10 mM $MgSO_4$, 100 mM KCl, 0.1 mM DTT, 4 μg LDH and aliquots of PK. The enzyme was preincubated with the reaction mixture excluding ADP at 30°C for 5 min, and the reaction was started by the addition of ADP. One unit of activity was defined as the amount of the enzyme

that oxidizes one micromole of NADH per minute.

Protein Determination

Protein determination was carried out by the method of Schaffner and Weissman, using bovine serum albumin as standard.[32]

Modification by BrPyr

In BrPyr modification experiments the R_4 form of the enzyme was used.[29,30] The enzyme eluted from the affinity column was concentrated by filtration through XM-10 filters, under nitrogen atmosphere, using Amicon model-12 ultrafiltration apparatus. For the complete removal of FDP from enzyme samples it was precipitated and washed twice with 80% saturated ammonium sulphate. The precipitate was dissolved in 25 mM Tris-acetate (pH 6.8) and reduced completely by incubating at 25°C for 2 h in the presence of 200 mM 2-mercaptoethanol (2-ME). The reduced enzyme was desalted on a Sephadex G-25 column (1 × 25 cm) equilibrated with 50 mM HEPES-KOH (pH 7.4) containing 0.1 mM EDTA. This reduced enzyme was used in all BrPyr modification experiments. PK was preincubated at 25°C in 50 mM HEPES-KOH, pH 7.4, containing 0.1 mM EDTA, for 15 min with or without addition of ADP, PEP or FDP and modification was started by the addition of different concentrations of BrPyr. Aliquots were drawn at certain time intervals and the remaining activity was determined in the absence of thiol reducing agents (dithiotreitol, 2-mercaptoethanol) and the allosteric activator, FDP.

Analysis of the Kinetic Data

The kinetic data were analyzed and kinetic constants were calculated by means of the nonlinear curve-fitting program of the statistical software package SYSTAT, version 5.03, 1991.

RESULTS

When inactivation and activity measurements were performed as described above, BrPyr inactivation of human erythrocyte PK followed pseudo-first order kinetics (Fig. 1). A plot of the reciprocals of the observed inactivation rate constants versus the reciprocals of BrPyr concentration gave a straight line, indicating formation of an enzyme–inhibitor complex before inactivation (inset of Fig. 1).

$$E + BrPyr \Leftrightarrow E \cdot \cdot \cdot BrPyr \Rightarrow E\text{–}BrPyr \quad (1)$$

$$k_{obs} = k_s[I]/(K_d + [I]) \quad (2)$$

$$1/k_{obs} = K_d/k_s \times 1/[I] + 1/k_s \quad (3)$$

where k_{obs} is the observed inactivation constant; K_d is the dissociation constant for E–I complex; k_s is the inactivation constant at saturated BrPyr concentration.

From the inset of Fig. 1, the values for k_s and K_d were calculated to be $1.84 \pm 0.15\ min^{-1}$ and 0.14 ± 0.03 mM, respectively. The correlation constant, r^2, of the line obtained was 0.99. The presence of either one of the substrates, ADP and PEP, or the allosteric activator, FDP, in the modification or activity measurement medium resulted in deviation from the pseudo-first order kinetics (Fig. 2). The protective effect of FDP against BrPyr modification was negligible. PEP and ADP both exerted partial protection although PEP was a better protective agent than ADP. When PEP was the varied substrate, at fixed saturated ADP and in the presence of the allosteric modifier, FDP, a linear Lineweaver–Burk plot was obtained with the unmodified enzyme. In the absence of FDP, however, Lineweaver–Burk plot showed a downward curvature indicating negative cooperativity (Fig. 3). On the other hand, a Lineweaver–Burk plot of the enzyme modified with BrPyr showed a similar curvature in the presence of FDP (Fig. 3), whereas no activity was detected in the absence of FDP (not shown). K_M of the unmodified enzyme was 0.24 ± 0.01 mM in the presence of FDP, and

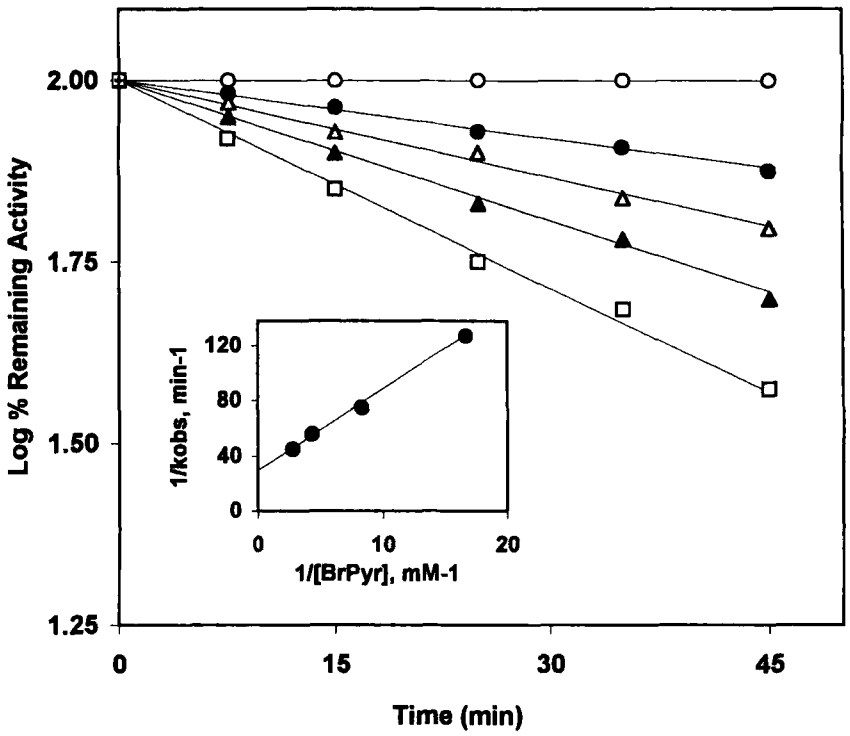

FIGURE 1 Bromopyruvate inactivation of human erythrocyte pyruvate kinase. [Bromopyruvate]: (—○—) 0, (—●—) 60, (—Δ—) 120, (—▲—) 240, and (—□—) 360 μM. Inset: $1/k_{obs}$ versus 1/[BrPyr]. Intercept, $1/k_s$, reciprocal of the saturation inactivation constant and the slope K_d/k_s. ($k_s = 1.84 \pm 0.15\,min^{-1}$ and $K_d = 0.14 \pm 0.03$ mM; $r^2 = 0.99$).

1.44 ± 0.06 mM in the absence of FDP. BrPyr modification decreased the affinity of the enzyme for PEP. K_M of the modified enzyme, in the presence of FDP was found to be 1.70 ± 0.11 mM.

When ADP was the varied substrate, hyperbolic Michaelis–Menten plots were obtained under all conditions (not shown). The affinity of human erythrocyte PK for FDP is very high and 40 μM FDP was sufficient for full activation of the enzyme (Fig. 4). When the differences in activities in the presence and absence of FDP versus 1/[FDP] were plotted, a straight line was obtained (inset to Fig. 4). From this graph K_M for FDP was calculated as 3.9 ± 0.7 μM.

DISCUSSION

The sensitivity of human erythrocyte PK to various sulfhydryl agents has been shown.[16–18,32] These properties are also shared by PKs purified from other sources.[13–15,26] The similarities of some properties of PKs obtained from PK deficient patients and normal enzymes in which cysteines had been modified, indicates the importance of the site of location and the function of cysteinyl residues.[18,28,33] BrPyr modification of human erythrocyte PK, in the absence of substrates and effector (FDP), resulted in inactivation with apparent first-order kinetics (Fig. 1). Addition of substrates and/or FDP to the preincubation medium or FDP to the activity measurement medium caused the inactivation kinetics to deviate from apparent first-order kinetics (Fig. 2). The same behavior was also observed with PK obtained from yeast.[26] When the $1/k_{obs}$ versus 1/[BrPyr] were plotted for yeast PK, a straight line was obtained and $1\,min^{-1}$ for k_s and 16 mM for K_d was reported.[26] By the same technique, a value of $1.84 \pm 0.15\,min^{-1}$ for k_s and

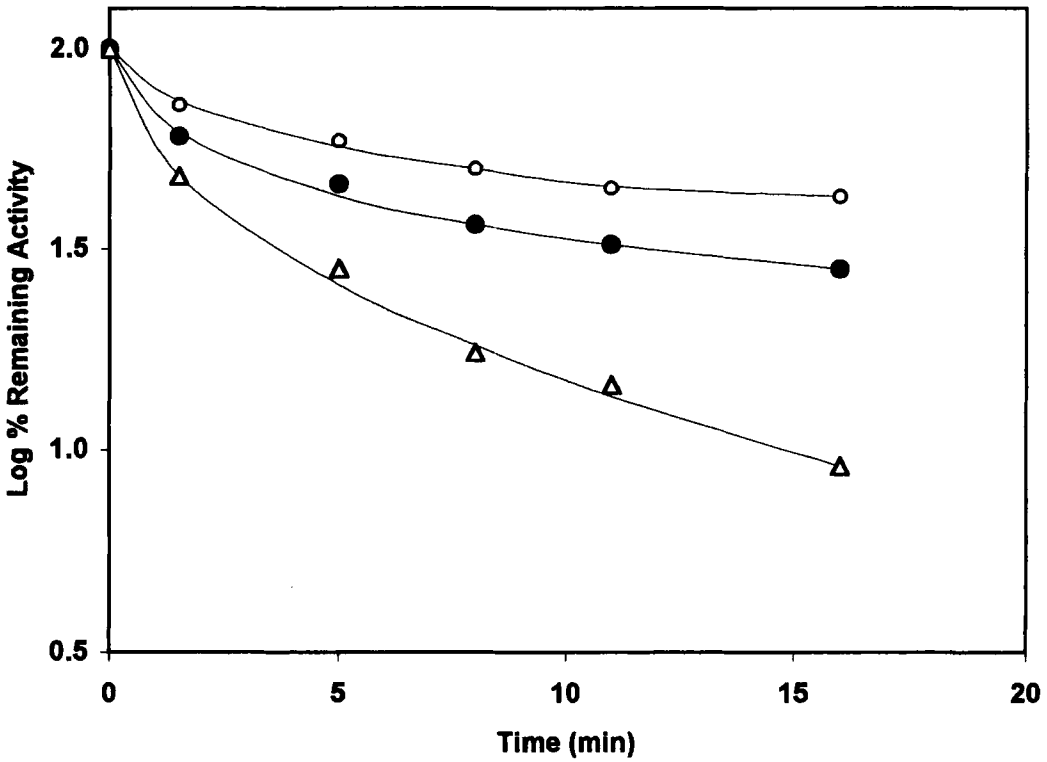

FIGURE 2 The effects of FDP, ADP and PEP on bromopyruvate inactivation of human erythrocyte pyruvate kinase. [Bromopyruvate] = 0.5 mM: (—○—) 2 mM PEP, (—●—) 4 mM ADP and (—Δ—) 0.05 mM FDP.

0.14 ± 0.03 mM for K_d were found for human erythrocyte PK (inset to Fig. 1). k_s values from the two sources are comparable, whereas K_d found for erythrocyte PK was about hundred times lower than the K_d of the yeast enzyme, indicating that specificity of binding of BrPyr to erythrocyte PK is one hundred times higher than that of yeast PK.[26] The dissociation constant of the complex between human erythrocyte PK and BrPyr was found to be lower than the dissociation constants reported for the BrPyr complexes of other enzymes of which pyruvate is a substrate or a product.[19,20,23,24] In this study, the value found for K_d in the presence of FDP (0.14 mM) is comparable to the K_M of the unmodified enzyme for PEP in the presence of FDP (0.24 mM), which was reported by others to be within the range of 0.1–0.3 mM.[2–4,17] These findings indicate that the group modified on human erythrocyte PK is more reactive than that of yeast PK. In fact, for human erythrocyte PK, the same inactivation rate was obtained with one-tenth of the concentration of BrPyr that was used to inactivate the yeast enzyme (Fig. 1). The modification of human erythrocyte PK with DTNB in the presence and absence of PEP resulted in the modification of 12 and 16 cysteines, respectively, and only cysteines protected by PEP were responsible from inactivation.[18] In BrPyr modification experiments the use of very low concentration of the reagent increases the probability of modification of cysteines located at the active site. When PEP, ADP or FDP were added to the preincubation medium or when the activity of the BrPyr-modified enzyme was measured in the presence of FDP, inactivation deviated from pseudo first-order kinetics (Fig. 2). From this Figure it is also seen that PEP was superior to ADP for protection

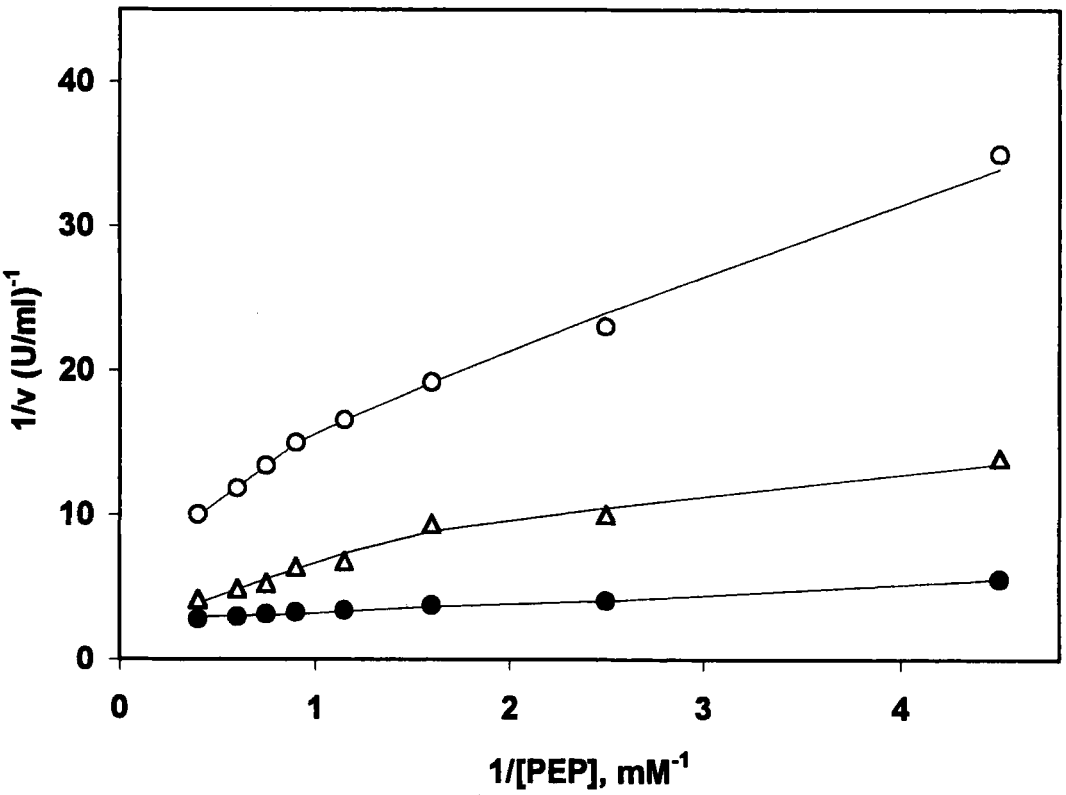

FIGURE 3 Lineweaver–Burk Plots of unmodified and modified human erythrocyte pyruvate kinase. No activity was observed with the bromopyruvate-modified enzyme in the absence of FDP. (—○—) unmodified in the absence of FDP, (—Δ—) modified and (—●—) unmodified in the presence of 100 μM FDP.

against BrPyr modification whereas FDP was ineffective in this respect. The partial protection offered by PEP is incompatible with the finding that PEP protects the human erythrocyte PK completely against specific cysteine modifiers such as DTNB, *N*-ethylmaleimide (NEM).[18,34] The partial protection by PEP may be explained by, (i) the tight complex formation between enzyme and the BrPyr: $K_d = 0.14$ mM, that is equal to the K_M for PEP; and (ii) the conformational change induced by added negative charge (due to BrPyr) which may result in the exposure of cysteine(s) located at the active site to BrPyr. In addition to PEP, ADP also shows protection against BrPyr modification but to a lesser degree (Fig. 2). The protection offered by ADP could also be explained by the repulsion of the negative charges on the phosphates of ADP. In modification assays with NEM, PEP exerted total protection whereas ADP remained ineffective.[34] Although the sizes of NEM and BrPyr are comparable, BrPyr carries a negative charge while NEM does not. Indeed, it was shown that in DTNB modification the partial protection exerted by ADP was due to steric hindrance and the same number of cysteines were modified in the presence and the absence of ADP.[18,34] These findings imply that the modified group is located at the PEP binding site. As in yeast PK the Lineweaver–Burk plot for the BrPyr modified human erythrocyte PK shows negative cooperativity both in the absence and presence of FDP[26] (Fig. 3). Due to the nature of the modifier, BrPyr, and remembering the partial protection exerted by both substrates ADP and PEP these cysteines must be responsible of either binding or the

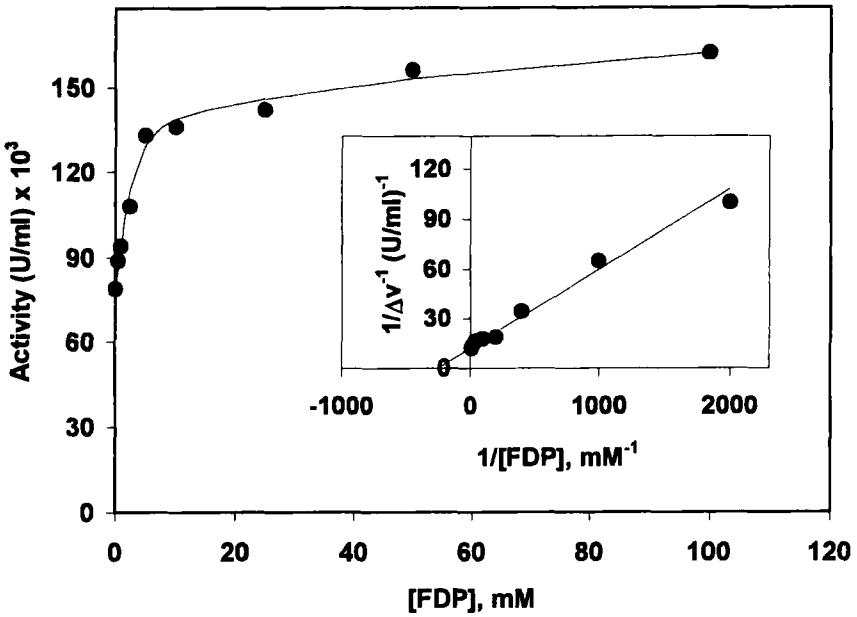

FIGURE 4 FDP saturation of unmodified human erythrocyte pyruvate kinase. Inset: Double-reciprocal plot of relative activity versus reciprocal of FDP. (Relative activity was obtained by subtracting the activity obtained in the absence of FDP from that in the presence of FDP).

formation of the active site. This hypothesis was supported by the reactivation of the totally inactive BrPyr modified enzyme in the presence of FDP (Fig. 3).

The identity of the BrPyr-modified group was not determined at the molecular level, however. The elucidation of the identity and the function of the BrPyr-modified group requires modification with radioactive BrPyr followed by amino acid analysis.

We hope that such studies may lead to an understanding of the molecular mechanisms underlying the diseases caused by defective PK.

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