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ON THE MOLECULAR BASIS OF PYRUVATE KINASE DEFICIENCY

II. ROLE OF THIOL GROUPS IN PYRUVATE KINASE FROM PYRUVATE KINASE-DEFICIENT PATIENTS

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SUMMARY

1. Human erythrocyte pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) from the class of pyruvate kinase-deficient patients, characterized by an increased affinity towards phosphoenolpyruvate and a loss of cooperative interaction towards this substrate, shows less affinity for the allosteric inhibitor ATP, when compared to pyruvate kinase from control persons. From the obtained kinetic data we can conclude that the loss of cooperativity towards phosphoenolpyruvate is a consequence of a shift in the R $\rightleftharpoons$ T equilibrium to the R state.

2. Incubation of pyruvate kinase, obtained from this class of pyruvate kinase-deficient patients with mercaptoethanol, changes the abnormal kinetics into normal kinetics, as can be concluded from the change in phosphoenolpyruvate dependency and ATP inhibition.

3. The effect of mercaptoethanol on the kinetics of pyruvate kinase from pyruvate kinase-deficient patients suggests that the alteration in the enzyme is a consequence of a modification of the –SH groups. It is suggested that pyruvate kinase deficiency is a secondary defect and that the process which causes the change in the –SH groups of pyruvate kinase, may also be responsible for the increased rate of haemolysis, found in these patients.

INTRODUCTION

Erythrocyte pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) deficiency is a well-known cause of hemolytic anemia. The deficiency comprises a heterogeneous group of disorders, characterized by both quantitative and qualitative enzyme abnormalities [1, 2]. By a comparison of the clinical and biochemical data we earlier suggested [2], that alteration in the kinetics of erythrocyte pyruvate kinase from pyruvate kinase-deficient patients might be a secondary defect. It was shown [2] that the primary defect, responsible for the abnormal pyruvate kinase kinetics, might be the redox state of the thiol groups in the red blood cell. Glutathione disulfide as

oxidizing agent was able to oxidize the thiol groups of the isolated erythrocyte pyruvate kinase, which resulted in a decreased affinity for one of the substrates, phosphoenolpyruvate and the allosteric activator Fru-1,6-P_2; kinetic properties also found with pyruvate kinase from most of the pyruvate kinase-deficient patients. This report deals with the effect of the reducing compound mercaptoethanol on pyruvate kinase, from pyruvate kinase-deficient patients, characterized by an increased affinity for the substrate phosphoenolpyruvate and a loss of the cooperative interaction towards this substrate [3, 4]. It will be shown, that in the altered kinetic properties of this class of patients thiol groups are also involved.

MATERIALS AND METHODS

Pyruvate kinase from erythrocytes was purified up to stage 4, as described by Staal et al. [5], except that during the isolation procedure mercaptoethanol was omitted. The final $(NH_4)_2SO_4$ precipitate was dissolved in 0.2 M Tris–maleate buffer pH 8.2 and divided into two fractions. One enzyme fraction was incubated with 10 mM mercaptoethanol at 10 °C for 1 h, whereas the other was incubated without further additions. Pyruvate kinase activity was assayed by following the decrease in absorbance at 340 nm in a coupled reaction with lactate dehydrogenase at 25 °C, according to Valentine and Tanaka [6]. The reaction mixture contained 0.2 M Tris–maleate buffer, pH 8.2, 2.0 mM ADP, 0.09 mM NADH, 65 mM KCl, 20 mM $MgSO_4$ and 0.1 mg lactate dehydrogenase in a final volume of 3.0 ml. The reported experiments were repeated after a three-month period, at which time a complete new population of erythrocytes is present, and these experiments were completely reproducible.

ADP, phosphoenolpyruvate, NADH and Fru-1,6-P_2 were obtained from Boehringer (Mannheim, Germany). Mercaptoethanol was obtained from Fluka, Basel. All other reagents were of an analytical grade purity.

RESULTS

Figs. 1A and 1B show the v vs [phosphoenolpyruvate] plot at [ADP] = 2 mM in the presence and absence of 0.5 mM Fru-1,6-P_2 for pyruvate kinase, obtained from normal red blood cells. Fig. 1B shows the activity curve of the enzyme after incubation with 10 mM mercaptoethanol, whereas for Fig. 1A this compound was omitted. In accordance with earlier reports [5, 6] a sigmoidal curve was obtained, in the absence of Fru-1,6-P_2, with an n value of 1.7 (see Hill plot insert Fig. 1A). Addition of 0.5 mM Fru-1,6-P_2 stimulates the enzymatic activity especially at low phosphoenolpyruvate concentrations and the sigmoidal curve was converted into a hyperbolic curve (n value of 1.0). Incubation of the enzyme with mercaptoethanol for 1 h does not alter the allosteric properties and exactly the same n values, 1.7 in the absence and 1.0 in the presence of Fru-1,6-P_2, were obtained.

Fig. 2A shows the v vs [phosphoenolpyruvate] plot of pyruvate kinase, obtained from a patient with increased hemolysis, which has been classified as pyruvate kinase deficient (cf. ref. 4 patient M.V.). In agreement with an earlier report [4] the enzyme from this patient has lost its allosteric properties towards phosphoenolpyruvate and an n value of 1.0 was obtained (insert Fig. 1A). Incubation of this enzyme

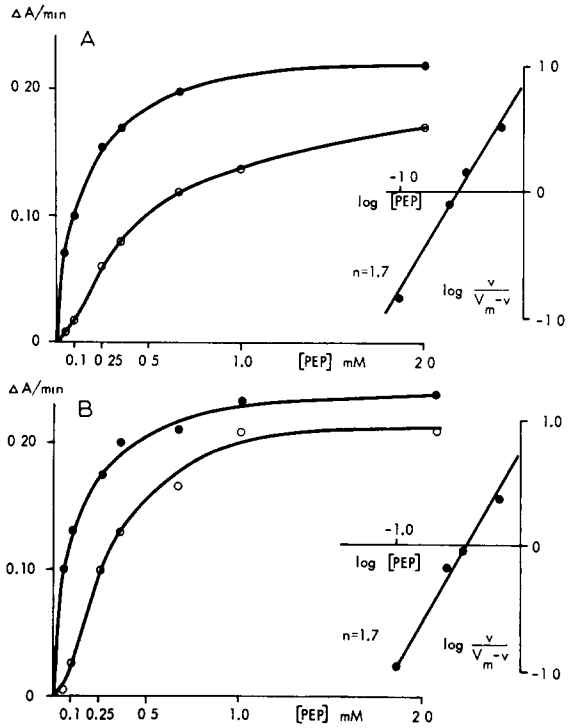

Fig. 1. The activity vs [phosphoenolpyruvate]plot of normal erythrocyte pyruvate kinase, measured in the presence and absence of Fru-1,6-P_2 (0.5 mM). The inserts are the Hill plots of the values obtained in the absence of Fru-1,6-P_2. The calculated Hill coefficients (n) are indicated. (A) The activity curve after incubation without mercaptoethanol, measured in the absence (○—○) and in the presence of Fru-1,6-P_2 (●—●). (B) Activity curve after a 1-h incubation with 10 mM mercaptoethanol at 10 °C, measured in the absence ○—○ and in the presence of Fru-1,6-P_2 ●—●.

in the presence of mercaptoethanol introduced an allosteric behavior towards phosphoenolpyruvate, as can be concluded from the obtained n value of 1.9 (insert Fig. 2B).

Figs 3A and 3B show the v vs [phosphoenolpyruvate] plot of the second, unrelated, patient P.R. Pyruvate kinase from this patient also shows a loss of its allosteric behavior towards phosphoenolpyruvate (n value of 1.0, insert Fig. 3A). By incubating the altered enzyme from this patient with mercaptoethanol, the normal kinetic behavior towards phosphoenolpyruvate was obtained (n value 1.7, insert Fig. 3B).

From Figs 1, 2 and 3 we can conclude that the abnormal kinetics of these mutant enzymes can be converted into a kinetic behavior towards phosphoenolpyruvate, which is identical with the normal enzyme. In order to investigate the nature of this conversion more closely, we studied the effect of ATP on the reduced and untreated enzyme of patient M.V. It can be seen (Fig. 4) that the untreated enzyme of this patient possesses a low affinity for the inhibitor ATP, whereas incubation with mercaptoethanol causes a shift in the inhibition curve; the affinity for ATP is increased, which is accompanied by a lowering of the n value (calculated by the method of Jensen and Nester [7]) from 2.5 to 1.6. With both enzymes Fru-1,6-P_2 (0.5 mM)

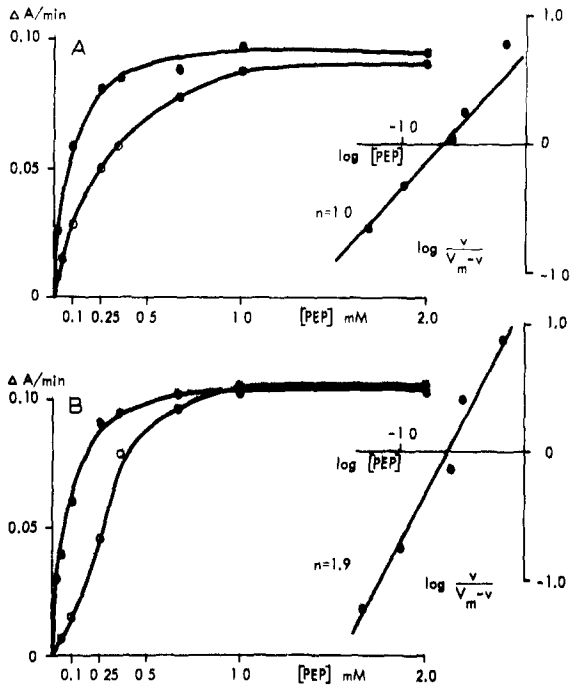

Fig. 2. The activity vs [phosphoenolpyruvate] plot of erythrocyte pyruvate kinase from a patient M.V., measured in the presence and absence of Fru-1,6-P_2 (0.5 mM). The inserts are the Hill plots of the values obtained in the absence of Fru-1,6-P_2. The calculated Hill coefficients (n) are indicated. (A) The activity curve after incubation without mercaptoethanol, measured in the absence (○—○) and in the presence of Fru-1,6-P_2 (●—●). (B) Activity curve after a 1 h incubation with 10 mM mercaptoethanol at 10 °C, measured in the absence (○—○) and in the presence of Fru-1,6-P_2 (●—●).

is able to abolish the inhibition, suggesting that this effector site is intact. With pyruvate kinase from patient P.R. the results obtained are qualitatively similar (Fig. 5), although the enzyme from this patient seemed less sensitive to ATP. Comparison of the ATP inhibition plots of patients and controls reveals the conclusion that the ATP inhibition, obtained with the patients' enzymes after incubation with mercaptoethanol, is similar to the inhibition plot of the controls (cf. ref. 4). This allows the conclusion that incubation with mercaptoethanol is able to convert the abnormal enzyme, isolated from the two patients into an enzyme which cannot be distinguished from pyruvate kinase from control persons.

From the phosphoenolpyruvate dependence and ATP inhibition of the mutant enzyme of the patients, it can be concluded that the mutant enzyme is characterized by a shift of the R ⇌ T equilibrium to the R state. Recently we showed that M-type pyruvate kinase from leucocytes [8] and liver [9] possess allosteric properties, similar to the L-type. To obtain more information about the molecular basis of pyruvate kinase deficiency we isolated leucocytes from the patients, described in this report, and characterized pyruvate kinase. No significant differences in the activity curve towards phosphoenolpyruvate could be detected. Also the alanine inhibition and the

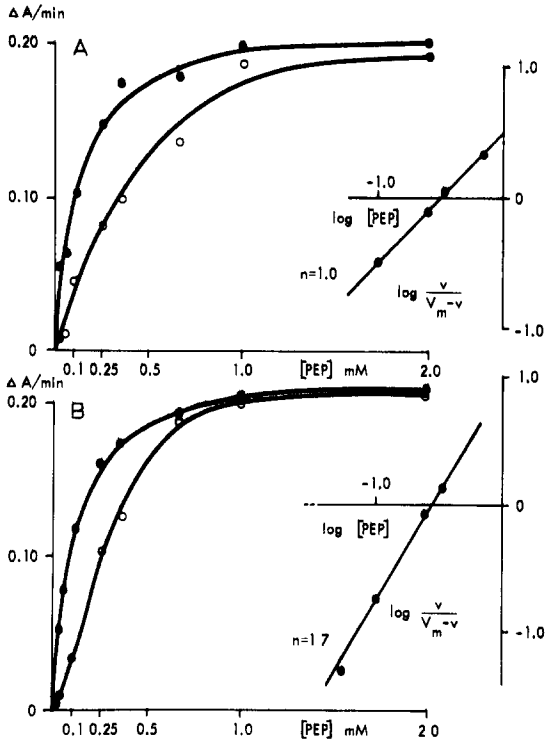

Fig. 3. The activity vs [phosphoenolpyruvate]plot of erythrocyte pyruvate kinase from a patient P.R., measured in the presence and absence of Fru-1,6-P_2 (0.5 mM). The inserts are the Hill plots of the values obtained in the absence of Fru-1,6-P_2. The calculated Hill coefficients (n) are indicated. (A) The activity curve after incubation without mercaptoethanol, measured in the absence (○—○) and in the presence of Fru-1,6-P_2 (●—●). (B) Activity curve after a 1-h incubation with 10 mM mercaptoethanol at 10 °C, measured in the absence ○—○ and in the presence of Fru-1,6-P_2 (●—●).

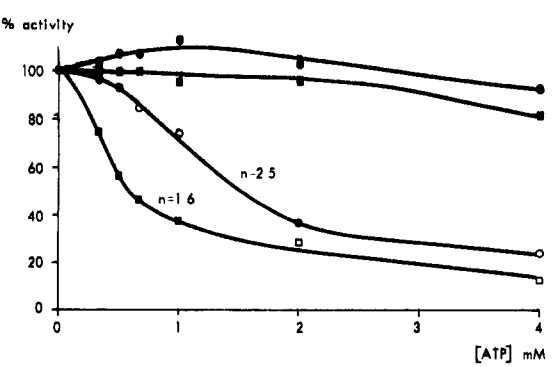

Fig. 4. The influence of ATP on the activity of erythrocyte pyruvate kinase of patient M.V. at 0.25 mM phosphoenolpyruvate, 0.5 mM ADP and pH 8.2. ○—○, the activity curve after incubation without mercaptoethanol. ●—●, the activity curve after incubation without mercaptoethanol measured in the presence of Fru-1,6-P_2 (0.5 mM). □—□, the activity curve after incubation with 10 mM mercaptoethanol for 1 h at 10 °C. ■—■, the activity curve after incubation with 10 mM mercaptoethanol for 1 h at 10 °C, measured in the presence of Fru-1,6-P_2 (0.5 mM). The calculated Hill coefficients (n) are indicated. The 100% value is the activity measured in the presence of various additions at [ATP] = 0 mM.

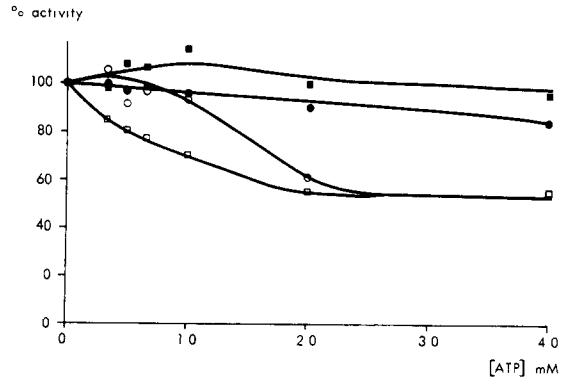

Fig. 5. The influence of ATP on the activity of erythrocyte pyruvate kinase of patient P R at 0 25 mM phosphoenolpyruvate, 0.5 mM ADP and pH 8 2. ○—○ the activity curve after incubation without mercaptoethanol. ●—●, the activity curve after incubation without mercaptoethanol, measured in the presence of Fru-1,6-P_2 (0 5 mM). □—□, the activity curve after incubation with 10 mM mercaptoethanol for 1 h at 10 °C. ■—■, the activity curve after incubation with 10 mM mercaptoethanol for 1 h at 10 °C, measured in the presence of Fru-1,6-P_2 (0 5 mM). The 100% value is the activity measured in the presence of various additions at [ATP] = 0 mM.

effect of Fru-1,6-P_2 was similar to the control values, which suggest that the R ⇌ T equilibrium of leucocyte M-type pyruvate kinase is not changed in the patients.

DISCUSSION

Since Valentine et al. [10] described in 1961 the first cases of non-spherocytic anemia with affected pyruvate kinase, more than 160 reports [1] about erythrocyte pyruvate kinase deficiency have been published. No quantitative correlation between the pyruvate kinase activity and the clinical severity, or between the pyruvate kinase activity and the degree of hemolysis could be demonstrated. Most of the cases showed a normal or even increased glycolytic rate, as can be concluded from the glucose consumption and lactate production [1]. Considering those facts, we suggested that the alteration in pyruvate kinase might be a secondary defect [2]. The heterogeneity, observed in the kinetics of pyruvate kinase from pyruvate kinase-deficient patients, suggest that this alteration can be the consequence of different primary defects. The most important condition for the primary defect must be that there exists a relation between this defect and the increased hemolysis, found in the patients.

Recently we showed [2] for the pyruvate kinase-deficient patients, characterized by a decreased affinity for phosphoenolpyruvate and Fru-1,6-P_2, that the alteration in the enzyme kinetics can be the consequence of oxidation of the thiol groups in the enzyme. This report shows that, also with patients characterized by an increased affinity for phosphoenolpyruvate, the thiol groups are involved in the enzyme modification. From the kinetic data obtained with the untreated mutant enzyme (a hyperbolic response to phosphoenolpyruvate and decreased sensitivity towards ATP), we can conclude that in the R ⇌ T model of Monod et al. [12], which is valid for erythrocyte pyruvate kinase [5, 12], this R ⇌ T equilibrium is shifted to the R state. It was speculated earlier [13] (based on the kinetics towards phosphoenolpyruvate) that the mutant enzyme, characterized by a hyperbolic activity curve

towards phosphoenolpyruvate in the absence as well in the presence of Fru-1,6-P_2, looks very similar to the conformation which can be obtained artificially with the normal enzyme, by lowering the pH and the temperature. The ATP inhibition curve, obtained with the patients, proves that this speculation was correct and that the molecular basis of the altered enzyme is a decrease of the allosteric constant L. Incubation of the mutant enzymes with mercaptoethanol converts the abnormal kinetics to normal kinetics. Since this compound affects the -SH groups of proteins, it seems likely that thiol groups are involved in this conversion. By restoring the thiol group(s) of the mutant enzyme to the "normal" reduction grade, the $R \rightleftharpoons T$ equilibrium is shifted to the equilibrium, found with the normal enzyme and the allosteric constant L reaches its normal value. At the moment the primary defect, responsible for the mutant enzymes described here, remains uncertain. However, it seems likely that the primary defect is related to the reduction state of the -SH groups in the red blood cell. This reduction state can form the link between alterations in pyruvate kinase and the increased hemolysis, found in these patients.

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