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ON THE MOLECULAR BASIS OF PYRUVATE KINASE DEFICIENCY

I. PRIMARY DEFECT OR CONSEQUENCE OF INCREASED GLUTATHIONE DISULFIDE CONCENTRATION

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SUMMARY

1. Human erythrocyte pyruvate kinase (ATP:pyruvate phosphotransferase EC 2.7.1.40) can be converted into an oxidized form by incubation with oxidized glutathione. The oxidized enzyme can be reduced again by incubation with mercaptoethanol, with reduced glutathione a partial reduction of the enzyme is obtained.

2. The oxidized enzyme shows a lower affinity for the substrate phosphoenolpyruvate and for the allosteric effector fructose 1,6-diphosphate. The thermolability of the oxidized enzyme is markedly increased, compared with the freshly isolated or reduced enzyme.

3. The data obtained with the oxidized enzyme are discussed in relation to the data obtained with pyruvate kinase from pyruvate kinase-deficient patients. It is concluded that erythrocyte pyruvate kinase deficiency can be a consequence of an increased oxidized glutathione concentration in the red blood cell.

INTRODUCTION

Erythrocyte pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) deficiency is a well-known cause of hemolytic anaemia. The deficiency comprises a heterogeneous group of disorders characterized by both quantitative and qualitative enzyme abnormalities[1]. Cases of hemolytic anemia attributed to pyruvate kinase abnormalities have been found with markedly decreased[2], moderately diminished[3–6], normal[3,7] or even increased[8] enzyme activity. The majority of the cases showed a decreased affinity for the substrate phosphoenolpyruvate[6,7,9–12], although also normal[3] or increased[13,14] affinities for phosphoenolpyruvate have been described. Most of the cases showed a normal or even increased glycolytic rate as can be concluded from the glucose consumption and lactate production[1]. The hemolytic anemia has been ascribed to the lowered ATP level found with the patients[1]. However, also

normal[15] or even increased ATP levels[16] have been reported. These data suggest that pyruvate kinase deficiency is a very heterogeneous molecular disorder and the lack of relation between the enzymatic activity and the degree of hemolysis suggested to us that the alteration in the pyruvate kinase activity might be a secondary defect. Reports have been published of cases with pyruvate kinase deficiency, which are combined with a decreased activity of glutathione reductase[17–19]. This prompted us to study the effect of oxidized glutathione on the kinetic behavior of the erythrocyte pyruvate kinase. Also the influence of oxidized glutathione on the stability of the enzyme is investigated, because it has been reported that the most common property in pyruvate kinase deficiency is the increased heat lability of the enzyme. The effect of oxidized glutathione is of special interest because the link between alterations in erythrocyte pyruvate kinase and the GSH/GSSG ratio might explain the increased hemolysis found in pyruvate kinase-deficient patients[20].

MATERIALS AND METHODS

Pyruvate kinase from erythrocytes, obtained from healthy volunteers (aged 20–35 years) was purified up to Stage 4 as described by Staal *et al.*[21], except that during the isolation procedure mercaptoethanol was omitted. The final $(NH_4)_2SO_4$ precipitate was dissolved in 0.25 M Tris–HCl, pH 8.0. Pyruvate kinase activity was assayed by following the decrease in absorbance at 340 nm in a coupled reaction with lactate dehydrogenase at 23 °C according to Valentine and Tanaka[22]. The triethanol–HCl buffer (0.4 M, pH 7.5) was replaced by Tris–HCl buffer (0.25 M, pH 8.0). Routinely about 0.08 mg enzyme was included in the assay mixture. The enzyme was oxidized by incubation with 2.5 mM oxidized glutathione at 4 °C for the time indicated in the legends to the figures. For the thermostability test 0.5 ml of the enzyme, containing precisely 2.0 mg/ml protein, was incubated at 53 °C. After the times indicated in Fig. 5 samples were taken and immediately assayed for pyruvate kinase activity at 5 mM phosphoenolpyruvate in the presence of 0.5 mM fructose 1,6-diphosphate (Fru-1,6-P_2).

ADP, phosphoenolpyruvate, NADH, Fru-1,6-P_2 and oxidized glutathione were obtained from Boehringer (Mannheim, Germany). Reduced glutathione was obtained from Sigma. All other reagents were of analytical grade purity.

RESULTS

Fig. 1 shows the v *vs* [phosphoenolpyruvate] plot at [ADP] = 2 mM in the presence or absence of 0.5 mM Fru-1,6-P_2 for the freshly prepared erythrocyte pyruvate kinase and the enzyme, which has been incubated with 2.5 mM oxidized glutathione for 8 h. With the freshly prepared enzyme we obtained in the absence of Fru-1,6-P_2 a $K_{0.5}$ value of 0.75 mM. Addition of 0.5 mM Fru-1,6-P_2 converted the sigmoidal curve into a hyperbolic one and the apparent K_m was now 0.15 mM. The enzyme which has been incubated with 2.5 mM oxidized glutathione showed a marked change in these activity curves. After an 8-h incubation the apparent K_m in the presence of Fru-1,6-P_2 was raised to 0.75 mM, and the $K_{0.5}$ in the absence of Fru-1,6-P_2 was about 5 mM. It could be observed that in the presence of Fru-1,6-P_2 the same maximal activity was measured, indicating that no loss of maximal activity occurred

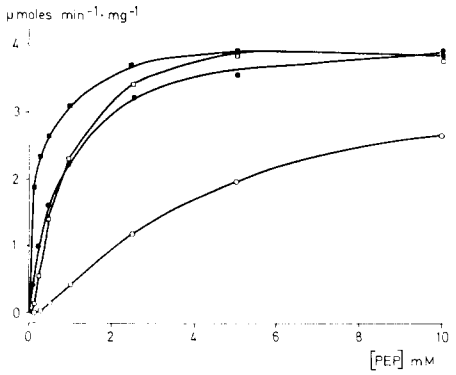

Fig. 1. The activity *vs* [phosphoenolpyruvate] plot of freshly isolated and partly oxidized human erythrocyte pyruvate kinase at [ADP] = 2 mM measured in the presence and absence of Fru-1,6-P_2 (0.5 mM). □—□, the activity of the freshly isolated erythrocyte pyruvate kinase; ■—■, the activity of the freshly isolated erythrocyte pyruvate kinase in the presence of Fru-1,6-P_2; ○—○, the activity of the erythrocyte pyruvate kinase after an 8-h incubation with 2.5 mM GSSG; ●—●, the activity of the erythrocyte pyruvate kinase after an 8-h incubation with 2.5 mM GSSG measured in the presence of Fru-1,6-P_2.

after partial oxidation of the enzyme. Prolonged oxidation caused a further decrease in the affinity of the enzyme for the substrate phosphoenolpyruvate both in the absence and presence of Fru-1,6-P_2 (Fig. 2). After a 24-h incubation at 4 °C in the presence of 2.5 mM oxidized glutathione the $K_{0.5}$ in the presence of 0.5 mM Fru-1,6-P_2 was increased to 1 mM, whereas the $K_{0.5}$ value in the absence of Fru-1,6-P_2 was higher than 10 mM. Fig. 2 also shows that oxidation of the erythrocyte pyruvate kinase is a reversible process. When the oxidized enzyme was incubated for 1 h at 10 °C with 1 mM mercaptoethanol the same kinetic data were obtained as with the freshly isolated enzyme. The $K_{0.5}$ in the absence of Fru-1,6-P_2 was lowered again

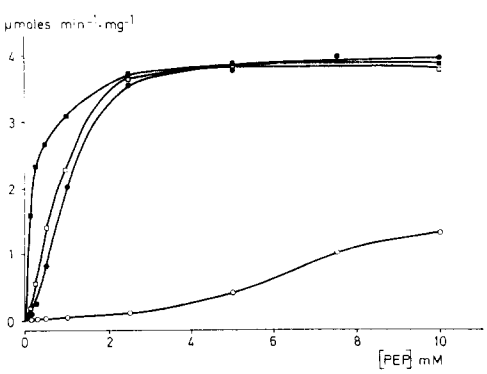

Fig. 2. The activity *vs* [phosphoenolpyruvate] plot of the oxidized erythrocyte pyruvate kinase and the oxidized erythrocyte pyruvate kinase after incubation for 1 h at 10 °C with 1 mM mercaptoethanol (reduced enzyme) measured in the presence and absence of Fru-1,6-P_2 at [ADP] = 2 mM. ○—○, the activity of the erythrocyte pyruvate kinase after a 24-h incubation with 2.5 mM GSSG; ●—●, the activity of the erythrocyte pyruvate kinase after a 24-h incubation with 2.5 mM GSSG measured in the presence of Fru-1,6-P_2; □—□, the activity of the reduced erythrocyte pyruvate kinase; ■—■, the activity of the reduced erythrocyte pyruvate kinase measured in the presence of Fru-1,6-P_2.

to 0.75 mM whereas in the presence of Fru-1,6-P_2 an apparent K_m value of 0.15 mM was obtained. Incubation of the enzyme without GSSG at 4 °C and pH 8.0 did not affect the enzyme, even if the incubation period was prolonged till 5 days. Also oxygen bubbling through the enzyme solution for 24 h did not result in an alteration of the enzyme.

Incubation of the oxidized enzyme with 5 mM reduced glutathione does not result in a complete restoration of the enzyme kinetics as shown in Fig. 3. After incubation of the oxidized enzyme with 5 mM reduced glutathione for 1 h the $K_{0.5}$ in the presence of 0.5 mM Fru-1,6-P_2 was 0.6 mM whereas in the absence of Fru-1,6-P_2 a $K_{0.5}$ value of about 8 mM was obtained. Extension of the incubation period with 5 mM GSH to 4 h did not influence the kinetic profile any further. However, if the partly-reduced enzyme (by 5 mM GSH) was treated for 1 h with 1 mM mercaptoethanol, the reduced enzyme was obtained (*cf.* Fig. 2).

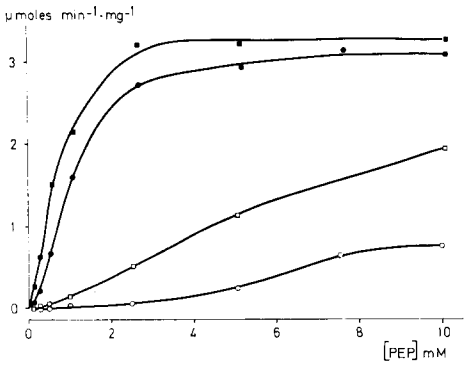

Fig. 3. The activity *vs* [phosphoenolpyruvate] plot of the oxidized erythrocyte pyruvate kinase and the oxidized erythrocyte pyruvate kinase after incubation for 1 h at 10 °C with 5 mM GSH measured in the presence and absence of Fru-1,6-P_2 at [ADP] = 2 mM; ○—○, the activity of the erythrocyte pyrvate kinase after a 24-h incubation with 2.5 mM GSSG; ●—●, the activity of the erythrocyte pyruvate kinase after a 24-h incubation with 2.5 mM GSSG measured in the presence of Fru-1,6-P_2; □—□, the activity of the oxidized erythrocyte pyruvate kinase which has been incubated with 5 mM GSH for 1 h at 10 °C; ■—■, the activity of the oxidized erythrocyte pyruvate kinase which has been incubated with 5 mM GSH for 1 h at 10 °C measured in the presence of Fru-1,6-P_2.

The activity of the oxidized erythrocyte pyruvate kinase as a function of the phosphoenolpyruvate concentration behaves in the same way as pyruvate kinase from patients with a lowered affinity for the substrate phosphoenolpyruvate. In most of these patients the affinity for Fru-1,6-P_2 is lowered[6,7,23]. Therefore, we investigated the activity of the normal and oxidized enzyme from control erythrocytes at increasing Fru-1,6-P_2 concentrations. Fig. 4 shows the pyruvate kinase activity of the freshly isolated, oxidized, partly reduced and reduced enzyme as a function of the [Fru-1,6-P_2] at 0.5 mM phosphoenolpyruvate. It can be seen that the oxidized enzyme showed a lower affinity for Fru-1,6-P_2 in comparison with the freshly isolated and reduced enzyme. The freshly isolated and reduced enzymes were already fully activated at 2 μM Fru-1,6-P_2 whereas with the oxidized enzyme full stimulation was obtained at 20 μM Fru-1,6-P_2. The curve obtained with the freshly isolated enzyme is identical with the activity curve obtained when the oxidized enzyme was incubated

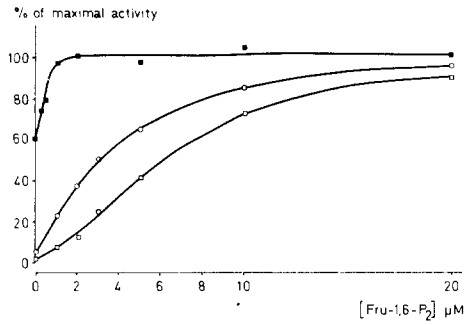

Fig. 4. The activity *vs* [Fru-1,6-P_2] plot of the freshly isolated, oxidized, partly reduced and reduced erythrocyte pyruvate kinase at [phosphoenolpyruvate] = 0.5 mM and [ADP] = 2 mM. ■—■, the activity of the freshly isolated erythrocyte pyruvate kinase and of the oxidized pyruvate kinase after incubation for 1 h at 10 °C with 1 mM mercaptoethanol (reduced enzyme); □—□, the activity of the erythrocyte pyruvate kinase after a 24-h incubation with 2.5 mM GSSG (oxidized enzyme); ○—○, the activity of the oxidized erythrocyte pyruvate kinase after incubation for 1 h at 10 °C with 5 mM GSH (partly reduced enzyme).

for 1 h with 1 mM mercaptoethanol at 10 °C. When the oxidized enzyme was incubated with 5 mM reduced glutathione an intermediate curve was obtained, which suggests that the oxidized enzyme cannot be reduced completely by GSH, at least in the concentration tested.

From Figs 1–4 we can conclude that the kinetics obtained with the enzyme which has been incubated with 2.5 mM GSSG are quite identical with the kinetics

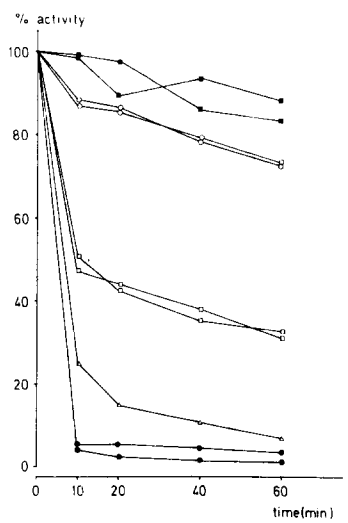

Fig. 5. The thermostability of the freshly isolated, partly oxidized, oxidized, partly reduced and reduced erythrocyte pyruvate kinase at 53 °C. The enzymatic activity was determined at [phosphoenolpyruvate] = 5 mM, [ADP] = 2 mM and [Fru-1,6-P_2] = 0.5 mM. ■—■, freshly isolated erythrocyte pyruvate kinase; □—□, erythrocyte pyruvate kinase which has been incubated for 4 h with 2.5 mM GSSG ($K_{0.5}$ for phosphoenolpyruvate about 3 mM); ●—●, erythrocyte pyruvate kinase which has been incubated for 24 h with 2.5 mM GSSG (oxidized enzyme); △—△, oxidized erythrocyte pyruvate kinase (24 h of oxidation with 2.5 mM GSSG) which has been incubated for 1 h with 5 mM GSH; ○—○, oxidized erythrocyte pyruvate kinase (24 h of oxidation with 2.5 mM GSSG) which has been incubated for 1 h with 1 mM mercaptoethanol.

obtained with most of the patients with the affected pyruvate kinase. It has been concluded earlier that the most common property of pyruvate kinase from pyruvate kinase-deficient patients is the increased lability of the enzyme at higher temperatures. Blume *et al.*[23] showed recently that the stability of the enzyme at 53 °C can serve as a useful tool for the diagnosis of pyruvate kinase deficiency. Therefore, the thermostability of the freshly isolated, partly oxidized, oxidised partly reduced and reduced enzyme at 53 °C was investigated (Fig. 5). Under the applied condition (see legend to Fig. 5) the freshly isolated enzyme showed, in accordance with Blume *et al.*[23], only a slight decrease in activity. The partly oxidized enzyme ($K_{0\,5}$ for phosphoenolpyruvate about 3 mM) showed an increased thermolability whereas the oxidized enzyme ($K_{0\,5}$ for phosphoenolpyruvate higher as 10 mM) lost, within 10 min, nearly all activity. When the oxidized enzyme was reduced with 5 mM GSH (*cf.* for the kinetics Figs 3 and 4) the thermolability was lowered, whereas reduction with 1 mM mercaptoethanol was able to restore the stability of the enzyme almost completely.

DISCUSSION

Since Valentine *et al.*[24] 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. Since a quantitative correlation could neither be demonstrated between the pyruvate kinase activity and the clinical severity nor between the pyruvate kinase activity and the degree of hemolysis, it was suggested earlier that a lowered pyruvate kinase activity might not be the primary lesion causing hemolysis[25,26]. The heterogeneity observed in the kinetics of pyruvate kinase from pyruvate kinase-deficient patients and the fact that the glycolytic rate is not affected in these patients, supports the idea that an alteration in pyruvate kinase activity might be a secondary effect. It has been reported[20] that the GSH/GSSG ratio can play an important rôle in the hemolysis of the red blood cell. Therefore, the effects of oxidized glutathione on the kinetics and stability of the erythrocyte pyruvate kinase are of special interest.

The presented kinetic data obtained with the oxidized enzyme are quite identical with the kinetic data described for most of the patients: the decreased affinities for phosphoenolpyruvate and Fru-1,6-P_2 (refs 6, 7, 9–12 and 23). Also the thermostability of the oxidized enzyme and the enzyme from pyruvate kinase-deficient patients seems identical[23]. Incubation of the oxidized enzyme with reduced glutathione only influences the kinetics obtained with the oxidized enzyme to a small extent and also the influence on the thermostability is suboptimal. This suggests that it is not the GSH/GSSG ratio which is important for the pyruvate kinase enzyme but only the GSSG concentration. However, since we applied only one GSH/GSSG ratio, the definite answer has to await further experimentation. Further support for the rôle of glutathione as a causative factor in most cases of pyruvate kinase deficiency is the description of several patients with both glutathione reductase and pyruvate kinase deficiency[17–19]. Deficiency in glutathione reductase increases the GSSG concentration, which affects pyruvate kinase. Also with three of the four pyruvate kinase-deficient patients described earlier by Staal and Koster[13,14], the glutathione reductase activity is lowered to 50% of controls with the same amount of young erythrocytes. Preliminary experiments with pyruvate kinase from these

patients suggest indeed that –SH groups are involved in the obtained abnormal kinetics (van Berkel, Th. J. C., Staal, G. E. J., Koster, J. F. and Nijessen, J. G., in preparation).

The question remains whether the obtained abnormal kinetics in pyruvate kinase-deficient patients are a reflection of the *in vivo* situation or a consequence of oxidation of the enzyme during the isolation procedure. The fact that in hemolysates from pyruvate kinase-deficient patients the enzyme is also altered, suggests that oxidation can occur in vivo. This would be a further support for the hypothesis[27] that –SH groups can play a rôle in the regulation of the pyruvate kinase activity *in vivo*.

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