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Characterization of 2,3-diphosphoglycerate phosphatase activity: electrophoretic study

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SUMMARY

Electrophoresis of 2,3-diphosphoglycerate phosphatase (EC 3.1.3.13) activity was carried out on starch gel.

The staining technique used the specific oxidation of NADH present in the incubation mixture. Thus, on a fluorescent background, the zones of migration of the enzyme appeared on the gel as bands of decreasing fluorescence.

The hemolysate of human origin or of different animal species gave, on migration, only one band of fluorescence decrease. In the case of heart tissue extracts two bands of activity appeared on the gel.

The distinction between 2,3-diphosphoglycerate phosphatase and 3-phosphoglycerate mutase (EC 2.7.5.3) activities has been obtained.

2,3-Diphosphoglycerate is the most abundant soluble organic phosphate in red cells. Since it plays an important role in the oxygen affinity of hemoglobin, the phosphatase which catalyses the hydrolysis of 2,3-diphosphoglycerate is of some interest. The general properties of erythrocyte 2,3-diphosphoglycerate phosphatase (EC 3.1.3.13) have been previously described[1], but its identification by electrophoresis, in hemolysates, has not been performed until now, most probably because this enzyme has a very low activity in red blood cells. Rose and Liebowitz[1] have demonstrated that this activity increases markedly in the presence of 2-phosphoglycolic acid. Starting from this point we were able to obtain sufficient activity to localize the enzyme on starch gel electrophoresis.

The present paper reports a simple method for detection of 2,3-diphosphoglycerate phosphatase on starch gel electrophoresis and its separation from 3-phosphoglycerate mutase (EC 2.7.5.3). This latter enzyme has been found, by several authors[2–4], to possess 2,3-diphosphoglycerate phosphatase activity. In the present work erythrocyte

2,3-diphosphoglycerate phosphatase activity from 200 human subjects and from some mammals has been studied.

Blood was collected in the presence of acid–citrate–dextrose solution. Hemolysates were made from washed red cells frozen and thawed three times and lysed with 1 vol. of distilled water and 0.5 vol. of toluene. Stroma were removed by centrifugation at 30 000 x *g* for 60 min at 4 °C.

Human white blood cells were prepared according to the method of Evans and Kaplan[5].

Human heart tissue extracts, obtained from two autopsies, were prepared by first homogenizing in saline solution and centrifuging at 30 000 x *g* for 30 min. The clear supernatant fluid was used for electrophoresis.

Starch gel electrophoresis was performed at 4 °C for 3 h in a horizontal system. Several buffers were tried: electrode compartments contained either 0.1 M triethanolamine-HCl buffer (pH 7.5) or 0.1 M Tris–HCl buffers of pH values between 8.0 and 9.0 or 0.1 M phosphate buffers of pH values 6.5 to 7.5.

An 11% starch gel was prepared in one-tenth dilution of electrode buffer. Samples were applied on rectangles of Whatman filter paper of 11 mm x 6 mm which were inserted into holes cut in the gel.

After completion of electrophoresis, the gel was sliced in two halves. One was stained for 2,3-diphosphoglycerate phosphatase activity and the other for 3-phosphoglycerate mutase activity.

(a) 2,3-Diphosphoglycerate phosphatase activity was detected on the gel, by using the fluorescence decrease due to oxidation of NADH to NAD^+, according to the following reactions:

$$\text{2,3-Diphosphoglycerate} \xrightarrow[\text{phosphatase}]{\text{2,3-diphosphoglycerate}} \text{3-Phosphoglycerate} + P_i$$

$$\text{3-Phosphoglycerate} + \text{ATP} \xrightarrow[\text{kinase}]{\text{phosphoglycerate}} \text{1,3-Diphosphoglycerate} + \text{ADP}$$

$$\text{1,3-Diphosphoglycerate} + \text{NADH} \xrightarrow[\text{dehydrogenase}]{\text{glyceraldehyde-3-phosphate}}$$

$$\text{Glyceraldehyde 3-phosphate} + NAD^+ + P_i$$

Details of the staining mixture are given in the legend of Fig. 1. The disappearance of NADH fluorescence of some zones of the gel was detected on illumination with ultraviolet light (approx. 340 nm) and indicated the areas to which 2,3-diphosphoglycerate phosphatase had migrated during electrophoresis. Photography was performed at the same wavelength, using a yellow filter and a Polaroid camera.

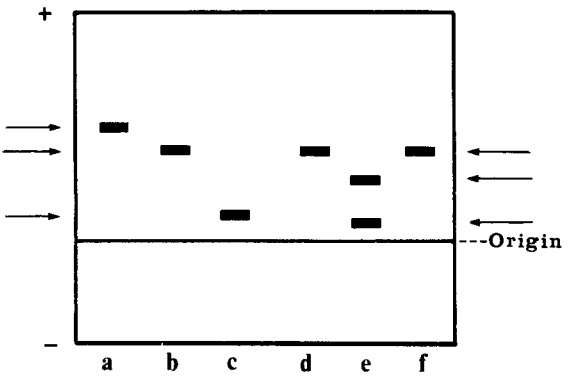

Fig. 1. Starch gel electrophoresis of 2,3-diphosphoglycerate phosphatase from: human (b, d, f), rat (c) and horse (a) erythrocytes and human heart tissue (e). The arrows indicate the position of 2,3-diphosphoglycerate phosphatase activity. Electrophoresis was performed in 0.1 M triethanolamine–HCl buffer (pH 7.5). The gel was incubated at 25 °C for approximately 60 min in a mixture of: 0.05 M triethanolamine–HCl buffer (pH 7.5); 2 mM 2,3-diphosphoglycerate; 3 mM Na_2ATP; 1 mM $MgCl_2$; 1.6 units per ml of glyceraldehyde-3-phosphate dehydrogenase; 1.6 units per ml of phosphoglycerate kinase and 0.25 mM NADH. 1 mM phosphoglycolic acid (Sigma) was used as an activator of 2,3-diphosphoglycerate phosphatase.

(b) 3-Phosphoglycerate mutase activity was revealed according to the following reactions:

$$\text{2-Phosphoglycerate} \xrightarrow[\text{mutase}]{\text{3-phosphoglycerate}} \text{3-Phosphoglycerate}$$

$$\text{3-Phosphoglycerate} + \text{ATP} \xrightarrow[\text{kinase}]{\text{phosphoglycerate}} \text{1,3-Diphosphoglycerate} + \text{ADP}$$

$$\text{1,3-Diphosphoglycerate} + \text{NADH} \xrightarrow[\text{dehydrogenase}]{\text{glyceraldehyde-3-phosphate}} \text{Glyceraldehyde 3-phosphate} + \text{NAD}^+ + \text{P}_\text{i}$$

Details of the staining mixture are given in the legend of Fig. 2.

The zone of migration of 3-phosphoglycerate mutase activity appeared on the gel as a band of fluorescence decrease due to transformation of NADH to NAD^+.

Fig. 1 shows the electrophoretic pattern of 2,3-diphosphoglycerate phosphatase activity. One zone of fluorescence decrease appeared on the gel within 30 to 60 min. This zone migrated towards the anode faster than hemoglobin, at all the pH values used between 6.5 and 9.0. In the 200 human subjects studied, the same electrophoretic pattern appeared on the gel for erythrocyte 2,3-diphosphoglycerate phosphatase activity. Cord blood erythrocyte 2,3-diphosphoglycerate phosphatase migrated to the same position as that of

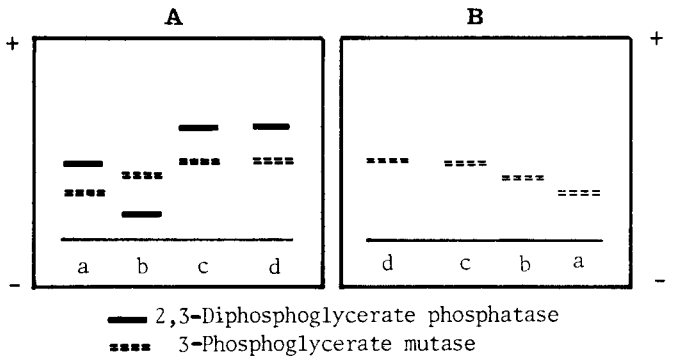

Fig. 2. Starch gel electrophoresis of erythrocyte 2,3-diphosphoglycerate phosphatase and of erythrocyte 3-phosphoglycerate mutase from: man (a), rat (b), horse (c) and pig (d). One side (B) of the gel was tested for 3-phosphoglycerate mutase activity. The other side (A) was tested for 2,3-diphosphoglycerate phosphatase activity; then, after its washing and blotting, it was used for the detection of 3-phosphoglycerate mutase activity. The staining mixture contained 0.05 M triethanolamine–HCl buffer (pH 7.5); 3 mM 2-phosphoglycerate; 1 mM 2,3-diphosphoglycerate; 1.6 units per ml of 3-phosphoglycerate kinase, 1.6 units per ml of glyceraldehyde-3-phosphate dehydrogenase; 0.25 mM NADH. All the enzymes and substrates came from Boehringer when not specified otherwise.

adult erythrocytes. Electrophoresis of hemolysates from pig, rat, rabbit, horse and dog also showed only one zone of 2,3-diphosphoglycerate phosphatase activity, but migrating to a different position for each species. No band of activity was seen for goat and sheep hemolysates.

In human white blood cells, no band corresponding to 2,3-diphosphoglycerate phosphatase activity was revealed.

On the contrary, in the case of tissue extract from human heart two bands of activity were visible. They migrated more slowly than the bands from human red cells and even appeared more quickly.

The fact that the zones of fluorescence decrease do in fact represent the areas of the gel to which 2,3-diphosphoglycerate phosphatase activity has migrated is supported by the following evidence:

(1) Omission of either 2,3-diphosphoglycerate or ATP or 3-phosphoglycerate kinase from the staining solution prevented the consumption of NADH. Thus, it is clear that NADH oxidation is linked to 2,3-diphosphoglycerate breakdown.

(2) 2,3-Diphosphoglycerate mutase cannot be responsible for this mechanism since Rapoport and Luebering[6,7] have given evidence that the reaction of 2,3-diphosphoglycerate mutase is not reversible.

(3) In order to exclude the possibility of unspecific reactions, we showed that inorganic phosphate, the second product of 2,3-diphosphoglycerate phosphatase reaction, was released. This was carried out after completion of electrophoresis by incubation of the gel for 60 min at 25 °C in a mixture of 0.05 M triethanolamine–HCl buffer (pH 7.5) and 2 mM 2,3-diphosphoglycerate. After blotting the gel, a mixture of phosphomolybdic acid and Gomori solution[8] was applied to the gel. Inorganic phosphate appeared as a slight blue

band which was localized at the same place as the band of fluorescence decrease appearing with the NADH staining method.

(4) Since purified 3-phosphoglycerate mutase from different sources has been said to possess 2,3-diphosphoglycerate phosphatase activity[2–4], it was also necessary to locate on the gel 2,3-diphosphoglycerate phosphatase activity with respect to 3-phosphoglycerate mutase activity. As can be seen in Fig. 2, in all species studied, at pH 7.5 the two red cell enzyme activities appeared as two distinct bands. Thus, our results are in agreement with those obtained by Rose and Liebowitz[1] who have purified human erythrocyte 2,3-diphosphoglycerate phosphatase from 3-phosphoglycerate mutase, and have only obtained one peak of activity of the former enzyme by chromatography.

On the other hand, our report does not confirm the results of Harkness *et al.*[9] who have found, by chromatography, two distinct peaks of 2,3-diphosphoglycerate phosphatase activity in human erythrocytes.

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