DIPHOSPHOGLYCERATE MUTASE AND 2,3-DIPHOSPHOGLYCERATE PHOSPHATASE ACTIVITIES OF RED CELLS : COMPARATIVE ELECTROPHORETIC STUDY

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SUMMARY - Erythrocyte diphosphoglycerate mutase (EC 2.7.5.4.) and 2,3-diphosphoglycerate phosphatase (EC 3.1.3.13.) activities of normal human adults, and DPG mutase deficient subject as well as of several animal species were subjected to electrophoretic study on starch gel. In U.V. light 2,3-diphosphoglycerate phosphatase activity was revealed as a band of fluorescence decrease on a fluorescent background, by the oxydation of NADH, whereas diphosphoglycerate mutase appeared as a fluorescent zone. It was found that the electrophoretic pattern of both DPG mutase and 2,3-DPG phosphatase activities was different from one species to the other, but that, in each species, 2,3-DPG phosphatase activity showed the same electrophoretic pattern as DPG mutase activity.

INTRODUCTION - The synthesis and breakdown of 2,3-diphosphoglycerate (2,3-DPG) constitute a bypass of the phosphoglycerate kinase step of glycolysis. Rapoport has demonstrated that, in red cells, this shunt is effected by two specific enzymes : diphosphoglycerate mutase (1)(DPG mutase)(EC 2.7.5.4.) and 2,3-diphosphoglycerate phosphatase (2)(2,3-DPG phosphatase)(EC 3.1.3.13.). These two enzymes have been purified from human red blood cells (RBC) and their properties have been studied by Z. Rose (3,4,5).

The electrophoretic pattern of human erythrocyte diphosphoglycerate mutase has been studied by Chen et al. (6). We described before (7) the technique of 2,3-DPG phosphatase electrophoresis in using, according to Z. Rose (5), the highly activating power of 2-phosphoglycolic acid. Here, in this paper, we give a report of a comparative study of the electrophoretic pattern of these two enzymes obtained from red cell hemolysates of man and several animal species.

EXPERIMENTAL - Erythrocyte DPG mutase and 2,3-DPG phosphatase present in blood hemolysates have been studied. Hemolysates from several animal species such as goat, sheep, rabbit, dog, pig, rat, chicken, as well as hemolysates from adult humans, were subjected to such study. In all cases, quantitative enzymatic assays were carried out concomitantly with the electrophoretic study of these enzymes. Hemolysates were prepared by washing red cells of fresh blood samples collected on heparin. These R.B.C, were then subjected to a process of freezing and thawing which was repeated for three times, and lysed with one volume of distilled water and a half volume of toluene. Stroma was eliminated by centrifugation at 30 000 x g for 60 min. at 4°C, leaving clear hemolysates.

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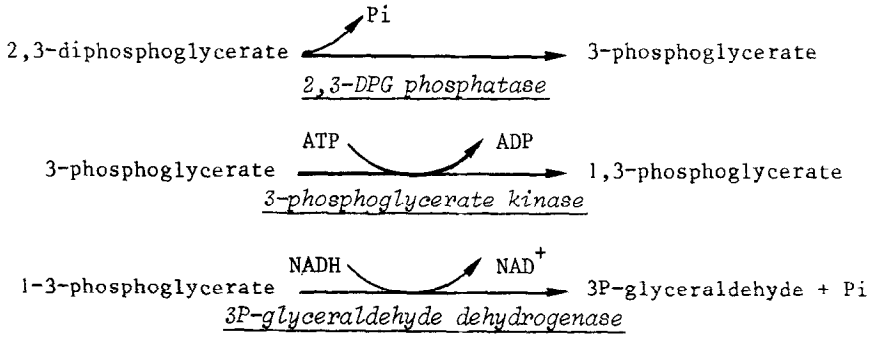
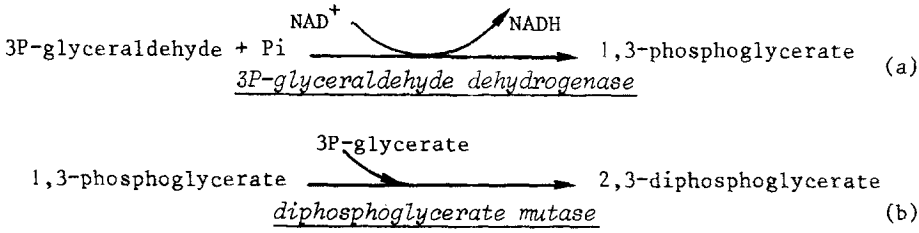
2,3-DPG phosphatase activity was determined according to Joyce and Grisolia (8) by a colorimetric determination of the released inorganic phosphate. DPG mutase activity was measured spectrophotometrically at 340 nm according to Schröter and Kalinowsky (9). Starch gel electrophoresis was performed at 4°C for 3 hours at 8 volts per cm, in a horizontal system. Several electrode buffers were tried of the following composition : either 0.1 M triethanolamine - HCl buffer pH 7.5 or 0.1 M Tris - HCl buffers of pH values between 8.0 and 9.5 or 0.1 M phosphate buffers of pH values between 6.5 and 7.5. A 11% starch gel was prepared in one tenth dilution of the electrode buffer. Samples were applied to rectangles of Whatman filter paper of 11 x 6 mm which were inserted into a sharp vertical cut in the gel. After completion of electrophoresis, the gel was horizontally sliced in two halves, one was stained for 2,3-DPG phosphatase activity and the other for DPG mutase activity. 2,3-DPG phosphatase activity was detected according to the technique recently described (7), by using the fluorescence decrease due to oxydation of NADH to NAD^+ according to the following sequence :



It can be realized that the NADH oxydation depends on 2,3-DPG phosphatase activity. Since the incubating mixture contains NADH (see legend figure 1), on a fluorescent background, on U.V. light, the zones of migration of the 2,3-DPG phosphatase activity appear on the gel as dark bands showing decreasing fluorescence. 2,3-DPG mutase activity was revealed according to the method described by Chen et al. (6). Their method is based on the following reactions :



Since there is no ADP in the staining mixture (see legend of figure 1), the 1,3-phosphoglycerate formed by the step (a) attains quickly a level sufficient to stop the reaction, i.e. to stop any more the reduction of NAD^+

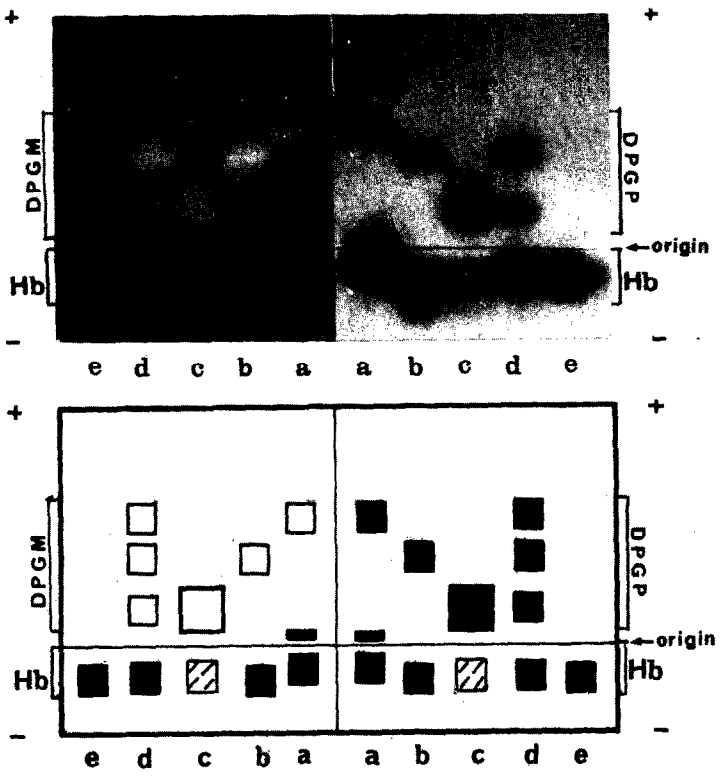


Figure 1 - Starch gel electrophoresis, in 0.1 M triethanolamine-HCl buffer pH 7.5, of DPG mutase (DPGM) and of DPG phosphatase (DPGP).
Red cell lysates of horse (a), man (b), rat (c), a mixture of horse, man and rat (d), and sheep (e) were tested. Details of migration are given in the text. For the detection of the DPG mutase activity, the gel was incubated at 25°C in a staining mixture prepared according to Chen et al. (6) and containing : 0.1 M Tris-HCl buffer pH 7.8, 3.5 mM glyceraldehyde 3-phosphate, 4 mM 3-phosphoglycerate, 10 mM K_2HPO_4, 1.5 mM NAD^+ and approximately 1 unit per ml of glyceraldehyde 3-phosphate dehydrogenase.

For 2,3-DPG phosphatase activity the gel was incubated at 25°C for approximately one hour in a mixture of 0.05 M triethanolamine-HCl buffer pH 7.5, 2 mM 2,3-DPG, 1 mM ATP, 1 mM $MgCl_2$; 0.25 mM NADH, 1.6 U/ml glyceraldehyde 3-phosphate dehydrogenase, 1.6 U/ml 3-phosphoglycerate kinase(7). 1 mM 2-phosphoglycolic acid was used as an activator of 2,3-DPG phosphatase.

On the two gel slices, the dark bands moving slightly towards the cathod are hemoglobin (Hb). On the left side, fluorescent bands are indicative of DPG mutase activity, whereas on the right side the corresponding dark bands represent DPG phosphatase activity of the same lysates. Neither DPG mutase nor DPG phosphatase appears in the case of sheep hemolysate.

to NADH, which begins to take place once the produced 1,3-phosphoglycerate is consumed by the second step (b) of the reaction. Consequently the zones

of migration of DPG mutase activity correspond to the bands of fluorescence appearing on the gel. After staining, the two halves of the gel were illuminated with U.V. light (approx. 340 nm) and zones of fluorescence, or fluorescence decrease, indicated respectively the areas to which DPG mutase and 2,3-DPG phosphatase had migrated during electrophoresis. Photography was accomplished at the same wave-length, using a yellow filter and a Polaroid camera.

RESULTS AND DISCUSSION - Figure 1 shows the comparative electrophoretic pattern of the 2,3-DPG phosphatase and DPG mutase activities of red cell hemolysates from horse, man, rat and sheep. Electrophoresis was performed at pH 7.5. Conditions of staining are given in the legend of figure 1. The slice of the gel tested for DPG mutase activity (left side of the figure) shows the zones of migration of this activity as fluorescent bands. Hemolysate of each of the tested species shows only one zone of fluorescence which migrated faster than hemoglobin, towards the anode. At the pH of our experiment the following data were obtained. In case of rat, the band corresponding to DPG mutase activity migrated slower than that of humans, in case of horse it migrated faster than that of humans, while in case of sheep, no band of DPG mutase activity was obtained. The other slice of the gel, tested for the 2,3-DPG phosphatase activity is seen on the right side of the figure 1. The zones of activity of the 2,3-DPG phosphatase appear as dark bands on a fluorescent background. The pattern of the electrophoretic mobility of the bands observed on this slice of the gel is exactly similar to that obtained on the other slice of the gel tested for the DPG mutase activity.

The possibility of artefacts at the level of revealing the DPG mutase activity was eliminated by the control experiments accomplished by Chen et al. (6). On the other hand, we afforded some pieces of evidence for eliminating the artefacts at the level of detecting the 2,3-DPG phosphatase activity (7). Several buffers have been tried of different pH values between 6.5 and 9.0. In every case the pattern of both enzymatic activities were strictly identical and always one and the same band of 2,3-DPG phosphatase and of DPG mutase was obtained for each species. In order to confirm the presence of 2,3-DPG phosphatase at the same level at which migrated the DPG mutase activity, we carried out the following experiment : after detection of the DPG mutase activity on the two slices of the gel, they were washed with distilled water. Then, one slice of the gel was blotted and reincubated with the staining mixture of 2,3-DPG phosphatase activity but without the addition of 2,3-DPG and NADH normally present in this mixture. Under these conditions we observed a gradual decrease of fluorescence of the DPG mutase fluorescent bands. At the same time, the second slice of the gel control, which was tested for the DPG

mutase activity and washed with distilled water did not show any decreasing fluorescence which led to an easy comparison. Our explanation is that 2,3-DPG as well as NADH produced during the detection of the DPG mutase activity served as substrates in the sequence of reactions detecting the 2,3-DPG phosphatase activity.

The electrophoretic study of both DPG mutase and 2,3-DPG phosphatase activities of other animal species have been performed. Two different results were obtained. In the case of the dog and the pig as well as man, horse and rat, both enzymatic activities could be revealed on the gel, whereas in the case of the chicken and the goat as well as sheep, no enzymatic activity could be revealed. It must be precised that goat, sheep and chicken possess a sufficiently low content of 2,3-DPG in their red cells (10,11), which conforms with the results obtained by quantitative enzymatic assays of DPG mutase and 2,3-DPG phosphatase, which indicated that the activities of these enzymes were too low to be detected in our conditions. These results led us to examine the 2,3-DPG phosphatase activity of a human subject, recently described as being partially deficient in red cell DPG mutase activity (12). The quantitative assays carried out with RBC of that subject gave the following results : 2,3- DPG : 1.5 µmoles/ml RBC (normal = 4 $\pm$ 0.5 µmoles), DPG mutase : 0.55 U/ml RBC (normal = 1.27 $\pm$ 0.19 U), 2,3-DPG phosphatase : 0.29 U/ml RBC (normal = 0.56 $\pm$ 0.077 U). Thus we conclude from the precited data that this subject has a partial and equal deficiency of both DPG mutase and 2,3-DPG phosphatase activities. Carrying out an electrophoretic study of DPG mutase and 2,3-DPG phosphatase of red blood cell hemolysate of this subject, we found an exactly identical electrophoretic mobility as for the normal subject.

In brief, both 2,3-DPG phosphatase and DPG mutase, present in blood hemolysate of each of the tested animal species and of the human mutant, were found to have the same electrophoretic mobility on gel electrophoresis. Such results lead to take into consideration the following hypotheses.

The first hypothesis is that 2,3-DPG phosphatase and DPG mutase represent two distinct molecular species having the same isoelectric point and could not be separated at least by our electrophoretic techniques. However, it was found that the electrophoretic mobility of both enzymes was different from one species to the other (fig.1) ; such difference in electrophoretic mobility is the proof of several mutations which led to some modifications of the structure of these enzymes during the phylogenetic evolution steps. It is improbable that such mutations exhibit the same modifications of structure leading to a similar charge carried by both enzymes in all tested species.

The second hypothesis is that 2,3-DPG mutase and 2,3-DPG phosphatase are

two distinct proteins carrying different electric charges but migrate together, because they are bound to a multienzyme complex as described for fatty acid synthetase complex and the electron carriers systems.

But such hypothesis may be discussed for two reasons : the first is that such an association is **not likely** to occur in cytoplasmic soluble enzymes ; the second is that the fact that a deficiency of one of these enzymes was systematically accompanied by a simultaneous deficiency of the other, as it was found in case of sheep, goat and chicken as well as a deficient human subject, cannot be explained unless we admit the same genetic control of both enzyme activities.

The last and most probable hypothesis is that DPG mutase and 2,3-DPG phosphatase activities represent two active sites on the same molecule as it was previously described for the threonine-sensitive homoserine dehydrogenase (13). Our results do not oppose those of Z. Rose who has effected independant purification of red cell DPG mutase (3) and 2,3-DPG phosphatase (5). She succeeded to obtain a preparation of each of these two enzymes free from the 3-phosphoglycerate mutase activity, but a checking test of the purified preparation of these two enzymatic activities on the purified preparation of the other, apparently was never attempted by Z. Rose.

Until now, no experimental evidence could oppose to our hypothesis that both DPG mutase and 2,3-DPG phosphatase represent two active sites on the same molecule ; which lead us to continue our research in that direction.

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