Molecular Aging and Phosphorylation of Erythrocyte Pyruvate Kinase

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ABSTRACT

Phosphorylation of two species of erythrocyte pyruvate kinase (PK, EC 2.7.1.40), PK-R_1 and PK-R_2, was analyzed. The light fraction of younger red cells which has a low PK-R_2/PK-R_1 ratio, and the dense fraction of older cells which has a high PK ratio were used as the enzyme source. On phosphorylation in the presence of cyclic AMP, PK of the dense fraction showed a larger amount of ^{32}P incorporation than that of the light fraction. Most of the radioactivity incorporated was recovered in the L' subunit of PK. The unphosphorylated PK of the dense fraction had a lower affinity for phosphoenolpyruvate (PEP) than that of the light fraction. Phosphorylation lowered the affinity of both fractions for PEP and the phosphorylated PK of the dense fraction still showed a lower affinity for PEP than that of the light fraction. These results indicate that PK-R_2 is more readily phosphorylated than PK-R_1 and the activities of both PK-R_1 and PK-R_2 are controlled by phosphorylation with a decrease of the affinity for PEP. The postsynthetic transformation of PK-R_1 into PK-R_2 is considered as a maturation process or molecular aging of the enzyme which involves protein degradation in erythrocytes.

Key words: erythrocyte; pyruvate kinase; phosphorylation

INTRODUCTION

Red blood cells are very unique tissue, because practically no proteins are synthesized once the cell has matured out of the reticulocyte stage. During aging of the red cell, its flexibility and mechanical and osmotic fragility decreased together and most of the enzymes including pyruvate kinase (PK, EC 2.7.1.40) decline in their activities[1]. Erythrocyte PK of the mature red cell is separated into 2 bands, PK-R_1 and

PK-R_2, electrophoretically, and the PK-R_2/PK-R_1 ratio increases during cell aging[2]. PK-R_2 has a higher Km and is more stable than PK-R_1[2]. Kahn et al. proposed that PK-R_1 is composed of 4 identical subunits (L'_4) and the L'_4 form is transformed by partial proteolysis during red cell aging into L'_2L_2 (PK-R_2)[3]. The L-type PK of the liver composed of 4 L subunits (L_4), which has kinetic, immunological and structural similarities to erythrocyte PK, is said to arise from its precursor enzymes, PK-R_1 and PK-R_2, by the drastic proteolytic system in the liver. They reported that the transformation of L'_4 into L_4 is mainly associated with sequential changes of the kinetic behavior of the enzyme and L'_2L_2 shows intermediate behavior between L'_4 and L_4[4]. However, the details and the physiological significance of the transformation of PK-R_1 to PK-R_2 and its sequential change of PK properties are not completely understood.

Marie et al. and we reported the intensive incorporation of ^{32}P into erythrocyte PK in the presence of cyclic AMP[5,6]. We also reported that the phosphorylated PK exhibited a lower affinity for phosphoenolpyruvate (PEP) than the unphosphorylated PK[6] and the phosphorylation could be involved in the physiological regulatory mechanism of erythrocyte PK. It can, therefore, be expected that two species of PK of erythrocyte, PK-R_1 and PK-R_2, differ in their ability to accept phosphate moiety and the phosphorylated forms change their catalytic activities. This type of analysis may give a clue to molecular aging of PK in erythrocyte. As reported previously[6], a crude sample containing PK fraction is used in our phosphorylation experiment and the recovery method of PK from the sample is simple and mild so we can minimize the structural change of PK accompanied by experimental procedures. Moreover, on the SDS-polyacrylamide gel electrophoresis of PK we can differentiate the L subunit from the L′ subunit. Therefore, our method is of use to analyze the relationship among PK-R_1, PK-R_2 and their subunits from the standpoint of phosphorylation. Using our method, this paper presents the results related to the phosphorylation and kinetic property of PK-R_1, PK-R_2 and their subunits.

MATERIALS AND METHODS

Light and dense fractions of red cells that include the younger and older cells, respectively, were obtained by the method described by Nakashima[2]. [γ-^{32}P]ATP was prepared from ADP and ortho[^{32}P]-phosphate by the method of Beutler et al.[7] Histone kinase was assayed according to Hosey et al.[8] PK was assayed by a slight modification of

the method of Beutler[9], as reported previously[6]. Thin layer polyacrylamide gel electrophoresis of erythrocyte PK and the detection of PK activity on the gel were performed according to Imamura et al.[10] SDS-polyacrylamide gel electrophoresis of erythrocyte PK was performed as described by Fairbanks et al.[11]

The partially purified samples containing cytosol protein kinase and PK were fractionated from the light and dense red cells by 15-40% saturation of ammonium sulfate[6] and used for the phosphorylation experiment. The partially purified PK showed the same electrophoretic pattern as that of the PK of fresh hemolysate.

The homogeneous PK was obtained by the modified method of Harada et al.[12], as follows:
Human blood was drawn with heparin and the white blood cells were removed carefully. Red cells were washed and hemolyzed. The fractionated sample by 15-40% saturation of ammonium sulfate, which showed the same electrophoretic pattern of PK as that of hemolysate, was applied on CM Sephadex column. PK was eluted with the phosphate buffer containing 2 mM PEP and 2 mM fructose-1, 6-diphosphate. The eluted PK showed a different electrophoretic pattern from that of hemolysate. All purification procedures were performed within 3 days at 4°C. The phosphate buffer in our experiment contained 2 mM 6-amino-n-caproic acid and 0.2 mM phenylmethylsulfonyl fluoride to get the homogeneous PK migrating as PK-R_2. Homogeneous PK-R_2 was immediately served for SDS-polyacrylamide gel electrophoresis.

Phosphorylation of erythrocyte PK was performed using the partially purified samples containing protein kinase and PK and [γ-^{32}P]ATP, as reported previously[6]. The PKs of light and dense fractions were completely dephosphorylated before phosphorylation experiment according to the previous report[6]. Erythrocyte PK was recovered by double antibody technique and the resulting immune complex containing erythrocyte PK was subjected to SDS-polyacrylamide gel electrophoresis. The stained and dried gel was used for autoradiography. Radioactivities incorporated into PK species were compared by scanning the autoradiogram using a Gilford 2400-S spectrophotometer at 600 nm.

RESULTS

As shown in Fig. 1, SDS-polyacrylamide gel electrophoresis of the purified PK-R_2 showed 2 bands, L′ and L subunits designated by Kahn et al[3]. On scanning Coomassie Blue stained gel at 600 nm, the density of the electrophoretic band of L′ subunit was 2.5 times of that of L

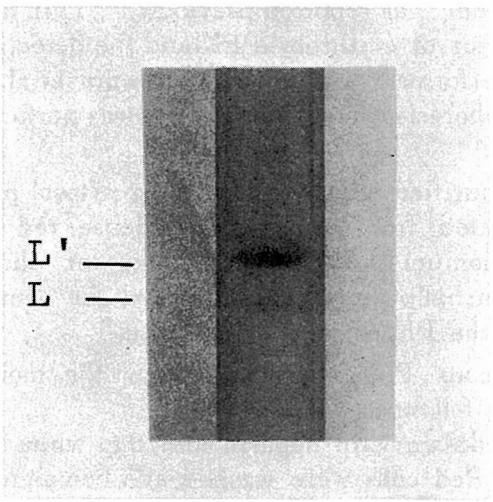

Fig. 1. SDS-polyacrylamide gel electrophoresis of purified PK-R_2. L′ and L are L′ subunit and L subunit of erythrocyte PK, respectively

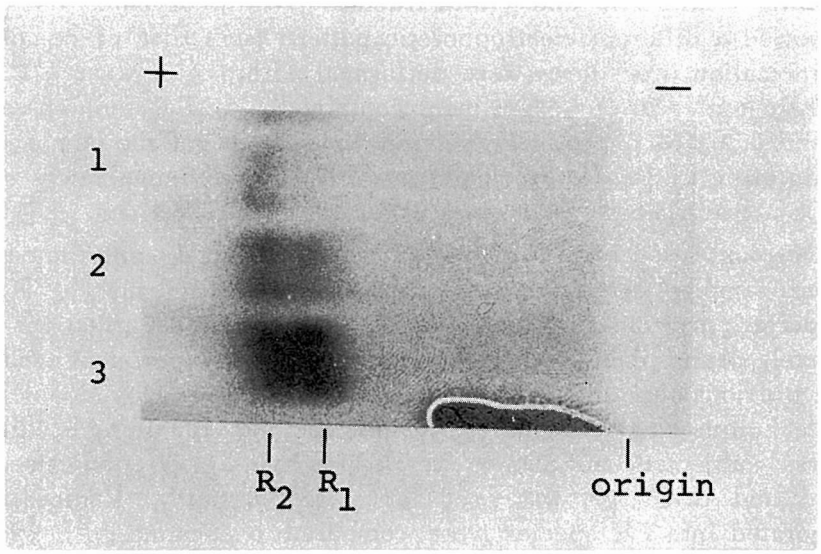

Fig. 2. Thin layer polyacrylamide gel electrophoresis of fresh hemolysate from dense, middle and light fractions of red cells. 1: erythrocyte PK of dense fraction; 2: erythrocyte PK of middle fraction; 3: erythrocyte PK of light fraction. R_1 and R_2 are PK-R_1 and PK-R_2, respectively.

subunit.

Thin layer electrophoretic patterns of PKs of fresh hemolysates from light, middle and dense fractions were shown in Fig. 2. Light fraction

with a 6.2% reticulocyte count showed a lower R_2/R_1 ratio (Fig. 2-3), while dense fraction with a 0.3% reticulocyte count showed a higher R_2/R_1 (Fig. 2-1). Middle fraction revealed an intermediate ratio between light and dense fractions (Fig. 2-2).

Using partially purified samples obtained from light and dense fractions shown in Fig. 2, the phosphorylation of erythrocyte PK was examined (Fig. 3). PK activity/g Hb of the dense fraction was 48% of that of the light fraction. Before starting phosphorylation, PK activities of both samples were adjusted to 3.7 U/ml. The intensive incorporation of ^{32}P into both PKs of light (Fig. 3A) and dense (Fig. 3B) fractions was detected at L′ position in the presence of cyclic AMP. Radioactivity /U PK was expressed with radioactivity of the light fraction in the absence of cyclic AMP as 1.0. In the presence of cyclic AMP, radioactivity/U PK of the dense fraction was higher than that of the light fraction. In addition, radioactivity/g Hb was obtained by using the quantitative relationship of PK activity/g Hb between light and dense fractions and expressed with radioactivity of the light fraction in the absence of cyclic AMP as 1.0.

$$\text{Radioactivity/g Hb of dense fraction : Radioactivity/g Hb of light fraction} = \frac{\text{Radioactivity/U PK of dense fraction}}{\text{Radioactivity/U PK of light fraction}} \times \frac{\text{PK activity/g Hb of dense fraction}}{\text{PK activity/g Hb of light fraction}}$$

Radioactivity/g Hb of the dense fraction was also higher than that of the light fraction in the presence of cyclic AMP.

On the other hand, at L position, only subtle radioactivity was detected in the presence of cyclic AMP in the light fraction (Fig. 3 A), but not in the dense fraction (Fig. 3 B).

Using these partially purified samples of both fractions the effect of phosphorylation on PK activities was shown in Fig. 4. In the absence of ATP, $K_{0.5S}$ (PEP) of unphosphorylated PK was higher in the dense fraction than in the light fraction. $K_{0.5S}$ (PEP) of PKs of light and dense fractions was elevated by phosphorylation and $K_{0.5S}$ of the phosphorylated PK of the dense fraction was higher than that of the light fraction. In the presence of 0.4 mM ATP the unphosphorylated PK of the dense fraction revealed a higher $K_{0.5S}$ (PEP) than that of the light fraction, and the phosphorylation elevated $K_{0.5S}$ of both fractions and the phosphorylated PK of the dense fraction showed a higher $K_{0.5S}$ than the phosphorylated PK of the light fraction.

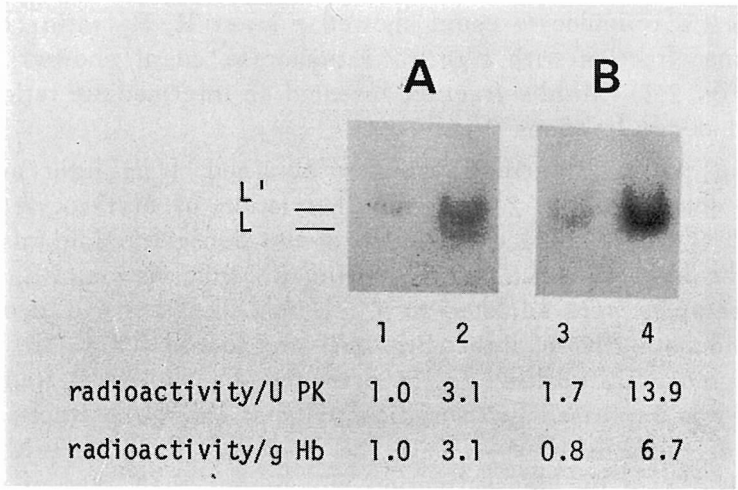

Fig. 3. Autoradiogram of SDS-polyacrylamide gel electrophoresis of phosphorylated erythrocyte PKs from light (A) and dense (B) fractions. L′ and L are L′ subunit and L subunit of erythrocyte PK, respectively. 1, 3 : cyclic AMP (−); 2, 4 : cyclic AMP (+).

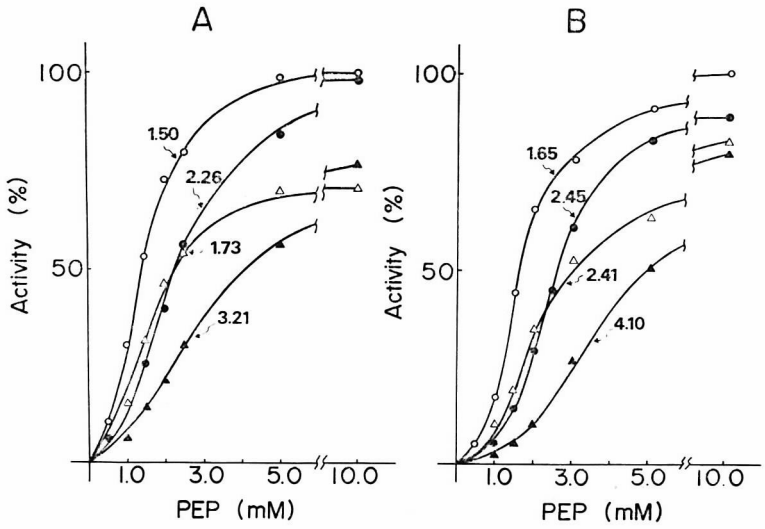

Fig. 4. Kinetic study of phosphorylated PKs from light (A) and dense (B) fractions. Open and solid symbols represent unphosphorylated and phosphorylated PKs, respectively. Assays were conducted in the absence (○, ●) and the presence (△, ▲) of 0.4 mM ATP. $K_{0.5S}$ is indicated in each curve.

DISCUSSION

The most reliable way to study the structure of erythrocyte PK is

to use the homogeneously purified enzyme. However, there have been many reports that erythrocyte PK showed a change in the electrophoretic pattern after purification, though it preserves kinetic and immunological properties similar to the native enzyme. Erythrocyte PK used in Fig. 1 revealed a single band migrating as PK-R_2 after complete purification, even though it showed 2 bands, PK-R_1 and PK-R_2, in hemolysate or in the sample fractionated by 15-40% saturation of ammonium sulfate. As far as we experienced, it was difficult to purify erythrocyte PK as PK-R_2 using the original method[12]. In most cases it migrated as L-type PK after purification. Our modification of the method made it possible to purify erythrocyte PK as PK-R_2. These observations suggest the structural change during purification. Thus, we used the partially purified sample in which erythrocyte PK showed the same electrophoretic pattern as that of the fresh hemolysate. The double antibody technique used here was valid to collect erythrocyte PK selectively from the partially purified sample[6]. Therefore, using the partially purified samples from the light and dense fractions of red cells, we could compare the phosphorylation between PK-R_1 and PK-R_2.

As shown in Fig. 3, radioactivity/U PK of the dense fraction is higher than that of the light fraction in the presence of cyclic AMP. PK activity/g Hb of the dense fraction was 48% of that of the light fraction. During red cell aging the degradation of erythrocyte PK might occur. This degradation might induce enzyme inactivation, but have no influence on the phosphorylatable site and immunological reactivity. When degradated PK lacks its activity but preserves phosphorylatable sites, radioactivity/U PK should considerably increase. The mature red cells are unable to synthesize new proteins and the hemoglobin content in the red cell remains constant throughout the life of the cell[13,14]. Therefore, radioactivity/g Hb is considered more reliable. As shown in Fig. 3, radioactivity/g Hb of the dense fraction was higher than that of the light fraction, indicating that PK-R_2 is more intensively phosphorylated than PK-R_1. Histone kinase activity/g Hb of the light fraction was about 3 times higher than that of the dense fraction, which may rule out the possibility of the lower ^{32}P incorporation in the light fraction due to the lower protein kinase activity.

As shown in Fig. 4, $K_{0.5S}$ (PEP) of both PK-R_1 and PK-R_2 was elevated by phosphorylation. In other words, erythrocyte PK preserves its property to be controlled by phosphorylation even after its transformation into PK-R_2. PK-R_2 is more intensively phosphorylated than PK-R_1, as discussed above. In the preliminary experiment, phosphorylation made erythrocyte PK more stable. Therefore, it is possible that PK-R_1

is transformed to PK-R_2 the activity and stability of which are more readily controlled by phosphorylation.

In addition, both unphosphorylated and phosphorylated PKs of the dense fraction were inhibited by 0.4 mM ATP, like unphosphorylated and phosphorylated PKs of the light fraction, as shown in Fig. 4. ATP controls PK activity in the red cell as an allosteric inhibitor[5]. Our results indicate that PK-R_2 as well as PK-R_1 are able to be controlled by ATP both in the unphosphorylated and phosphorylated forms.

According to the postsynthetic transformation theory proposed by Kahn et al.[3], PK-R_1 is composed of 4 identical subunits (L'_4) and the L'_4 form is transformed by partial proteolysis into the L'_2L_2 form (PK-R_2). They showed that purified PK-R_2 was dissociated by SDS into two types of subunits, one migrating as L′ subunit, the other as L subunit. As shown in Fig. 1, the homogeneously purified PK migrating as PK-R_2 was actually dissociated by SDS into 2 bands. However, the electrophoretic band of L′ was denser than that of L′ which contradicts their hypothesis that PK-R_2 is composed of 2 L′ and 2 L subunits.

As discussed above, PK-R_2 is composed of L′ and L subunits, though the ratio of L′/L in PK-R_2 is higher than that expected on the postsynthetic transformation theory. As shown in Fig. 3 B, no radioactivity was detected at L position in the presence of cyclic AMP, even though the dense fraction had a high R_2/R_1 ratio as shown in Fig. 2. As protein kinases transfer the terminal phosphoryl group from ATP to a seryl or threonyl hydroxyl group of a protein, this result may be due to the lack of such phosphorylatable sites in the L subunit or the conformation of the L subunit which affects these phosphorylatable sites. The interaction among the subunits of PK-R_2 may lower the ability of the L subunit of PK-R_2 to be phosphorylated. Likewise, a larger amount of ^{32}P incorporation into L′ position of the dense fraction than into that of the light fraction in the presence of cyclic AMP may be explained by the subunit interaction of PK-R_2 or the conformation of L′ subunit.

Considering red cell aging, the lower affinity of PK-R_2 for PEP than of PK-R_1 may result from the degradation of the enzyme accompanied by molecular aging. The postsynthetic transformation may be in this sense considered as an inevitable and passive process. However, during the 120-day life span of the red cell, no new enzyme is synthesized. Therefore, this process may have an active role in maintaining red cell metabolism. As discussed above, PK-R_2 preserves its properties to be phosphorylated and to be controlled by ATP, or rather PK-R_2 is more phosphorylatable and more stable than PK-R_1. That is to say, PK-R_1 is transformed into PK-R_2 to maintain red cell metabolism during its life

span, the activity and stability of which are more readily controlled by phosphorylation. Thus, the postsynthetic transformation of PK-R_1 into PK-R_2 may be considered as a maturation process of the enzyme or molecular aging of the enzyme which involves protein degradation in erythrocytes.

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