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Proteolytic processing of human erythrocyte pyruvate kinase : Study of normal and deficient enzymes

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SUMMARY

The L' subunits, phosphorylatable precursors of L-type pyruvate kinase, can be proteolyzed in vivo and in vitro, and transformed into several molecular species.

Trypsin induces the appearance of a phosphorylatable form similar to liver L-type enzyme.

Subtilisin splits the phosphorylatable site and induces the appearance of a form similar to the minor form occuring with red cell aging.

Sensitivity to proteolysis of some pyruvate kinase variants is modified.

INTRODUCTION

We have previously shown that erythrocyte pyruvate kinase was synthesized in the erythroblasts under the form of a precursor L'_4 molecule which could be transformed by a mild tryptic attack into a L_4 tetramer similar to liver L-type enzyme (1,2). In the mature red cells the L'_4 enzyme coexists with a heterotetramer composed of 2 L' and 2 partially proteolysed L-type subunits (1). The proteolytic maturation of L'_4 into L_4 has been shown to cause a marked improvement of the regulatory properties of L-type pyruvate kinase (1,3 and Sprengers et al, in preparation). Besides, Bergström et al have reported that liver L-type pyruvate kinase is sensitive to subtilisin which splits the phosphorylatable site and provokes a dramatic shift toward an allosteric T form (4,5). In our laboratory we have found that subtilisin induces the same phenomena with L'_4 than with liver L-type enzyme (6, and unpublished data). Finally, we have reported in previous papers that some mutant pyruvate kinase variants exhibited an altered sensitivity to in vivo and in vitro proteolytic processing (3,7,8).

The goal of this work was, therefore, to characterize further the different types of proteolytic cleavages of normal and mutant pyruvate kinase variants.

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METHODS

Pyruvate kinase from control and patient red cells was purified by the 2-step method reported in (1,8). The overall yield of this procedure was from 30-60 % ; it was obviously lower for the unstable variants than for normal pyruvate kinase. For three patients we started from blood enough (20 to 50 ml) to purify pyruvate kinase before and after treatment with trypsin. In ten other cases in which not enough starting material was available, enzyme was only purified after tryptic treatment.

The $PK\text{-}R_1$ and $PK\text{-}R_2$ forms (9,10) from normal erythrocytes were separately purified as follows : after $(NH_4)_2SO_4$ fractionation (1,8,11) enzyme was fixed on Blue Dextran Sepharose 4B equilibrated with a 50 mM Tris/HCl buffer (pH 7.5)/40 mM KCl/2 mM EDTA/2 mM β-mercaptoethanol/0.1 mM diisopropylfluorophosphate. The column was washed with this buffer, KCl concentration of which was raised to 60 mM, until all absorbance at 280 nm has disappeared.

The absorbent was then equilibrated against the Tris/HCl buffer without KCl, and pyruvate kinase was eluted by fructose 1,6 P_2 and a linear gradient of ionic strength between 6 volumes of the Tris/HCl buffer and 6 volumes of the same buffer plus 60 mM KCl, both solutions containing 0.02 mM fructose 1,6 P_2 . Two peaks of activity were clearly separated ; it was proven by polyacrylamide slab gel electrophoresis (1,9,10) that they corresponded to $PK\text{-}R_2$ and $PK\text{-}R_1$ respectively.

Proteolytic attack of the enzymes by trypsin (1-3) and subtilisin (4,5) was carried out as described previously, using the preparations fractionated by $(NH_4)_2 SO_4$ precipitation. The preparations were usually treated for 30 min at 37° with 10 µg/ml trypsin or 0.25 µg/ml subtilisin.

Phosphorylation of pyruvate kinase was performed either on whole red cells, as recently described (6), or using a partially purified preparation of pyruvate kinase and of soluble cyclic AMP-dependent red cell protein kinases (Marie et al, in preparation). Phosphorylated enzyme was studied by autoradiography.

SDS-polyacrylamide slab gel electrophoresis was performed according to Laemli (12), except that the running gel was a 9-24 % (W/V) polyacrylamide gradient gel. Preparation of the gels and electrophoresis were performed using "Uniscil" apparatus (Universal Scientific Limited, London, GB). The molecular weights (Mr) were determined using rabbit muscle phosphorylase and aldolase, human muscle phosphofructokinase, bovine serum albumin, human leukocyte glucose phosphate isomerase and bovine liver catalase as standards.

RESULTS

1. Subunit structure of the different forms resulting from in vivo and in vitro proteolysis of L'_4 (figure 1)

After separation by chromatography on Dextran Blue-Sepharose column, $PK\text{-}R_2$ (first peak) and $PK\text{-}R_1$ (second peak) were analyzed by SDS-polyacrylamide gel electrophoresis. As already reported (1), $PK\text{-}R_1$ is only composed of L' subunits (Mr : 63000) while $PK\text{-}R_2$ is composed of two major types of subunits, L' and a lighter form of 57000-58000 daltons ; a very faint protein band can also be detected in an intermediate position (fig. 1). In the normal red cells, L' represents about 85 % of the total subunits.

Subtilisin converts L' into subunits with similar molecular weight to the

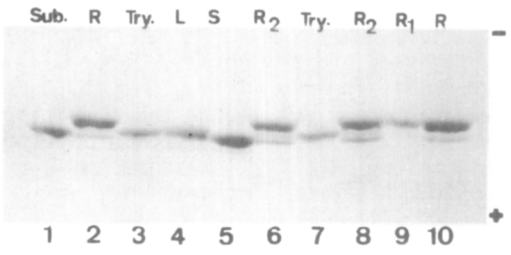


Fig. 1 : SDS-polyacrylamide gradient gel analysis of various forms of human L-type pyruvate kinase.
5 µg protein per sample, staining with coomassie blue.
1 and 5 : subtilisin-treated red cell enzyme ; 2 and 10 : unfractionated red cell enzyme ; 3 and 7 : trypsin-treated red cell enzyme ; 4 : liver L-type enzyme ; 6 and 8 : erythrocyte enzyme, peak 1 ; 9 : erythrocyte enzyme, peak 2.
Peak 1 of red cell pyruvate kinase is mainly composed of PK-R2 while peak 2 corresponds to PK-R1. All these enzymes were, before dissociation, fully active.

light subunits of red cell enzyme, while trypsin causes the transformation of L' into a form with intermediate Mr (59000-60000) (fig. 1).

As previously reported (1,2), the trypsin-treated erythrocyte L-type subunits have the same Mr as liver L-type subunits.

After treatment by subtilisin liver L-type pyruvate kinase, L'_4 and trypsin-treated erythrocyte enzyme have all a single type of subunits, corresponding to the light 57000-58000 dalton form. Trypsin is inactive on the subtilisin-induced form.

2. Susceptibility of the phosphorylatable site to proteolysis

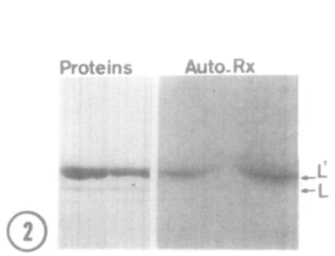
After phosphorylation, erythrocyte pyruvate kinase is only labelled on its L' subunits as judged by autoradiography (figure 2). The trypsin-induced form is still labelled, while the subtilisin-induced form has lost its phosphorylated site.

3. Transient form occuring upon sequential tryptic attack (figure 3)

Figure 3 shows that a sequential tryptic attack of the L' subunits results first in the transitory appearance of a form of 60000-61000 daltons, then in the definitive conversion into the 59000-60000 dalton species.

4. Abnormal tryptic cleavage of some mutant enzymes(figures 4 and 5)

Among the three variants purified before and after tryptic attack, one



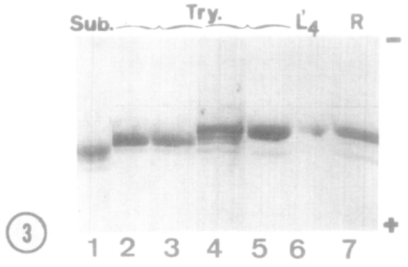
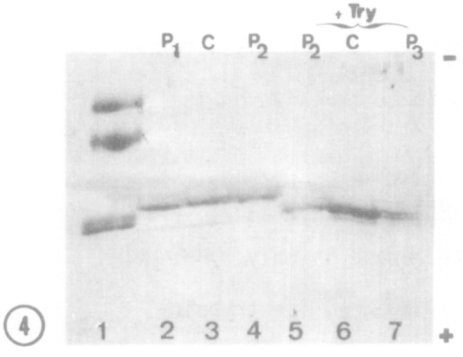


Fig. 2 : Autoradiography of SDS-dissociated, phosphorylated erythrocyte pyruvate kinase
Pyruvate kinase from a partially purified preparation (see "methods") was phosphorylated from $|\gamma^{32}P|$ATP by red cell soluble cyclic AMP-dependent protein kinase(s) ; then it was totally purified and analysed by SDS-polyacrylamide gradient gel electrophoresis.
In the left : Coomassie blue staining.
In the right : Autoradiogram.
A similar result was obtained with pyruvate kinase endogenously phosphorylated by incubation of the whole red cells with exogenous $|\gamma^{32}P|$inorganic phosphate and cyclic nucleotides (6).

Fig. 3 : Progressive proteolytic attack of red cell pyruvate kinase by trypsin. Analysis by SDS-polyacrylamide gradient gel electrophoresis.
About 5 µg protein per sample, staining with coomassie blue.
1 : subtilisin-treated red cell enzyme ; 2 : red cell pyruvate kinase treated by 10 µg/ml trypsin for 30 min. : 3 : idem, 10 µg/ml trypsin for 10 min. ; 4 : idem, 1 µg/ml trypsin for 10 min. ; 5 : idem, 0.1 µg/ml trypsin for 10 mn ; 6 : L'_4 (i.e. peak 2) : 7 : untreated, unfractionated red cell pyruvate kinase.



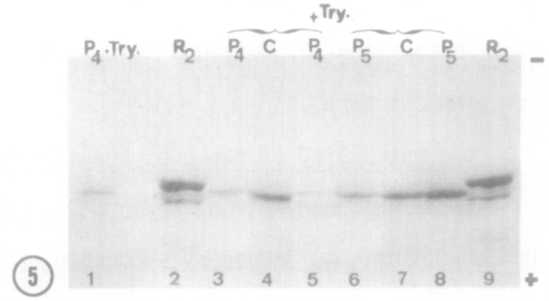


Fig. 4 : Sensitivity to trypsin of mutant pyruvate kinase variants ; analysis by SDS-polyacrylamide gradient gel electrophoresis
About 5 µg protein per sample, staining with coomassie blue.
1 : protein markers (Phosphorylase, human muscle phosphofructokinase and catalase) ; 2 : Patient 1, untreated enzyme ; 3 : control, untreated enzyme ; 4 : patient 2, untreated enzyme ; 5 : patient 2, trypsin-treated enzyme ; 6 : control, trypsin-treated enzyme ; 7 : patient 3, trypsin-treated enzyme.

Fig. 5 : Sensitivity to trypsin of mutant pyruvate kinase variants ; analysis by SDS-polyacrylamide gradient gel electrophoresis. Staining with Coomassie blue.
1, 3 and 5 : patient 4, trypsin-treated enzyme.
2 and 9 : untreated normal red cell enzyme, peak 1 (see fig. 1).
4 and 7 : control, trypsin-treated enzyme ; 6 and 8 : patient 5, trypsin-treated enzyme.

exhibited an abnormal behavior in that the native enzyme was only composed of L' subunits. Trypsin induced the appearance of the expected 59000-60000 dalton species, but its action was incomplete, and some L' subunits were not cleaved (figure 4, patient 2). The richness in reticulocytes of blood from this patient did not seem sufficient to explain these findings since they were not observed in other patients with similar reticulocytosis (fig. 4, patient 1), or in non deficient patients suffering from another type of hemolysis (not shown). Sensitivity to trypsin of the two other variants was normal (not shown).

Ten additional pyruvate kinase variants were purified only after treatment with trypsin. In four of them, proteolysis resulted in abnormal forms. In the two first observations (fig. 4, patient 3) we observed two discrete forms with similar Mr to normal, trypsin-treated subunits. In two other observations the trypsin treated subunits were slightly heavier than for control enzyme (fig. 5, patient 4).

DISCUSSION

From the data reported above it appears that the precursor L' subunits can be transformed by limited proteolysis into several molecular species. In order of decreasing Mr we can characterize at least three major forms, or, better, families of molecular forms (it is, indeed, probable that the species obtained by cleavage in vitro are not exactly the same as those physiologically occuring ; moreover some of the forms obtained by proteolytic attack in vitro seem to be themselves microheterogeneous) : trypsin transforms L' into La, them into Lb ; subtilisin cleaves L' or Lb and yields the light Lc form. A sequential proteolysis by trypsin, then subtilisin, or vice versa, yields the same Lc form.

L', La and Lb, but not Lc, are phosphorylatable. Thus we may conclude that subtilisin splits the same portion of the pyruvate kinase molecules as trypsin, plus the phosphorylatable site which is not cleaved by trypsin.

The liver L-type subunits are phosphorylatable, and seem to correspond to the trypsin-induced Lb subunits.

Fig. 6 : Schematic representation of the different forms of L-type pyruvate kinase occuring upon in vitro and in vivo proteolytic cleavages
Try = trypsin treatment
Sub = subtilisin treatment.

In the red cells the light, partially proteolysed subunits occuring with cell aging are not phosphorylatable ; they seem to correspond to the subtilisin-induced Lc subunits. In normal conditions this proteolysed form amounts to less than 20 % of the L-type subunits of unfractionated red cell populations, but this percentage can artefactually increase during purification without proteolysis inhibitors, and is probably higher in the older red cells. The formula of PK-R_2, abundant in the old erythrocytes, can be designated as L'_2Lc_2. Fig. 6 summarizes our current concepts about in vivo and in vitro postsynthetic maturation of L-type pyruvate kinase.

As we have previously suspected from electrophoretic data (7,8), some mutant molecules have an abnormal behaviour when subjected to proteolysis with trypsin: either the L' subunits are incompletely transformed into Lb, or proteolysis is qualitatively abnormal. It could be hypothesized that the tryptic form with high Mr obtained for four variants corresponds to the normally transient La form. In the patient whose pyruvate kinase was transformed by trypsin into two discrete bands, two hypotheses could be put forward : either a heterozygosis (with 2 types of molecules, one sensitive, and the other partially resistant to trypsin), or an incomplete proteolysis of the mutant molecules, a part of them being only transformed into a La form.

In conclusion, it is possible to describe several types of in vitro proteolytic cleavages of erythrocyte pyruvate kinase which mimic some in

vivo processes. The tryptic cleavage can be regarded as an essential maturation process, transforming the L'_4 precursor into a L_4 tetramer similar to the liver enzyme. The subtilisin-type cleavage splits the phosphorylatable site and results in an enzyme with altered kinetics ; it is sometimes an "in vitro" artefactual phenomenon, but also a minor process of molecular aging which takes place in the old red cells. Some mutant pyruvate kinase variants exhibit an altered sensitivity to in vitro, and probably in vivo, proteolysis. We hypothesize that some of the conformational changes associated with the mutation could mask some of the normal cleavage points.

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REFERENCES

1. Kahn, A., Marie, J., Garreau, H., and Sprengers, E.D. (1978). Biochim. Biophys. Acta 523, 59-74.
2. Marie, J., Garreau, H., and Kahn, A. (1977). FEBS Letters 78, 91-94.
3. Kahn, A., and Marie, J. (1979), in "Model for the study of inborn errors of metabolism" Hommes F.A. ed., Elsevier/North-Holland, Amsterdam - pp 191-204.
4. Bergström, G., Ekman, P., Dahlqvist, U. and Engström, L. (1975). FEBS Letters 56, 288-290.
5. Dahlqvist-Edberg, U. (1978)FEBS Letters 88, 139-143.
6. Marie, J., Tichonicky, L., Dreyfus, J.C. and Kahn, A. (1979). Biochem. Biophys. Res. Commun. 87, 862-868.
7. Kahn, A., Marie, J., and Vives-Corrons, J.L. (1979) Hum. Genet. 47, 339-342.
8. Marie, J., Zanella, A., Vives-Corrons, J.L., Najman, A., and Kahn, A. (1979). Clin. Chim. Acta 93, 61-69.
9. Imamura, K., and Tanaka, T. (1972) J. Biochem. (Tokyo) 71, 1043-1051.
10. Nakashima, K., Miwa, S., Oda, S., Tanaka, T., Imamura, K.,and Nishina, T. (1974) Blood 43, 537-548.
11. Marie, J., Kahn, A., and Boivin, P. (1977). Biochim. Biophys. Acta 481, 96-104.
12. Laemli, U.K. (1970) Nature 227, 680-685.