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# THE FUNCTION OF PYRROLINE-5-CARBOXYLATE REDUCTASE IN HUMAN ERYTHROCYTES

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SUMMARY: Pyrroline-5-carboxylate reductase, which converts pyrroline-5-carboxylate to proline, has been identified for the first time in human erythrocytes. The function of the enzyme in erythrocytes may be related to glucose metabolism through the hexosemonophosphate-pentose pathway. In both intact erythrocytes and erythrocyte extracts, pyrroline carboxylate added to the medium markedly stimulated the activity of the HMP pathway. The effect of pyrroline-5-carboxylate on HMP activity is mediated by the generation of $NADP^+$ accompanying the reduction of pyrroline-5-carboxylate to proline.

Pyrroline carboxylate (PC), the intermediate in proline biosynthesis and degradation is derived enzymatically from a number of amino acids (1), from proline by proline oxidase (EC number not assigned), from ornithine by ornithine aminotransferase (EC 2.6.1.13) and from glutamic acid by pyrroline carboxylate synthase (EC number not assigned) (2). Thus pyrroline carboxylate (PC), can be derived from intermediates of the TCA and urea cycles as well as proline. We recently showed that PC is a potent stimulator of the hexosemonophosphate-pentose (HMP) pathway in cultured human fibroblasts (3). These studies suggest that pyrroline-5-carboxylate reductase (EC 1.5.1.2) (PCR), which oxidizes NADPH coupled to the conversion of PC to proline, activates the hexosemonophosphate-pentose pathway by generating the $NADP^+$ required as cofactor for glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (4) and perhaps by removing the inhibition of glucose-6-phosphate

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dehydrogenase by NADPH (5). Although PCR has been studied in a variety of animal tissues (6,7) few measurements have been made in human cells (8,9). We now report that pyrroline-5-carboxylate reductase activity has been identified for the first time in normal human erythrocytes. The other proline metabolic enzymes, i.e. proline oxidase, ornithine aminotransferase, PC synthase and PC dehydrogenase (EC 1.5.1.12.) are undetectible in erythrocytes. Our findings suggest that in erythrocytes the function of PC reductase is closely linked to glucose metabolism through the HMP pathway.

## MATERIALS AND METHODS:

Human venous blood was obtained from nonfasting normal adults, anticoagulated with heparin (1:1000, 0.1 ml/5 ml blood). The erythrocytes were separated from plasma and leukocytes by standard techniques (10) and kept at 4° throughout the procedure. Intact erythrocytes as well as erythrocyte extracts were used for measurements of HMP activity by a previously described method (3). For measurement of proline metabolic enzyme, we prepared an extract in which erythrocytes were suspended in two volumes of 0.5 M potassium phosphate buffer, pH 8.0 and sonicated for 40 sec. on a Branson sonifier at a setting of 20. The activity of PCR in these extracts was not cold-labile (7) but decreased with freezing and thawing. Thus PCR activity was determined on the day of extract preparation. All other enzyme activities were assayed within one week on extracts kept frozen at -20°. The activities of PC reductase (11), PC dehydrogenase (12), ornithine aminotransferase (13) and proline oxidase (14) were measured by previously published radioisotopic methods. Protein determination was measured by the method of Lowry (15). Glucose labeled in the C-1 position was from Amersham. L-pyrroline-5-carboxylic acid and L-PC-$^{14}$C were synthesized and purified by a previously published method (16). All other chemicals were from Sigma Chemical Company.

## RESULTS AND DISCUSSION

We found that the activity of PC reductase in normal human erythrocytes was 67 IU/g hemoglobin, a level comparable to the activities reported for a number of red cell enzymes with important and well-defined physiologic functions (17). PC reductase was the only proline metabolic enzyme detectible in erythrocytes. Using sensitive, specific radioisotopic assays for PC dehydrogenase, proline oxidase and ornithine aminotransferase, we were unable to detect any activity of these enzymes in erythrocytes.

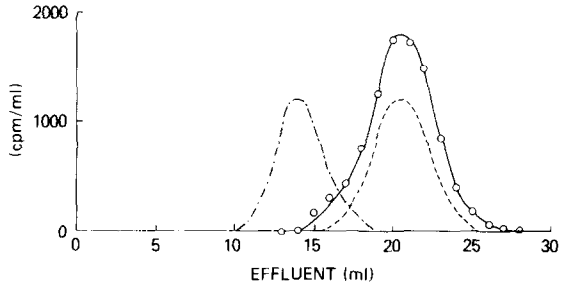


Figure 1.

Proline production by extracts of human erythrocytes. The incubation conditions are as described for the PC reductase assay (11). The reaction mixture contained in a total volume of 0.25 ml, 0.1 M potassium phosphate, pH 6.8; L-PC, .04 mM; L-PC-$^{14}$C, .05 μCi; NADH, 0.68 mM and erythrocyte extract corresponding to 0.83 μl of RBC's. The duration of incubation was 10 min. After incubation, 50 μl of o-aminobenzaldehyde (30 mg/ml in 10% ethanol, 90% 6N HCl) was added. The o-aminobenzaldehyde traps unreacted PC. After centrifugation, a 0.2 ml aliquot was applied to a 3-ml bed volume Dowex-50W columm, eluted with 1 N HCl, and the effluent collected in 1 ml fractions. The elution profile of the reaction product (o——o) was compared with that for a proline standard (- - - -) and for a PC standard (—·—·—). An unreacted blank showed no radioactivity in the proline region.

The characteristics of PC reductase in red cells were similar to those described for the enzyme from other sources (18,19). Formation of product proline was dependent on the presence of NAD(P)H and was a saturable function of PC concentration. Furthermore, PC-dependent oxidation of NADPH was observed and product proline was identified by ion exchange column chromatography (figure 1). Conforming to an enzyme-mediated reaction, the formation of product was linear with increasing duration of incubation and with increasing amount of added extract. The activity was completely destroyed by heating at 100° for 5 minutes.

The measured erythrocyte PC reductase activity was not due to contamination by other blood elements. Erythrocytes contained more

than 99% of the enzyme activity measured in whole blood. Based on determinations of PC reductase activity in preparations of isolated leukocytes and platelets, we showed that only a trivial fraction of measured enzyme activity is due to these elements. There was no activity found in plasma.

In animals, the level of PC reductase is especially high in tissues with a high demand for proline for protein and collagen synthesis, e.g. cartilage and bone (20). However, in erythrocytes where proteins are not synthesized, the functional end-point of the enzyme cannot be related to protein synthesis. One possibility is that erythrocytes are a carrier system for proline. Pyrroline-5-carboxylate excreted by certain tissues, e.g., liver or kidney, may be converted to proline by erythrocytes and delivered to peripheral tissues. Although free PC has not been identified in the peripheral venous plasma (21), detailed studies of PC in selected tissue effluents have not been reported.

Another possibility is that PC reductase in erythrocytes is linked to glucose metabolism through the redox state (3). Erythrocytes metabolize glucose via two major pathways, the Embden-Meyerhof pathway which converts glucose-6-phosphate to lactate thereby generating ATP and the hexosemonophosphate-pentose pathway which converts glucose-6-phosphate to ribose phosphates and generates NADPH, the major source of reducing potential for red cells. Using the conversion of glucose-1-$^{14}C$ to $^{14}CO_2$ by intact cells as a measure of HMP activity, we found that PC added to the incubation medium strikingly increased the activity of the pathway. Recovered $^{14}CO_2$ increased linearly with increasing duration of incubation with or without PC. More importantly, the magnitude of the PC stimulation increased with increasing concentrations of PC (Figure 2). The effect plateaued at a PC concentration of 3 mM with a 15 fold increased in HMP activity. The PC effect on HMP activity was specific and could not be produced by other amino acids. Proline, glutamate or a

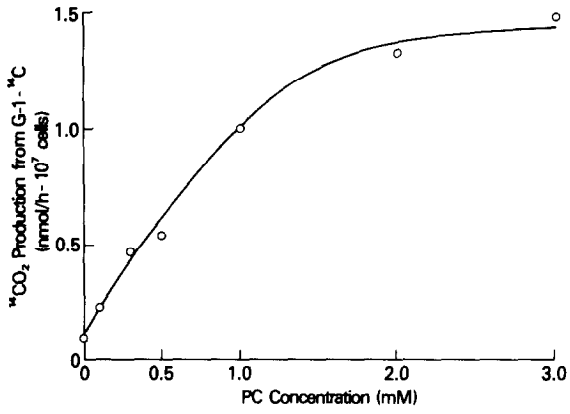


Figure 2.

Glucose-1-$^{14}C$ oxidation to $^{14}CO_2$ as a function of medium PC concentration. Intact human erythrocytes (50 µl) were incubated for 1 h with glucose-1-$^{14}C$ (~ 2 µCi) in 2 ml of Earle's balanced salt solution. Glucose concentration was 2.5 mM. L-PC was added to the medium at the indicated final concentrations.

mixture of nonessential amino acids were without effect. Even when erythrocytes were incubated in a growth medium (Eagle's MEM) containing both essential and nonessential amino acids, PC increased HMP activity by a magnitude similar to that seen in Earle's balanced salt solution. Thus, PC, the substrate for PC reductase was a potent and specific stimulator of HMP activity in red cells.

We sought evidence that the PC effect was on the HMP pathway and not on the formation of glucose-6-phosphate. By using cell-free erythrocyte extracts we found that PC at a concentration of 0.3 mM greatly stimulated $^{14}CO_2$ production from glucose-6-phosphate-1-$^{14}C$ with NADPH in the reaction mixture. However, PC had little effect on $^{14}CO_2$ production in the presence of $NADP^+$ (Table 1). This result suggested that the effect of PC on HMP activity involved the enzymes of the HMP pathway. The generation of $NADP^+$ by PC reductase appeared to be the mechanism.

We further explored the linkage between PC and the HMP pathway in intact cells by using methylene blue. Methylene blue, an auto-oxidant,

TABLE 1

Oxidation of Glucose-6-phosphate-1-$^{14}C$ by Human Erythrocyte Extracts

| | $^{14}CO_2$ production from G-6-P-1-$^{14}C$ (nmol/h-$10^7$ cells) |
|---|---|
| $NADP^+$ | 15.0 |
| $NADP^+$ + PC | 16.7 |
| NADPH | 0.05 |
| NADPH + PC | 6.06 |

We prepared erythrocyte extracts by suspending packed cells in equal volumes of .025 M phosphate buffer pH 7.4, sonicating for 40 secs and centrifuging at 25,000 X g for 10 min. We then incubated 0.1 ml of the supernatant with glucose-6-phosphate-1-$^{14}C$ ( 2 uCi) at 37° for 1 hour with $NADP^+$ or NADPH in the presence and absence of PC. The incubation medium was 1 ml of .025 M phosphate buffer, pH 7.4 and the concentration of glucose-6-phosphate was 0.2 mM. $NADP^+$, NADPH and PC were added at a final concentration of 0.3 mM. Hyamine was used to trap evolved $^{14}CO_2$.

is known to stimulate the HMP pathway by increasing the availability of $NADP^+$. We found that the effect of PC was comparable in magnitude to that produced by saturating concentrations of methylene blue. More importantly, the effects of methylene blue and PC at saturating concentrations were not additive (Table 2). Thus, PC, stiumlated the HMP

TABLE 2

Oxidation of Glucose-1-$^{14}C$ in Intact Erythrocytes

| | $^{14}CO_2$ Production from G-1-$^{14}C$ (nmole/hr-$10^7$ RBC) |
|---|---|
| CONTROL | 0.22 |
| PC (2.0 mM) | 2.95 |
| METHYLENE BLUE (0.05 mM) | 4.27 |
| PC (2.0 mM) + METHYLENE BLUE (0.05 mM) | 4.30 |

Intact human erythrocytes (50 ul) were incubated for 1 h with glucose-1-$^{14}C$ ( 2 uCi). The incubatíon medium was 2 ml of Earle's balanced salt solution and the concentration of glucose was 2.5 mM. Methylene blue and PC were added as indicated. We used hyamine to trap evolved $^{14}CO_2$.

pathway by a mechanism similar to that of methylene blue. It was likely that the oxidation of NADPH accompanying the conversion of PC to proline by PC reductase generated the $NADP^+$ for supporting the catalytic capacities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase.

In erythrocytes, the proposed linkage between PC reductase and the HMP pathway promotes the formation of pentose phosphates and proline at the expense of reducing potential. Although reducing potential is critical for maintenance of red cell integrity (21), the capacity of the HMP pathway exceeds these requirements. PC reductase by providing oxidizing potential would promote the generation of PRPP, a vital intermediate for purine nucleotide formation via the salvage pathway (22). On the other hand, the HMP pathway can support the production of proline for other cells. The reducing potential in the erythrocyte can be used for the production of proline for protein and collagen synthesis by peripheral tissues. In other tissues, which have mitochondrial proline oxidase (liver, kidney, heart and brain) (7), proline produced by erythrocytes may be a substrate for oxidative phosphorylation by a mechanism independent of NADH oxidation (23).

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