Pyrroline-5-Carboxylate Reductase in Human Erythrocytes

A COMPARISON OF DIFFERENTIAL REGULATION

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ABSTRACT Pyrroline-5-carboxylate reductase, which converts pyrroline-5-carboxylate to proline, has been identified in human erythrocytes. The level of pyrroline-5-carboxylate reductase activity in these cells is comparable to the activity levels of major erythrocyte enzymes. The physiologic function of the enzyme in erythrocytes cannot be related to its function in other tissues, i.e., producing proline for protein synthesis. We examined the kinetic properties of erythrocyte pyrroline-5-carboxylate reductase and compared them to the properties of the enzyme from proliferating cultured human fibroblasts. We found that the kinetic properties and regulation of the erythrocyte enzyme are distinctly different from those for human fibroblast pyrroline-5-carboxylate reductase. These characteristics are consistent with the interpretation that the function of the enzyme in human erythrocytes may be to generate oxidizing potential in the form of $NADP^+$.

INTRODUCTION

Pyrroline-5-carboxylate reductase (PCR) (EC 1.5.1.2), which catalyzes the final step in proline synthesis, converts pyrroline-5-carboxylic acid (PC) to proline in a NAD(P)H-dependent reaction (1, 2). Although a number of studies have characterized the enzymes from bovine and rat liver (3–5), there have been few studies on PC reductase from normal human tissues. We recently reported that PC reductase activity is found in human erythrocytes. Moreover, PC reductase is the only proline metabolic enzyme present in these cells; the activities of PC dehydrogenase, proline oxidase, and ornithine aminotransferase are undetectable (6). Although the level of PC reductase activity in human erythrocytes is comparable to the activity levels of major erythrocyte enzymes, the physiologic function of PCR in erythrocytes cannot be related to that in other tissues, i.e., the production of proline for protein synthesis. Instead, we have shown that the oxidizing potential generated by PCR accompanying the conversion of PC to proline greatly stimulates the metabolism of glucose through the hexosemonophosphate-pentose pathway in intact erythrocytes (6). These findings led us to examine the kinetic properties of erythrocyte PC reductase and to compare them to the properties of the enzyme from proliferating cultured human fibroblasts where protein synthesis is a prominent feature. We now report that the kinetic properties and regulation of the erythrocyte enzyme are distinctly different from that found in human fibroblasts. These characteristics support the interpretation that the function of the enzyme in human erythrocytes may be to generate oxidizing potential in the form of $NADP^+$.

METHODS

Materials. [U-^{14}C]Ornithine (260 μCi/mmol) was purchased from New England Nuclear (Boston, Mass.). Ornithine, L-proline, NADH, NAD^+, NADPH, $NADP^+$, and *o*-aminobenzaldehyde were from Sigma Chemical Co. (St. Louis, Mo.). Cation-exchange resin, 200–400 mesh (AG-50, 8% cross-linked, hydrogen form) was from Bio-Rad Laboratories (Richmond, Calif.).

Extract preparation. Erythrocytes: venous blood was obtained from nonfasting normal adults and anticoagulated with heparin (1,000 U/ml, 0.1 ml/5 ml blood). Samples were processed immediately and all processing was performed at 4°C. After centrifugation at 500 *g* for 10 min, plasma and the buffy layer were removed from the packed erythrocytes. Erythrocytes were washed three times with cold 0.85% sodium chloride solution (4:1, saline:cells). With each wash the suspension was centrifuged for 10 min at 500 *g* and the top layers of erythrocytes discarded to ensure complete removal of leukocytes (12). The washed erythrocytes were

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suspended in 2 vol of 0.5 M potassium phosphate buffer, pH 8.0, and sonicated for 40 s on a Branson sonifier (Branson Sonic Power Co., Danbury, Conn.) at a setting of 20. Extracts were aliquoted and stored at −20°C and assayed for PCR activity within 1 wk of preparation. About 90% of the enzyme activity remained after 1 wk in storage at −20°C.

Fibroblasts. Normal human fibroblasts were grown from forearm skin obtained by punch biopsy. Our methods for culturing, harvesting, and preparing extracts from fibroblasts have been described in detail (13). Assay for PCR activity was determined within 1 wk of extract preparation.

Other blood cellular elements. Preparations of separated mononuclear cells, granulocytes (14), and platelets (15) were obtained by standard procedures. Extracts were prepared in a manner similar to that for erythrocytes.

Preparation of substrates. We synthesized L-PC[1] and L-[U-^{14}C]PC by a previously reported method (8). Unlabeled ornithine or [U-^{14}C]ornithine together with α-ketoglutarate were incubated from ornithine ketoacid aminotransferase purified from rat liver. The resultant L-PC or L-[U-^{14}C]PC were purified by cation exchange column chromatography. PC concentrations were determined by the method of Strecker (11).

Enzyme assays. PCR activity was assayed by a published specific radioisotopic method (9). In brief, an aliquot of erythrocyte or fibroblast extract was incubated with L-[^{14}C]PC, L-PC (0.040 mM), and NADH or NADPH at defined concentrations in 0.1 M phosphate pH 6.8 buffer. The volume of the incubation mixture was 0.25 ml. After incubation, 50 μl of *o*-aminobenzaldehyde (30 mg/ml in 10% ethanol and 90% 6 N HCl) was added. Precursor PC combines with *o*-aminobenzaldehyde, forming a dihydroquinazolinium compound which binds tightly to cation exchange resin. Product [^{14}C]-proline was then eluted with 1 N HCl. Protein determination was measured by the method of Lowry et al. (10).

RESULTS

Normal values of PC reductase activity in normal adults. Erythrocyte PC reductase activity was determined in 12 healthy adults, 6 male and 6 female, age range 28–41 yr. In the standard assay ([PC] = 0.04 mM, [NADH] = 0.68 mM), the range in this population was 25.5–58.4 nmol/h·mg protein with a mean±SD of 43.2±4.1 nmol/h·mg protein. Since these conditions optimized recovery of labeled product, the activity levels did not reflect those at maximum velocity (V_{max}) conditions. With [PC] = 2.0 mM and [NADH] = 3.2 mM, the activity was 67 IU/ml cells, a level which compares favorably with activity levels reported for a number of erythrocyte enzymes (7).

Possible contamination by other blood elements. Although blood elements other than erythrocytes have PC reductase activity, contribution by these elements to measured erythrocyte activity was insignificant for the following reasons. We first showed that the activity per cell in whole blood was essentially the same as in isolated erythrocytes. Second, isolated erythrocytes after defibrination, which removes the majority of platelets, showed no difference in activity per volume of erythrocytes. Finally, we assayed the activity of PC reductase in isolated mononuclear cells, granulocytes, platelets, and plasma and found that the contribution of these elements to whole blood is <1%.

Unlike rat liver PCR (5), erythrocyte PCR activity is not cold sensitive nor can it be activated by heat (3). The activity in cultured fibroblasts is similarly stable to cold. Under our standard assay conditions extracts of both erythrocytes and fibroblasts formed product proline linearly with increasing duration of incubation and with increasing amounts of added extract.

Our strategy was to characterize PCR from erythrocytes (EPCR) by comparing its affinities for PC, NADH, and NADPH to those for the PCR from fibroblasts (FPCR). In addition we sought differential regulation of the enzyme from the two sources.

Affinity for pyridine nucleotides. Since PCR can use either NADH or NADPH for supporting the reduction of pyrroline-5-carboxylate, we first compared enzyme from the two sources in their affinities for reduced pyridine nucleotides. PCR activity was measured at five different concentrations (0.034, 0.068, 0.136, 0.34, and 0.68 mM) of either NADH or NADPH. Each data point was based on four determinations from two separate experiments. Using Lineweaver-Burk transformations and computer calculated linear regressions, we determined the respective K_m for each reduced pyridine nucleotide for each enzyme. In FPCR, we found that the affinities for NADPH and NADH were similar, K_m = 0.09±0.018 mM (mean±SD) and 0.12±0.013 mM, respectively. In marked contrast, EPCR showed an affinity for NADPH, (K_m = 0.02 ±0.004 mM) which was 20 times greater than that for NADH, (K_m = 0.39±0.044 mM). Thus, EPCR not only showed a preferential affinity for NADPH over NADH but also its affinity for NADPH was much higher than that of FPCR for NADPH.

Affinity for substrate PC. These differences in affinities for reduced pyridine nucleotides, led us to compare the affinities for substrate pyrroline-5-carboxylate using either NADH or NADPH. In these experiments we chose a concentration of the reduced pyridine nucleotide, which was three times the observed K_m for the respective nucleotide for that enzyme. Under these conditions, the affinity of FPCR for substrate PC was similar with either reduced pyridine nucleotide. The K_m for PC were 0.41±0.118 and 0.20±0.017 mM (mean±SD), respectively, with NADH or NADPH. However, with EPCR, we observed a striking difference in the affinity for substrate PC depending on which reduced pyridine nucleotide was used. With NADPH the affinity for PC (K_m PC = 0.09±0.018 mM) was almost five times higher than that with NADH (K_m PC = 0.48±0.096 mM). Not only does EPCR ex-

[1] *Abbreviations used in this paper*: PC, Δ[1]-pyrroline-5-carboxylic acid; PCR, pyrroline-5-carboxylate reductase; EPCR, PCR from erythrocytes; FPCR, PCR from fibroblasts.

hibit a preferential affinity for NADPH over NADH, but also the affinity for substrate PC is higher with NADPH than with NADH. The fibroblast enzyme on the other hand, shows little difference in affinity for PC with NADPH or NADH.

Differential regulation of PCR activity. Since previous studies showed regulation of fibroblast PCR activity by product proline (13), we compared the sensitivities of EPCR and FPCR to feedback inhibition. Proline at a concentration of 1 mM inhibited the activity of FPCR by 88±2% but was without effect on EPCR activity (inhibition = 6±2%). Thus, a striking difference was seen in the two enzymes in their sensitivities to inhibition by proline. Since the characteristics of EPCR relative to reduced pyridine nucleotides suggested that the erythrocyte enzyme is linked preferentially to NADPH, we tested the sensitivity of PCR activity to inhibition by $NADP^+$ which is known to inhibit hepatic PCR activity (3). We found that $NADP^+$ at a concentration of 1 mM inhibited EPCR activity by 67±3% but was only inhibited FPCR by 5±1%. Previous investigators showed that hepatic PCR is inhibited by other adenine nucleotides (3, 4). However, EPCR is not affected by these nucleotides (Table I). The inhibition of EPCR increased with increasing concentrations of $NADP^+$ (Fig. 1). Kinetics studies showed that the inhibition is competitive in type and occurs with either NADH or NADPH (Fig. 2). Thus, EPCR is regulated by $NADP^+$ whereas FPCR is regulated by product proline.

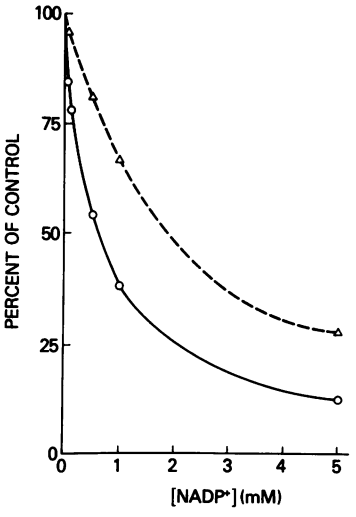

FIGURE 1 The inhibition of erythrocyte PC reductase by increasing concentrations of $NADP^+$. The incubation conditions are as described under Methods. Erythrocyte extract corresponding to 0.83 μl of erythrocytes was used to catalyze the reaction. The duration of incubation was 10 min. The data is expressed as percent control EPCR activity with NADH (0.68 mM) (○) and with NADPH (0.068 mM) (△) at different concentrations of $NADP^+$. Each point represents the average of two determinations.

DISCUSSION

PCR catalyzes the conversion of PC to proline with the concomitant oxidation of NADH or NADPH. This enzymatic step is the final and committed step in proline biosynthesis for bacterial as well as mammalian cells. In most mammalian tissues, PCR produces proline for protein synthesis (2). However, in human

TABLE I
Inhibition of EPCR by Nucleotides

Nucleotide	Concentration	Percentage of control
	mM	
AMP	2	105
ADP	2	100
ATP	2	98
dATP	2	100
NAD^+	2	100
$NADP^+$	2	18

The assay mixture contained in a volume of 250 μl of PO_4 buffer pH 6.8, 0.04 mM PC, erythrocyte extract equivalent to 0.83 μl of erythrocytes, 0.68 mM NADH and various adenine nucleotide inhibitors with 5 mM $MgSO_4$. The duration of incubation was 10 min. All addends were adjusted to pH 6.8. Data is expressed as percentage of control from control values. Each point represents the average of duplicate determinations.

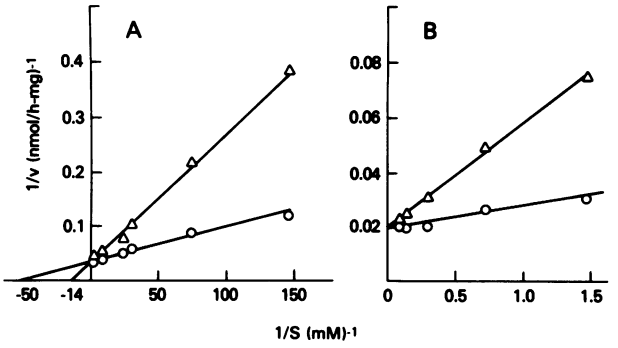

FIGURE 2 Kinetics of inhibition by $NADP^+$. (A) Interaction with NADPH. The assay conditions are as described under Methods. EPCR is expressed as nanomoles per hour per milligram protein. Activity was determined over a 100-fold of NADPH concentration in the presence (△) and absence (○) of 1 mM $NADP^+$. Lineweaver-Burk transformations of the data are shown. Each point represents the average of two determinations. (B) Interaction with NADH. The conditions are identical to that for A except that NADH was used. The activity in the presence (△) and absence (○) of 1 mM $NADP^+$ are shown. Each point represents the average of two determinations.

erythrocytes, the absence not only of protein synthesis but also of the other enzymes of proline metabolism, suggests that PCR either serves a different function or else is a vestige of erythrocyte development and maturation. We sought insight into the possible physiologic significance of EPCR by comparing the kinetic properties of erythrocyte PC reductase to the properties of the enzyme from proliferating cultured human fibroblasts.

Two lines of evidence suggest that PCR serves a special function in erythrocytes. First the enzyme has a preferential affinity for NADPH; the K_m for NADPH is 20 times lower than the K_m for NADH. In addition, with NADPH as coenzyme the affinity of the enzyme for PC is five times higher than the affinity for PC with NADH. Thus, the reduction of PC especially at low PC concentrations is linked to the oxidation of NADPH rather than NADH. These features distinguish EPCR from FPCR. Second, EPCR is regulated by a mechanism distinct from that for FPCR. In fibroblasts, product proline regulates PCR activity by feedback inhibition (13). With 1 mM proline, the activity is inhibited 88%. In contrast EPCR activity is unaffected by proline. Instead, EPCR is sensitive to feedback inhibition by product $NADP^+$. With kinetic analysis, we showed that $NADP^+$ is a competitive inhibitor of both NADH or NADPH mediated reactions. On the basis of these findings, the function of EPCR appears related to NADPH oxidation rather than proline production. In fact, we recently reported that PC stimulates the activity of the hexosemonophosphate-pentose pathway 15-fold in intact erythrocytes (6).

The characteristics of PCR from erythrocytes are distinct not only from cultured fibroblasts but also from other cells and tissues. The lack of sensitivity to feedback inhibition by proline is especially striking. PCR from cultured cells derived from a variety of tissue sources is uniformly proline-sensitive (13). The enzyme from peripheral tissues, e.g., xiphoid and epiphyseal cartilage is also inhibited by proline (unpublished data). In contrast, PCR from bovine or rat liver is relatively insensitive to proline inhibition (4). Furthermore resting human lymphocytes isolated from peripheral blood have the proline-insensitive form of the enzyme. Accompanying lectin-induced mitogenesis, PCR is not only induced but also becomes highly sensitive to proline inhibition (16). The sensitivity to proline inhibition may be a characteristic of PCR from proliferating cells in which the amino acid is required for protein synthesis.

The enzyme from erythrocytes is only sensitive to inhibition by $NADP^+$. In previous studies using partially purified rat liver PCR, Smith and Greenberg (3) found that the activity of the enzyme was inhibited by a variety of nucleotides. ATP, ADP, and $NADP^+$ were especially potent but NAD^+ was also inhibitory. In the context of these previously reported findings, the properties of EPCR are unique. EPCR is regulated singularly by $NADP^+$. Adenine nucleotides and proline are ineffective in inhibiting the formation of product proline.

Although the conversion of PC to proline by PCR can be supported by either reduced pyridine nucleotide, differences are emerging in their use by PCR from various sources. Previous workers have emphasized the in vitro finding that at any concentration of reduced pyridine nucleotide, proline formation is much greater with NADH than with NADPH (5). In contrast we find that EPCR is a NADPH-preferring enzyme. The markedly higher affinity for NADPH as well as the affinity for PC, which is higher with NADPH than with NADH, strongly suggest that the enzyme *in situ* is coupled to the oxidation of NADPH rather than NADH.

Two aspects of these differential characteristics for PCR from various sources are noteworthy. First, these patterns of regulation and affinities for pyridine nucleotides can be explained best by the hypothesis that there are different isozymes of PCR. Whether the putative isozymes are gene products or are interconvertible forms is a topic of intense investigation in our laboratory. Second, the possibility of PCR from contaminating erythrocytes in tissue extracts and even partially purified preparations from specific tissues should be considered by the investigator. Such contamination may obscure kinetic patterns of the enzyme from the presumed specific tissue source.

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