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LINKAGE OF THE HMP PATHWAY TO ATP GENERATION BY THE PROLINE CYCLE

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SUMMARY: The reactions catalyzed by proline oxidase and pyrroline-5-carboxylate reductase form a catalytic cycle linking the hexose-monophosphate pentose (HMP) pathway to mitochondrial ATP generation. The cycling of proline and pyrroline-5-carboxylate couples glucose oxidation to ATP generation by a mechanism independent of the Embden-Meyerhof pathway and the tricarboxylic acid cycle.

The metabolism of proline has features which are unique among 5-carbon amino acids (1,2). Proline is neither transaminated nor decarboxylated. Instead, the oxidation of proline by mitochondrial-bound proline oxidase (EC number not assigned) is linked to mitochondrial electron transport probably at the level of a flavoprotein (3,4). The product of proline oxidase, pyrroline-5-carboxylate (PC), may be recycled to proline by PC reductase (EC 1.5.1.2), a cytosolic enzyme which *in vitro* requires either NADH or NADPH as cofactor (5,6). We recently showed that PC markedly stimulates the metabolism of glucose in intact cells but only through the HMP pathway (7). This finding suggested that the in situ conversion of PC to proline by PC reductase is linked to the oxidation of NADPH. These metabolic relationships led us to the hypothesis that the interconversions of proline and PC form a catalytic cycle linking the HMP pathway to mitochondrial ATP generation.

To support our hypothesis we showed *in vitro* that the proposed proline cycle can generate the ATP and $NADP^+$ necessary for the oxidation of glucose through the HMP pathway. We combined rat

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kidney mitochondria which contain proline oxidase as well as the necessary components for oxidative phosphorylation with extracts of human erythrocytes which have PC reductase (8), hexokinase and the enzymes of the HMP pathway. Erythrocyte extract was chosen because it provided several advantages. Human erythrocytes have PC reductase activity with a high affinity for NADPH but without sensitivity to proline feedback inhibition (Yeh, G.C. et al., unpublished results). In addition, human erythrocytes cannot synthesize PC and are without PC dehydrogenase activity. Lacking mitochondria, they are also without proline oxidase (8). Thus, PC metabolism in human red cells is restricted to the conversion of PC to proline. By incubating intact mitochondria, erythrocyte extract and glucose-(1)-^{14}C in the presence of ADP and NADPH we used the production of $^{14}CO_2$ as an end point which can occur only if ATP and $NADP^+$ both have been generated endogenously in the reaction mixture.

MATERIALS AND METHODS

Mitochondria were prepared on the day of the experiment from the kidneys of 200 g male Sprague-Dawley rats by the method of Chappell and Hansford (9). For some experiments, kidney mitochondria from PRO/Re or C57/B6 mice were prepared. The source of erythrocytes was heparinized venous blood from normal volunteers. Erythrocytes were harvested, washed X 3 with 0.85% saline to remove plasma and other formed elements and were suspended in two volumes of 0.5 M potassium phosphate buffer, pH 8.0. Cells were lysed by sonication for 60 secs and cell membranes were removed by centrifugation at 25,000 x g for 10 min. Bovine serum albumin (Fraction V, fatty acid-free) was from Miles Lab. Inc. and 2,3-butanediol was from Aldrich Chemical Co. Hexokinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase (yeast) and all other chemicals were from Sigma Chemical Company. Glucose labeled in either C-1 or C-6 position was from Amersham and L-pyrroline-5-carboxylic acid was prepared by a previously published method (10).

RESULTS AND DISCUSSION

In the presence of erythrocyte extract and rotenone-treated mitochondria, $^{14}CO_2$ production from glucose-(1)-^{14}C occurred only when both ATP and $NADP^+$ were available (Table I). This was ex-

TABLE I
Glucose Oxidation in the
Mitochondria-Erythrocyte Extract System

	G-(1)-^{14}C to $^{14}CO_2$ per incubation		
	CPM X 10^{-3}	nmols	Per Cent
I. COMPLETE	25.3 ± 0.8	14.4 ± 1.3	100
minus ATP	0.7	0.1	2
minus $NADP^+$	0.4	0.3	1
II. SUBSTITUTED	1.8 ± 0.2	1.0 ± 0.2	6

The reaction mixture contained in a volume of 1 ml:sucrose, 0.25 M; KCl, 20 mM; $MgCl_2$, 5 mM; triethanolamine, 15 mM, pH 7.0; potassium phosphate, 12 mM; bovine serum albumin, 0.5% rotenone 0.5 µg/ml (dissolved in 2,3-butanediol such that the final concentration of butanediol was 0.9 mM); mitochondria, ∿300 µg protein (220-460 µg); erythrocyte extract equivalent to 12 µl of cells; glucose, 1 mM; and glucose-(1)-^{14}C, 1 µCi. In Part I, ATP 0.2 mM, $NADP^+$ 0.1 mM and AMP 2.0 mM were added to the mixture. In part II, ATP and $NADP^+$ were replaced by equimolar ADP and NADPH, respectively. The duration of incubation was 30 minutes and $^{14}CO_2$ was trapped in hyamine. Data are expressed per incubation.

pected because ATP is necessary for the phosphorylation of glucose by hexokinase and $NADP^+$ is required as cofactor for both glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. The reaction system did not support the production of $^{14}CO_2$ from

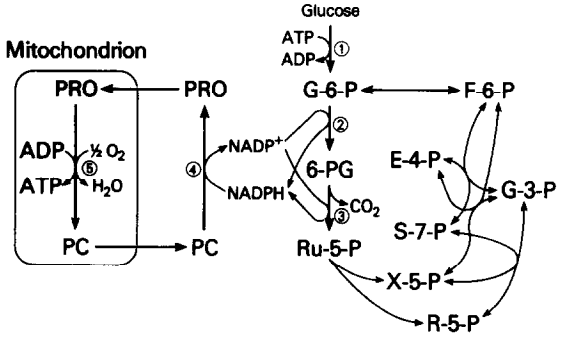

Figure 1.

The proposed linkage of the HMP pathway to ATP generation by the proline catalytic cycle. Enzymes are designated as follows: 1) hexokinase; 2) G-6-P dehydrogenase; 3) 6-PG dehydrogenase; 4) PC reductase; 5) proline oxidase.

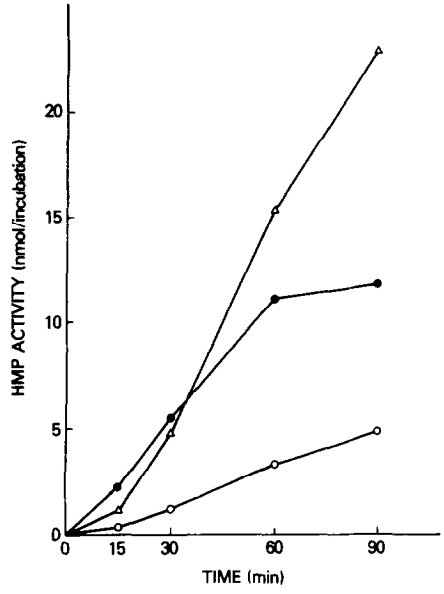

Figure 2. Time course of glucose oxidation through the hexose-monophosphate pentose pathway. The conditions are as described for Table I. Mitochondria containing 315 µg of protein were used. The concentrations of PC and proline were 0.3 and 10 mM, respectively. The amount of $^{14}CO_2$ (nmols) recovered from G-(1)-^{14}C under substituted conditions (O--O), plus PC (● --●) or proline (Δ --- Δ) are shown. Each point represents the average of at least duplicate determinations.

glucose-(6)-^{14}C. Thus, with glucose-(1)-^{14}C, the sole source of $^{14}CO_2$ was from the HMP pathway. When ATP and $NADP^+$ were replaced by ADP and NADPH, respectively, and with AMP added to minimize ATP production by adenylate kinase, little glucose-(1)-^{14}C was oxidized. However, when either PC or proline in addition to ADP and NADPH was present, the oxidation of glucose was more rapid (figure 2). The requirements of the system allowed us to conclude that the generation of $NADP^+$ depended on PC and the generation of ATP depended on proline (Table II). The effect of PC required the presence of PC reductase. With yeast hexokinase, G-6-P dehydrogenase and 6-PG dehydrogenase added in quantities shown to

TABLE II
Proline Cycle
Linkage to Glucose Oxidation

	G(1)-^{14}C to $^{14}CO_2$ per incubation	
	nmols	Per Cent
I. COMPLETE	14.4 ± 1.3	100
II. SUBSTITUTED	Blank	0
plus proline	3.6 ± 0.9	25
plus PC	4.1 ± 0.4	28
plus glutamate	0.1	0.7
Plus PC, minus mitochondria	Blank	0
Plus PC, minus erythrocyte extract	Blank	0
Plus PC, minus erythrocyte extract, plus hexokinase G6P dehydrogenase and 6PG dehydrogenase	Blank	0
III. PC-DEPENDENT "$NADP^+$ GENERATION"	14.8	100
IV. PROLINE-DEPENDENT "ATP GENERATION"	2.3	26

The incubation conditions are identical to those described for Table I. For Part I, ATP, 0.2 mM and $NADP^+$, 0.1 mM were present. For Part II, ATP and $NADP^+$ were replaced by equimolar ADP and NADPH, respectively. PC, 0.3 mM, proline, 5 mM or glutamate 5 mM were added as indicated. A mixture of yeast enzymes: hexokinase, 1.0 IU; G6P dehydrogenase, 0.2 IU and 6-PG dehydrogenase, 0.2 IU was added where shown. Part III had ATP, 0.2 mM and PC, 0.3 mM added to the substituted mixture. Part IV had proline, 10 mM and $NADP^+$, 0.1 mM added to the substituted mixture. The data for parts III and IV are expressed as an increase over controls without PC or proline, respectively. The "$NADP^+$ generation" and "ATP generation" denotes the amount of glucose oxidation dependent on endogenously produced $NADP^+$ and ATP, respectively.

support rapid glucose oxidation, PC was without effect unless erythrocyte extract containing PC reductase was present. Thus, the mitochondrial metabolism of PC to glutamate and tricarboxylic acids was not the mechanism for stimulating glucose oxidation. Furthermore, added glutamate was without effect. To ensure that proline-dependent ATP generation was not due to a process in-

TABLE III
Dependence of Glucose Oxidation
On Proline Oxidation in Mitochondria

	G-(1)-^{14}C to $^{14}CO_2$ nmol per incubation	
	C57/B6 (normal)	PRO/Re (proline oxidase deficient)
I. COMPLETE	32.9 ± 1.3	32.5 ± 0.5
II. SUBSTITUTED	2.8 ± 0.8	3.0 ± 0.1
plus proline	32.5 ± 4.0	2.9 ± 0.1
III. PROLINE-DEPENDENT "ATP GENERATION"	25.9	0

The incubation conditions were similar to that described for Table I except that the duration of incubation was 90 minutes. Aliquots of mitochondrial suspension from C57/B6 and PRO/Re kidneys containing 270 and 240 µg of protein, respectively, were used. Proline, where indicated, was present at a concentration of 10 mM. For part III, $NADP^+$, 0.1 mM was included. The proline dependent glucose oxidation is expressed as the increase in $^{14}CO_2$ production over the control without proline.

dependent of proline oxidase, we used mitochondria from PRO/Re mice, an inbred strain lacking this enzyme (11). Under conditions where rat mitochondria or normal mouse mitochondria produced the ATP required for glucose oxidation, the mitochondria from PRO/Re mice could not. PRO/Re mice mitochondria showed no glucose oxidation mediated by proline-dependent ATP generation (Table III). Thus, we conclude that it is the oxidation of proline which was necessary for augmenting ATP generation by mitochondria. Based on these findings we formulated the hypothesis shown schematically in figure 1. The $NADP^+$ generated from reaction 4 (PC reductase) served as cofactor for reactions 2 (glucose-6-phosphate dehydrogenase) and 3 (6-phosphogluconate dehydrogenase). Product proline was transported into mitochondria and was oxidized back to PC by mitochondrial proline oxidase accompanied by the generation of ATP (reaction 5).

The proposed scheme predicts a stoichiometry which would allow for an efficient production of ATP from glucose oxidation. It is well recognized that glucose-6-P cycled in the HMP pathway can be oxidized to CO_2; the overall reaction is represented by the following equation:

$$6\ \text{G-6-P} + 12\ \text{NADP}^+ \longrightarrow 5\ \text{G-6-P} + 12\ \text{NADPH} + 6\ CO_2 \quad (1)$$

If the NADPH thus generated is coupled to PC reductase, proline production will accompany the generation of oxidized pyridine nucleotide.

$$12\ \text{PC} + 12\ \text{NADPH} + 12\ H^+ \longrightarrow 12\ \text{PRO} + 12\ \text{NADP}^+ \quad (2)$$

Assuming that the protons from proline oxidation are contributed to the electron transport chain at the level of a flavoprotein, then:

$$12\ \text{PRO} + 6\ O_2 + 24\ \text{ADP} \longrightarrow 12\ \text{PC} + 6\ H_2O + 24\ \text{ATP} \quad (3)$$

Combining equations 1-3, we get:

$$6\ \text{G-6-P} + 24\ \text{ADP} + 6\ O_2 + 12\ H^+ \longrightarrow 5\ \text{G-6-P} + 6\ CO_2 + 6\ H_2O + 24\ \text{ATP}$$

For each mole of G-6-P completely oxidized by cycling through the HMP pathway, the proline cycle would allow for the generation of 24 moles of ATP, a stoichiometry which compares favorably to ATP production from the metabolism of G-6-P in both the Embden-Meyerhof pathway and the tricarboxylic acid cycle. We emphasize that we have yet to demonstrate the predicted stoichiometry. Reconstitution with purified enzymes e.g. PC reductase may be necessary to confine the substrates to the designated pathways. Nevertheless, our studies show that the proposed linkage does occur readily in an *in vitro* system.

Although our model links the proline cycle to the HMP pathway through NADPH, a link through NADH to NAD^+-dependent pathways may be also possible. In insect flight muscle, a coupling between

NADH oxidation and a "proline shuttle" has been demonstrated (12). The PC reductase in insect flight muscle is absolutely dependent on NADH as cofactor; NADPH is ineffective in supporting proline production from PC. In this regard mammalian species have the advantage of versatility in their PC reductase. Isozymes of PC reductase remain a likely possibility. Preferential affinity for NADPH as well as differential levels of activity catalyzed by saturating concentrations of the two pyridine nucleotides have been found in PC reductase from different mammalian cells (unpublished results). Thus, a NADH-preferring PC reductase, coupled to NAD^+-dependent metabolic pathways may yet be found for certain mammalian tissues. Nevertheless, we emphasize that our proposal, linkage of the HMP pathway to ATP generation by the proline cycle may be a specialization in higher animals i.e. mammalian species.

The physiologic consequence of the proline cycle at present remains obscure. However, we can speculate that for tissues with proline oxidase (liver, kidney, heart and brain) (13), the HMP pathway for glucose utilization may either augment ATP production under physiologic conditions with increased energy demand or may serve as a mechanism to substitute for a dysfunctional Embden-Meyerhof pathway and/or tricarboxylic acid cycle in pathophysiological states.

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