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Biochemistry

Regulation of proline oxidase activity by lactate

(proline metabolism/amino acid metabolism/gluconeogenesis/lactic acid)

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ABSTRACT **We found that proline oxidase, the first enzyme of the proline degradative pathway, is inhibited by lactate. The K_m of the enzyme for proline increases with increasing concentrations of lactate. Since proline can be a source for gluconeogenesis, regulation of proline degradation by lactate may serve as a mechanism for allocation of metabolic fuel sources. The marked inhibition of proline oxidase at levels of lactate that commonly occur in both genetic and acquired lactic acidosis may cause the previously unexplained hyperprolinemia seen in these metabolic disorders.**

Mammalian organisms have a number of endogenous sources of metabolic fuels. The regulated interaction between certain peripheral tissues and the liver is important in the utilization of these sources. With acute starvation, when tissue proteins serve as a source of metabolic fuel, the rate of muscle proteolysis and release of amino acids determines the rate of hepatic gluconeogenesis (1). In states characterized by high rates of peripheral glucose consumption, e.g., exercise, lactate formed by glycolyzing muscle serves as the main source of glucose produced by the liver (2). Since the conservation of tissue proteins is important, the regulation of the hepatic degradation of amino acids when alternative fuel sources are available would benefit the organism. We now report such a regulation: the inhibition of hepatic proline oxidase activity by lactate.

Proline oxidase catalyzes the first step in proline degradation and thus initiates a sequence of reactions whereby proline can directly contribute its carbons to the tricarboxylic acid cycle as α-ketoglutarate (Fig. 1). We have found that lactate increases the K_m of proline oxidase for substrate proline and thereby inhibits proline degradation. Physiologic fluctuations in lactate levels may regulate proline oxidase activity, thus providing a mechanism whereby the rate of proline degradation may be adjusted in accordance with the availability of alternative fuel sources.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (6 weeks af age) weighing 100–120 g were obtained from Zivic-Miller Co. Rats were maintained on ad lib. chow and water until they were killed.

Reagents. L-Lactic acid (crystalline), sodium pyruvate, sodium succinate, α-ketoglutarate, acetoacetate, fumarate, cytochrome *c* from equine heart muscle, iodonitrotetrazolium, and Dowex-50W, hydrogen form, 100–200 mesh, were obtained from Sigma Chemical Co. Bio-Beads SM-2 were from Bio-Rad Laboratories. L-Proline was obtained from Calbiochem. [*U*-^{14}C]Proline (230 mCi/mmol) was obtained from New England Nuclear.

Tissue Preparation. Mitochondria were prepared by a modification of the method of Chappell and Hansford (3). In brief, about 500 mg of rat liver was homogenized in 8 volumes of ice-cold 0.25 M sucrose, 3.4 mM Tris·HCl/1 mM EDTA at pH 7.4 with a Dounce tissue homogenizer. The homogenate was centrifuged at 500 × *g* for 10 min and the supernatant was centrifuged at 9000 × *g* for 7 min. The resulting pellet was resuspended in sucrose and recentrifuged at 9000 × *g* for 7 min. The washing process was repeated twice and the resultant mitochondrial pellet was dispersed in 0.5–1.0 ml of sucrose buffer and stored frozen at −20° prior to assay. These preparations have 2- to 2.5-fold enrichment of proline oxidase activity relative to whole homogenates. The activity of proline oxidase in these preparations was stable for several weeks.

Enzyme Solubilization. Proline oxidase was solubilized by the method of Kramar (4) with some modifications. Mitochondrial pellets containing about 30 mg of protein were dispersed into 2 ml of Triton X-100, 16 mM in 50 mM potassium phosphate/1 mM EDTA at pH 7.4, and were incubated at 4° for 1 hr. After incubation, the suspension was centrifuged at 100,000 × *g* for 1 hr. The solubilized enzyme preparation then was applied to a 1 × 5 cm column of SM-2 beads (1.5 g dry weight) to remove Triton X-100 (5). The enzyme was eluted with 50 mM potassium phosphate/1 mM EDTA at pH 7.4 at a flow rate of 0.3 ml/min and collected in 0.5-ml fractions. The fractions containing protein were pooled and stored in 0.1-ml aliquots at −20°.

Enzyme Assays. Proline oxidase was assayed by a previously published radioisotopic method (6). With [^{14}C]proline as the substrate, the reaction product, [^{14}C]pyrroline-5-carboxylate was reacted with *o*-aminobenzaldehyde, forming a dihydroquinazolinium compound which is recovered by Dowex column chromatography. Mitochondrial preparations containing 20–40 μg of protein were routinely assayed. For assay of activity in the solubilized preparation, iodonitrotetrazolium was added at a final concentration of 0.33 mM (4). Lactate was added in 50 μl of a stock solution which was neutralized with NaOH to the reaction pH of 7.2. Control tubes received 50 μl of H_2O. The methods for assaying the activities of ornithine-ketoacid aminotransferase (7), pyrroline-5-carboxylate reductase (8), and pyrroline-5-carboxylate dehydrogenase (9) have been published.

Protein Determination. The method of Lowry *et al.* was used (10).

RESULTS

Proline oxidase activity decreased with increasing lactate concentrations (Fig. 2). Lactate reduced proline oxidase activity by 50 and 95% at lactate concentrations of 1.4 and 10 mM, respectively. Although these observations suggested a role for lactate as an effector of this enzyme, additional characterization

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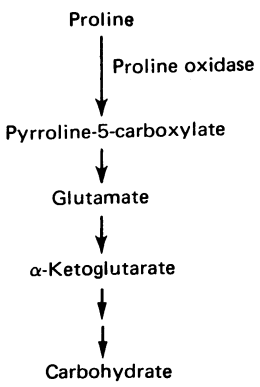

FIG. 1. Schematic representation of the degradative pathway for proline.

of the interaction was needed to rule out nonspecific and artifactual effects.

To verify that mitochondrial proline oxidase was inhibited by lactate, we showed that product formation with and without lactate was linear for the duration of incubation and protein concentration (data not shown). Thus, lactate depressed product formation under a variety of assay conditions. Furthermore, these results suggested that lactate itself, rather than a product derived from lactate, was the inhibitor.

The lactate effect was specific for proline oxidase. Lactate, even at high concentrations, had no effect on a number of other enzymes in the proline metabolic pathway (Table 1). Of note, the activity of ornithine-ketoacid aminotransferase, another mitochondrial enzyme, was not inhibited by lactate. Thus, nonspecific interference *in vitro* with mitochondrial integrity is unlikely as the basis for the effect on proline oxidase.

Proline oxidase is tightly bound to mitochondrial membranes and is functionally associated with the cytochrome *c* complex; therefore we considered the possibility that lactate was nonspecifically damaging a component of this enzyme complex. Mitochondria were incubated with 5 mM lactate for 30 min at 37°. To remove the lactate, we centrifuged mitochondrial particles at 9000 × *g* for 7 min, resuspended them in fresh sucrose, and recentrifuged. After this washing procedure the recoveries of proline oxidase activity from mitochondria incubated with or without lactate were comparable. Thus, the lactate inhibition is completely reversible, ruling out nonspecific destruction of the enzyme (Table 2).

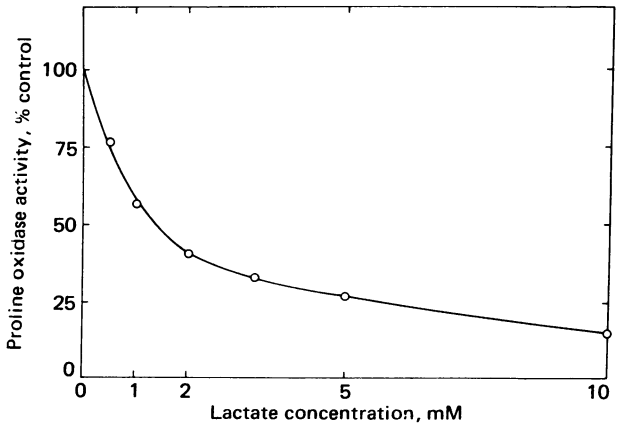

FIG. 2. Inhibition of proline oxidase activity by lactate. Proline oxidase activity in rat liver mitochondria (~20 μg of protein) was assayed with various concentrations of lactate. Proline concentration was 0.1 mM, and the duration of reaction was 20 min at 37°. The averages of at least two determinations are shown.

Table 1. Lactate effect on the enzymes of proline metabolism

Enzyme	Activity with lactate, % of control
Proline oxidase	26
Pyrroline carboxylate dehydrogenase	110
Ornithine-ketoacid aminotransferase	105
Pyrroline carboxylate reductase	80

Rat liver whole homogenate was used as the source of enzyme. Tissue extract containing 25–100 μg of protein was assayed in the presence and absence of 5 mM lactate. The activity in the presence of lactate is expressed as percent of control activity without lactate. Each value represents the average of at least two determinations.

With the validity of the lactate effect established, we considered whether the effect could be generalized to other intermediates of carbohydrate metabolism. We found that other than lactate, only pyruvate and succinate inhibited proline oxidase activity (Table 3). All other substances tested were without significant effect.

Since proline oxidase is bound to mitochondrial membranes, lactate inhibition of proline entry into mitochondria might have caused the apparent decrease in proline oxidase activity. Therefore, we examined the lactate effect on enzyme solubilized into 100,000 × *g* supernatants. The specific activity of proline oxidase in the solubilized preparation (14 nmol/hr per mg of protein) was less than that in mitochondria (82 nmol/hr per mg of protein). Nevertheless, the solubilized enzyme was inhibited by lactate (Fig. 3). In fact, the sensitivity to lactate of the solubilized enzyme was somewhat greater than that in mitochondria. This observation suggests that lactate inhibits proline oxidase by interacting with the catalytic complex rather than by blocking proline entry into mitochondria.

We determined the sensitivity of the solubilized enzyme to pyruvate and succinate, which inhibited enzyme activity in mitochondrial preparations (Table 3). Although significant inhibition was seen at high concentrations, pyruvate and succinate were much less effective than lactate in inhibiting proline oxidase activity (Fig. 3). Lactate produced 50% inhibition at a concentration of 0.5 mM, whereas pyruvate produced a comparable inhibition at about 3.0 mM.

The effect of succinate on proline oxidase appeared to be

Table 2. Recovery of proline oxidase activity after incubation with lactate

Conditions	Recovered enzyme: Activity, %	Recovered enzyme: Inhibition by lactate, %
Mitochondria incubated with lactate	71	83
Mitochondria incubated without lactate	57	82

Rat liver mitochondria (1.58 mg of protein) were incubated in 1.0 ml of 0.1 M phosphate buffer (pH 7.2) for 20 min at 37° in the presence or absence of 5 mM lactate. After incubation, 2 ml of ice-cold sucrose buffer (0.25 M sucrose/3.4 mM Tris/1 mM EDTA at pH 7.4) was added. The mitochondria were harvested by centrifugation at 9000 × *g* for 7 min. The resultant pellet, after two additional washes in sucrose buffer, was suspended in 0.5 ml of sucrose buffer. With this recovered enzyme, proline oxidase activity was determined in the presence and absence of lactate (5 mM). Each value represents the average of at least two determinations.

Table 3. Effect of various metabolic intermediates on proline oxidase activity

Additions	Proline oxidase activity, %
None	100
Lactate	18
Pyruvate	43
Succinate	56
α-Ketoglutarate	99
Fumarate	99
Oxalacetate	85
Acetoacetate	83
β-Hydroxybutyrate	88
α-Ketoisocaproate	85
α-Ketoisovalerate	85
α-Ketomethylvalerate	84

Rat liver mitochondria (10–20 μg of protein) were incubated in the presence or absence of the metabolic intermediates at a concentration of 5 mM. Proline concentration was 0.1 mM. Each value represents the average of at least two determinations.

indirect, at least in the solubilized preparation. Succinate (5 mM) inhibited enzyme activity less in 10-min reactions (6%) than in 20-min reactions (18%). In contrast, the percentage of inhibition with lactate and pyruvate was constant with reaction time. Furthermore, the presence of succinate greatly increased iodonitrotetrazolium reduction, whereas lactate and pyruvate did not (data not shown). Succinate-dependent iodonitrotetrazolium reduction presumably was catalyzed by succinate dehydrogenase. With the assumption that the succinate effect on proline oxidase activity was due to succinate-dependent depletion of iodonitrotetrazolium, we compared the effects of lactate, pyruvate, and succinate on proline oxidase at a higher concentration of iodonitrotetrazolium (Table 4). The results clearly showed that the succinate effect was decreased when the iodonitrotetrazolium concentration was increased from 0.33 to 0.50 mM. The lactate and pyruvate inhibition, on the other hand, was unaltered by the higher concentration.

We found that lactate inhibited proline oxidase by decreasing the affinity of the enzyme for substrate proline. Using Lineweaver–Burk analysis of Michaelis–Menten kinetics, we showed that 5 mM lactate increased the K_m from 2.3 to 15.1 mM, while V_{max} remained unchanged (Fig. 4). In fact, determinations of

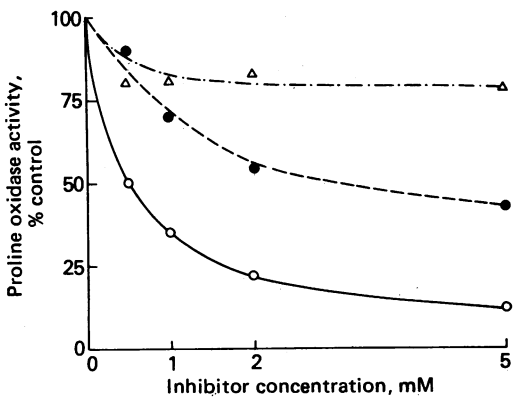

FIG. 3. Inhibition of solubilized proline oxidase. Proline oxidase was solubilized and the activity of the solubilized preparation was determined in the presence of various concentrations of (○) lactate, (●) pyruvate, or (△) succinate. About 50 μg of protein was used. Proline concentration was 0.1 mM, iodonitrotetrazolium was 0.33 mM, and the duration of incubation was 20 min. The values shown represent the average of duplicate determinations.

Table 4. Inhibition of solubilized proline oxidase

[Iodonitrotetrazolium], mM	Inhibition of proline oxidase activity, %		
	Lactate	Pyruvate	Succinate
0.33	88	54	18
0.50	95	59	5

Proline oxidase activity was assayed with either 0.33 or 0.50 mM iodonitrotetrazolium in the presence of inhibitors. The concentration of proline was 0.1 mM; the concentration of inhibitors was 5.0 mM. The duration of incubation was 20 min at 37°. Data are expressed as percent inhibition of control activity. Each value is the average of at least two determinations.

the K_m over a range of lactate concentrations showed a linear increase in K_m for proline with increasing lactate concentrations (Fig. 4 *Inset*). Thus, in the physiologic range of lactate and proline concentrations, the rate of proline degradation varies inversely with the concentration of lactate.

DISCUSSION

These studies show that lactate regulates proline degradation by decreasing the affinity of proline oxidase for proline and thereby inhibiting the activity of the enzyme. The lactate effect is specific to proline oxidase; other enzymes involved in proline metabolism, located either in cytosol or mitochondria, were unaffected by lactate. We demonstrated that lactate inhibited proline oxidase in solubilized preparations as well as mitochondria. In addition, the effect could be reversed with the removal of lactate. Thus, lactate appears to interact with the proline oxidase complex *per se* to produce the regulatory changes in its K_m for proline. Interference of proline entry into mitochondria or a nonspecific compromise of mitochondrial integrity are most unlikely as the basis for the lactate effect.

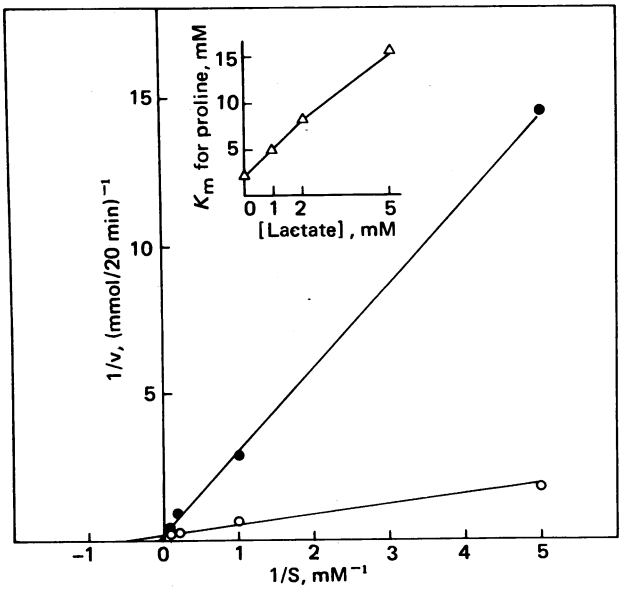

FIG. 4. Kinetics of lactate inhibition. Proline oxidase activity of rat liver mitochondria was determined at various concentrations of proline in the presence and absence of 5 mM lactate. Duration of incubation was 20 min, and about 20 μg of protein were assayed. A Lineweaver–Burk plot of the reciprocal of the activity, v, against the reciprocal of proline concentration, S, is shown. Control (○) and samples in the presence of 5 mM lactate (●) had the same V_{max} but different K_m values. (*Inset*) Based on Lineweaver–Burk analysis performed at various concentrations of lactate, the K_m of proline oxidase for substrate proline is shown as a function of lactate concentration during the assay.

The K_m of proline oxidase for substrate proline increases linearly with increasing lactate concentrations in the range of 0–5 mM. This observation reinforces the physiologic relevance of the effect since plasma lactates in normal human beings will increase from a resting level of about 1 mM to an exercise level of 4–5 mM (11). In addition, hepatic levels of lactate will vary within a similar concentration range (12). Thus, proline oxidation is sensitive to physiologic fluctuations of lactate. With increasing lactate, the decrease in affinity of the enzyme for proline will reduce markedly the rate of proline degradation.

Although pyruvate also inhibited proline oxidase activity, the inhibition observed *in vitro* is probably unimportant physiologically. Unlike lactate, pyruvate does not attain *in vivo* levels sufficiently high to produce significant inhibition (11). Additional studies are required, however, to define the effects of pyruvate on the enzyme.

The physiologic relevance of the lactate effect on proline oxidase that we have shown *in vitro* is supported by studies *in vivo* in patients with inherited and acquired lactic acidosis. If our *in vitro* observations are valid, the hyperlactacidemia in these patients would block proline degradation and produce hyperprolinemia. Indeed, in studies of two infants with inherited metabolic disorders resulting in lactate levels 8- to 10-fold normal, Haworth *et al.* found that plasma proline levels were elevated 2- to 3-fold (13). Similarly, Marliss *et al.* reported eight patients with lactic acidosis secondary to a variety of diseases. These patients had proline levels 5- to 6-fold normal in the face of elevated plasma lactates (14). Thus, in patients with pathophysiological elevations in plasma lactates, plasma proline was concomitantly elevated. We recognize, however, that the metabolic derangements in these patients are complex and the causal relationship between lactic acidosis and hyperprolinemia has not been established. Nevertheless, the demonstrated effect of lactate on proline oxidase seems a plausible explanation for the *in vivo* correlation.

Lactate regulation of proline oxidase may be important not only for hepatic disposition of proline, but also for hepatic carbohydrate formation from this amino acid. As noted, proline, undergoing sequential dehydrogenations, yields α-ketoglutarate, an intermediate of the tricarboxylic acid cycle, and a glucose precursor. Indeed, Greth *et al.*, injecting [^{14}C]proline into animals, found that disappearance of the label from plasma was followed by reappearance of ^{14}C in the circulation primarily as glucose (W. Greth, S. O. Thier, and S. Segal, unpublished). Preliminary work in our laboratory suggests that liver minces *in vitro* readily incorporate proline carbons into free glucose released into the incubation medium (E. M. Kowaloff, A. S. Granger, and J. M. Phang, unpublished). Our recent demonstration that glucocorticoids, known stimulators of gluconeogenesis, induce proline oxidase further supports the role of proline as a glucose source (15).

The physiologic relationship of proline to alanine, the principal amino acid precursor for hepatic gluconeogenesis (1), remains unclear. Since alanine taken up by liver is converted to pyruvate and lactate, inhibition of proline oxidation would be an expected concomitant of increased alanine uptake by the liver. The possibility that gluconeogenesis from these two amino acid precursors might be interrelated, perhaps in a reciprocal fashion, deserves additional study.

Considered as a model of amino acid degradation and the regulated provision of metabolic fuel, the lactate effect may play a role in coordinating hepatic fuel allocation with muscle requirements during and immediately after exercise. In exercise the major fuel consumed by working muscle is glucose supplied largely by glycogenolysis. In the face of accelerating lactate production by working muscle, plasma lactate in human beings increases 4- to 5-fold (11). This increased lactate is taken up by liver and converted to glucose. Thus, with exercise, the Cori cycle is accelerated. On the other hand, hormonal changes accompanying exercise, i.e., increased catecholamines, increased glucagon, and decreased insulin levels, results in decreased muscle proteolysis and decreased release of alanine and glutamine. Kipnis has suggested that these hormone-mediated changes in muscle preserve muscle protein in the face of decreased rates of protein synthesis in muscle secondary to depressed insulin levels (16). In accord with these adaptive changes in muscle and liver during exercise, a lactate-mediated inhibition of hepatic amino acid degradative enzymes would harmonize fuel allocation with muscle needs and would spare amino acids for protein synthesis after exercise. This model suggests that other amino acid degrading systems also may be sensitive to lactate inhibition. In fact, patients with pathological lactic acidosis have plasma elevations and depressed hepatic degradation of a number of amino acids (14).

In addition to the regulation of proline degradation, lactate inhibition of proline oxidase may affect the metabolic fate of lactate itself. Recent studies in isolated hepatocytes suggest that glycogen formation from lactate requires the presence of glutamate or glutamine. Without these amino acids, glycogen was not formed from lactate. In contrast, glucose formation from lactate does not have this amino acid requirement (17). Glutamate and glutamine are products of proline degradation. The inhibition of proline oxidase with increased lactate uptake by the liver, e.g., with exercise, would decrease glutamate and glutamine production and lead to decreased glycogen deposition. Thus, lactate would be channeled into glucose to meet the acute demands of peripheral tissues.

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