Enzymes Related to Lactate Metabolism in Green Algae and Lower Land Plants[1]

Peter J. Gruber,[2] Sue Ellen Frederick,[2] and N. E. Tolbert
Department of Biochemistry Michigan State University, East Lansing, Michigan 48823

ABSTRACT

Cell-free extracts of *Chlorella pyrenoidosa* contained two enzymes capable of oxidizing D-lactate; these were glycolate dehydrogenase and NAD+-dependent D-lactate dehydrogenase. The two enzymes could be distinguished by differential centrifugation, glycolate dehydrogenase being largely particulate and NAD+-D-lactate dehydrogenase being soluble. The reduction of pyruvate by NADH proceeded more rapidly than the reverse reaction, and the apparent Michaelis constants for pyruvate and NADH were lower than for D-lactate and NAD+. These data indicated that under physiological conditions, the NAD+-linked D-lactate dehydrogenase probably functions to produce D-lactate from pyruvate.

Lactate dehydrogenase activity dependent on NAD+ was found in a number of other green algae and in the green tissues of a few lower land plants. When present in species which contain glycolate oxidase rather than glycolate dehydrogenase, the enzyme was specific for L-lactate rather than D-lactate. A cyclic system revolving around the production and utilization of D-lactate in some species and L-lactate in certain others is proposed.

The enzyme glycolate dehydrogenase occurs in many species of green algae, where an analogous enzyme, glycolate oxidase, is present in certain other species of green algae, as well as in green tissues of lower and higher land plants (3, 15). Although the two enzymes both oxidize glycolate to glyoxylate and thereby participate in the glycolate pathway, they differ in several of their biochemical properties, one of these being their capacity to use stereoisomers of lactate as alternate substrates (15). Whereas glycolate oxidase can oxidize L-lactate but not D-lactate in addition to glycolate, glycolate dehydrogenase can oxidize D-lactate but not L-lactate. In fact, in a number of algal species containing glycolate dehydrogenase, levels of activity with D-lactate are higher than those with glycolate (3).

There have also been reports that some algae contain an NAD+-dependent D-lactate dehydrogenase (17, 26). However, the relationship between this activity and glycolate dehydrogenase has remained unclear. This study was undertaken to ex-

amine whether NAD+-linked D-lactate dehydrogenase and glycolate dehydrogenase, both of which can oxidize D-lactate, represent identical or separate enzymes. Further work on the NAD+-dependent D-lactate dehydrogenase activity led to a survey of its occurrence among green plants. The combined results have provided some insight into the possible physiological role and significance of lactate metabolism in many green algae.

MATERIALS AND METHODS

Stocks of *Chlorella pyrenoidosa* Chick (No. 395) and *Dunaliella tertiolecta* Butcher (No. LB 999) were obtained from the Indiana University Culture Collection (21). The two species were cultured autotrophically and as axenically as possible, *C. pyrenoidosa* in the mineral medium V of Norris *et al.* (16) and *D. tertiolecta* in the mineral medium of McLachlan (14). For experiments, the algae were grown in 2800-ml Fernbach flasks at 18 to 20 C under continuous light of approximately 600 ft-c provided by both incandescent and fluorescent lamps. Flasks containing *C. pyrenoidosa* were shaken, while flasks containing *D. tertiolecta* remained stationary; both were gassed by bubbling air into the culture medium. For the survey (Tables III and IV), various species of green algae and land plants were employed; the source, method of culture, and procedure of homogenization of these plants are described elsewhere (3).

Cell-free extracts of *C. pyrenoidosa* were prepared according to the following routine. The algae were collected by centrifugation and rinsed two to three times with deionized water. The washed cells, suspended in 5 to 10 volumes of 0.05 M K-phosphate (pH 7.0), were then passed twice through a chilled French pressure cell at 12,000 to 14,000 p.s.i. The resulting homogenate was centrifuged at 500g for 10 min to remove debris and unbroken cells. The 500g supernatant was either used directly for enzymatic determinations or further fractionated by differential centrifugation and sometimes by $(NH_4)_2SO_4$.

The reaction catalyzed by NAD+-dependent lactate dehydrogenase was assayed in both directions; assays were performed spectrophotometrically at 25 C by following changes in absorbance at 340 nm. For the determination of pyruvate reduction, the 1.0-ml reaction sample contained 0.1 M K-phosphate (pH 6.2), 0.01% Triton X-100, 0.09 mg/ml NADH, enzyme preparation, and 10 mM K-pyruvate. For the measurement of the reverse reaction, that involving the oxidation of either D-lactate or L-lactate, the 1.0-ml reaction sample contained 0.1 M Na-pyrophosphate (pH 9.2), 0.01% Triton X-100, 2 mg/ml NAD+, enzyme preparation, and 50 mM D(−)-lactate (Li salt) or 50 mM L(+)-lactate (Li salt). For both directions, the endogenous rate was monitored for 2 to 3 min, then the reaction was initiated by addition of substrate. Glycolate dehydrogenase activity was measured as described by Frederick *et al.* (3). The assay for NADH-hydroxypyruvate reductase was the same as for pyruvate reductase, but the substrate was 10 mM

[2] Present address: Department of Biological Sciences, Mount Holyoke College, South Hadley, Mass. 01075.

Table I. *Pyruvate Reductase and Lactate Dehydrogenase Activities in Chlorella Extracts*

A fraction of the supernatant containing 0.15 to 0.35 mg of protein from the homogenate after centrifuging at 12,000g for 30 min was used for these determinations.

Substrate	Cofactor	
		nmoles/min·mg protein
Pyruvate	NADH	73
D-Lactate	NAD+	13
L-Lactate	NAD+	0

Li-hydroxypyruvate. Protein was determined according to Lowry et al. (13).

RESULTS

Cell-free preparations from *Chlorella* were found to have the enzymatic capacity to reduce pyruvate using NADH. Lactate, the product of this reaction, was not analyzed directly for stereoisomerism in our studies, but previous work has indicated that it has the D configuration (26). When the reverse enzyme reaction was measured employing NAD+ as oxidant of lactate, activity was observed with D-lactate but not with L-lactate (Table I). These results indicate that the enzyme under consideration was the NAD+-linked D-lactate dehydrogenase of *Chlorella* already described by Warburg et al. (26). In this paper, it is referred to as a reductase or dehydrogenase, depending upon the assay direction.

Experiments were then conducted to establish whether this enzyme is distinct from glycolate dehydrogenase, the algal enzyme which also oxidizes D-lactate (3, 15). It was possible to separate NAD+-linked D-lactate dehydrogenase activity from that of glycolate dehydrogenase by differential centrifugation. In the case of *Chlorella*, 75 to 80% of the glycolate dehydrogenase activity, measured in terms of either glycolate or D-lactate oxidation with DCPIP[3] as the electron acceptor, remained particulate even following harsh preparative procedures, wheras the NAD+-dependent D-lactate dehydrogenase activity, including the reaction in the other direction using pyruvate and NADH, was almost totally solubilized (Table II). Studies with (NH4)2SO4 fractionation reinforced this finding, as most of the glycolate dehydrogenase but less than 10% of the NAD+-D-lactate dehydrogenase activity was precipitated by 35% saturated (NH4)2SO4 at 4 C. In another green algae, *Dunaliella*, glycolate dehydrogenase activity was again largely particulate. When homogenates of this species were centrifuged on sucrose gradients, at least 50% of the activity entered into the gradient, and the profile of activity followed closely that of the mitochondrial marker, cytochrome *c* oxidase (data not shown). The possible mitochondrial location of glycolate dehydrogenase will have to be confirmed by further electromicroscopic and cytological investigations.

With *Dunaliella*, it is possible to distinguish glycolate dehydrogenase and NAD+-lactate dehydrogenase further by their relative stabilities in homogenates prepared by sonication. After this treatment, glycolate dehydrogenase activity measured with either glycolate or D-lactate as substrate remained stable over a period of at least several hours; on the other hand, 85% of the NADH-pyruvate reductase activity (only this direction of the reaction was measurable in *Dunaliella*) vanished within 1 hr (Fig. 1).

Chlorella extracts were used to determine certain parameters

relating to the NAD+-linked D-lactate dehydrogenase reaction. With 37,000g supernatants, the apparent K*m* values in the direction of pyruvate reduction were 1 mM for pyruvate and 60 μM for NADH; in the direction of D-lactate oxidation, they were 40 mM for D-lactate and 0.5 mM for NAD+. Attempts were made to detect glycolate oxidation with NAD+ as a cofactor, but no reaction whatsoever was observed at pH 9.2, even with final concentrations of glycolate as high as 60 mM. Fractions enriched in NAD+-D-lactate dehydrogenase always retained some capacity to oxidize D-lactate using DCPIP, but since these fractions also retained a proportional ability to oxidize glycolate using DCPIP, this activity can be attributed to

Table II. *Distribution of Glycolate Dehydrogenase and NAD+-Linked D-Lactate Dehydrogenase Activity following Differential Centrifugation*

The homogenate from *Chlorella* was centrifuged at 500g for 10 min. The resulting supernatant was centrifuged at 37,000g for 45 min.

| Enzyme	Substrates	Fraction	Total Activity		Specific Activity
			nmoles/min	%	*nmoles/min·mg protein*
Glycolate dehydrogenase	Glycolate, DCPIP	500g supernatant	1780	100	18
		37,000g pellet	1330	75	22
		37,000g supernatant	429	24	10
	D-Lactate, DCPIP	500g supernatant	1570	100	16
		37,000g pellet	1270	81	21
		37,000g supernatant	356	23	8
NAD+-D-lactate dehydrogenase	Pyruvate, NADH	500g supernatant	3690	100	37
		37,000g pellet	151	4	3
		37,000g supernatant	3600	98	84
	D-Lactate, NAD+	500g supernatant	1100	100	11
		37,000g pellet	40	4	1
		37,000g supernatant	1050	94	24
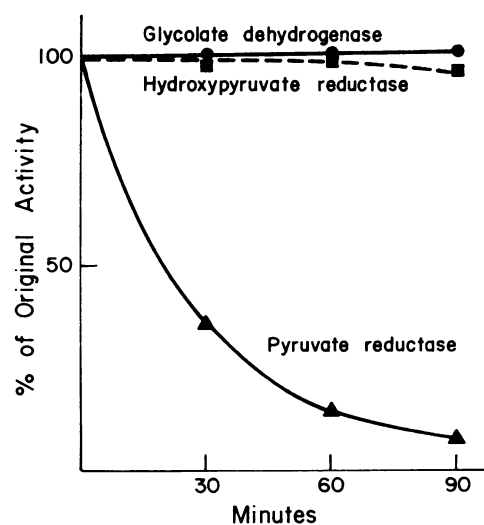
FIG. 1. Relative stabilities of three enzymes in an extract of *Dunaliella*. Cells suspended in 0.05 M Tricine, pH 7.4, were sonicated for 30 sec, then centrifuged at 270g for 5 min. The 270g supernatant was assayed immediately (time zero) and at subsequent intervals during storage at 4 C. Glycolate dehydrogenase (●); NADH-pyruvate reductase (▲); hydroxypyruvate reductase (■).

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[3] Abbreviation: DCPIP: 2,6-dichlorophenolindophenol.

contamination by glycolate dehydrogenase. Thus, it is probable that the NAD⁺-linked D-lactate dehydrogenase does not use DCPIP as electron acceptor.

The possibility that NADH-pyruvate reductase activity was catalyzed by the enzyme hydroxypyruvate reductase, which is highly active in all green tissues, was also explored. With a preparation from *Dunaliella*, hydroxypyruvate reductase activity was stable over a 90-min test span, whereas pyruvate reductase activity was not stable (Fig. 1). When *Chlorella* extracts were fractionated by $(NH_4)_2SO_4$, twice as much of the pyruvate reductase as the hydroxpyruvate reductase activity was precipitated by 55% saturated $(NH_4)_2SO_4$. These and similar unreported data give preliminary indications that pyruvate reductase and hydroxypyruvate reductase in algae are different enzymes as they are in higher plants (23).

In conjunction with studies on the distribution of glycolate dehydrogenase and glycolate oxidase among green plants (3), species representative of various levels of evolutionary advancement were also surveyed for their ability to reduce pyruvate using NADH. As shown in Table III, pyruvate reductase activity was detected in many but not all of the algal species which contain glycolate dehydrogenase; likewise, it was found in several but not all of the algal species which have glycolate oxidase. A few lower land plants also had NADH-pyruvate reductase activity in their green tissues but the majority of them, as well as several angiosperms, did not contain such activity.

Extracts of some green algae and lower land plants having NADH-linked pyruvate reductase activity were assayed in the reverse direction to establish whether the enzyme involved is stereospecific for D- or L-lactate. Generally, the oxidation of

Table III. *Levels of NADH-Pyruvate Reductase Activity in the Green Tissues of Various Land Plants and in Green Algae*

Species	Enzyme Activity	Type of Glycolate Oxidizing Enzyme (ref. 3)
	nmoles/ min·mg protein	
Liverworts		
Marchantia sp.	0	Glycolate oxidase
Porella sp.	22	Glycolate oxidase
Pellia sp.	69	Glycolate oxidase
Mosses		
Leptodictyum riparium	17	Glycolate oxidase
Polytrichum sp.	0	Glycolate oxidase
"Fern Allies"		
Psilotum nudum	17	Glycolate oxidase
Selaginella apoda	0	Glycolate oxidase
Equisetum sp.	0	Glycolate oxidase
Ferns		
Osmunda sp.	0	Glycolate oxidase
Anthyrium sp.	0	Glycolate oxidase
Onoclea sensibilis	0	Glycolate oxidase
Angiosperms		
Myriophyllum sp.	0	Glycolate oxidase
Lemna minor	0	Glycolate oxidase
Spinacia oleracea	0	Glycolate oxidase
Green Algae		
Chlorella pyrenoidosa	30–80	Glycolate dehydrogenase
Chlorella vulgaris	21	Glycolate dehydrogenase
Dunaliella tertiolecta	75	Glycolate dehydrogenase
Eremosphaera viridis	54	Glycolate dehydrogenase
Oocystis polymorpha	25	Glycolate dehydrogenase
Protosiphon botyroides	0	Glycolate dehydrogenase
Codium sp.	0	Glycolate dehydrogenase
Microspora sp.	83	Glycolate dehydrogenase
Stigeoclonium helveticum	0	Glycolate dehydrogenase
Coleichaete scutata	29	Glycolate oxidase
Klebsormidium flaccidum	20	Glycolate oxidase
Nitella sp.	0	Glycolate oxidase
Spirogyra varians	21	Glycolate oxidase
Netrium digitus	0	Glycolate oxidase

Table IV. *Stereospecificities of NAD⁺-Lactate Dehydrogenase*

Species	D-Lactate	L-Lactate
	nmoles/min·mg protein	
Porella sp.	0	+
Psilotum nudum	0	3
Chlorella pyrenoidosa	11	0
Microspora sp.	8	0
Dunaliella tertiolecta	0	0
Eremosphaera viridis	0	0
Coleochaete scutata	0	4
Klebsormidium flaccidum	0	4

either D- or L-lactate proceeded very slowly or, as in the case of *Dunaliella* and *Eremosphaera*, not at all. The results obtained from the species with measurable activity indicate that the enzyme is a D-lactate dehydrogenase in at least two species but an L-lactate dehydrogenase in certain others (Table IV). From this limited survey and that conducted previously on the enzymes which oxidize glycolate (see Table III), an interesting, though necessarily tentative, correlation emerges: the NAD⁺-linked lactate dehydrogenase present in those species containing glycolate dehydrogenase is specific for D-lactate, while the NAD⁺-linked lactate dehydrogenase present in those species containing glycolate oxidase is specific for L-lactate.

DISCUSSION

Our findings indicate that there are two distinct enzymes capable of reaction with D-lactate in certain algal species such as *Chlorella*. One enzyme, glycolate dehydrogenase, is particulate (see also 6, 12, 27) and is probably associated with a membrane; its oxidation of D-lactate can be coupled to DCPIP but not NAD⁺. The second enzyme, D-lactate dehydrogenase (or pyruvate reductase, if the other direction of the reaction is considered), is soluble and utilizes NAD⁺ but not DCPIP as electron acceptor. Despite some differences in substrate and electron acceptor specificity, the algal glycolate dehydrogenase appears related to the class of NAD⁺-independent D-lactate dehydrogenases found in various other organisms. Enzymes in this category include the D-lactate dehydrogenase present in bacterial plasma membranes (9, 10), the D-lactate cytochrome *c* reductase from membrane preparations of aerobically grown yeasts (7, 8, 20), and the "mitochondrial" D-α-hydroxy acid dehydrogenase from mammals (1, 24, 25). The algal NAD⁺-D-lactate dehydrogenase, on the other hand, clearly belongs to a different class of enzymes, representatives of which occur also in bacteria (2, 22), in a slime mold (4), in fungi (5, 18), and in certain invertebrates (11, 19). These invariably are dependent on NAD⁺ and are soluble.

In extracts of *Chlorella* and other species having lactate dehydrogenase activity, the rate of pyruvate reduction with NADH at the physiological pH is much faster than the rate of lactate oxidation with NAD⁺ in the reverse direction. The apparent Km values for the pyruvate reductase reaction are also considerably lower than those for the reverse reaction, as determined from *Chlorella*. These observations suggest that the physiologically operative direction of this reaction is in the production of D-lactate from pyruvate. That *Chlorella* suspensions grown anaerobically produce D-lactate (26) provides evidence that the reduction of pyruvate does, in fact, take place in intact cells.

It is our view, then, that in certain green algae there occurs one enzyme which produces D-lactate and a second enzyme, glycolate dehydrogenase, which can metabolize it. On the other
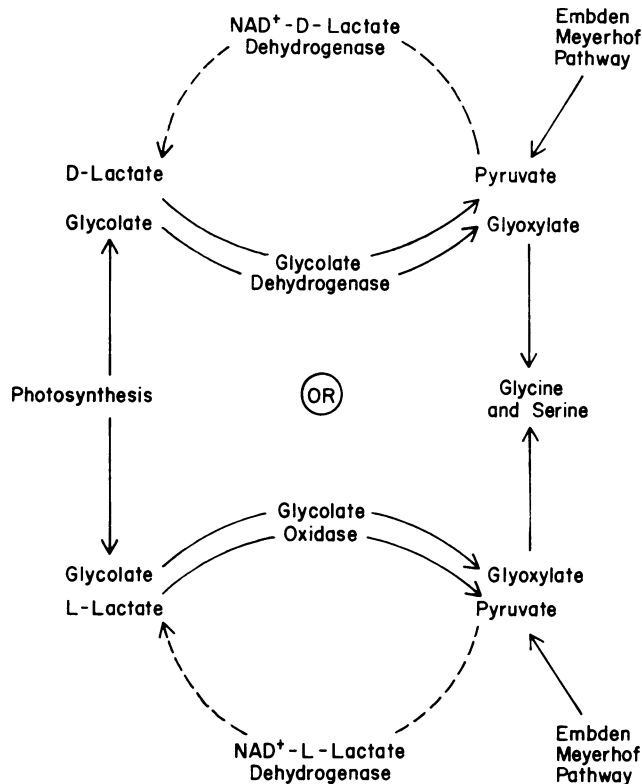
FIG. 2. Proposed cyclic systems mediated by glycolate oxidizing enzymes and NAD$^+$-lactate dehydrogenases.

hand, when an NAD$^+$-linked lactate dehydrogenase is present in a plant containing glycolate oxidase, it is specific for L-lactate rather than D-lactate. Glycolate oxidase can use L-lactate but not D-lactate as an alternative substrate and presumably would be able to oxidize the L-lactate produced by the L-lactate dehydrogenase. Based on these observations, it is proposed that in certain green algae and a limited number of lower land plants, a cyclic system exists, revolving around the production and utilization of one or the other stereoisomer of lactate (Fig. 2). According to this scheme, glycolate dehydrogenase and glycolate oxidase would each serve dual roles: (a) they would oxidize glycolate to glyoxylate and thus be involved in the glycolate pathway, and (b) they would, under certain conditions at least, oxidize D-lactate or L-lactate to pyruvate and thereby participate with NAD$^+$-linked lactate dehydrogenase in some form of a pyruvate-lactate shuttle.

While it is known that the oxidation of glycolate comprises a key step in the metabolism of a major photosynthetic product, the physiological function of lactate-pyruvate interconversions in green cells remains to be established. A bacterial system has been detailed, however, which provides a useful model for interpreting the enzymic situation exemplified by Chlorella. In E. coli, a membrane-associated enzyme which oxidizes D-lactate to pyruvate occurs along with a soluble enzyme, NAD$^+$-D-lactate dehydrogenase, which produces D-lactate from pyruvate. The shuttle operated by these two enzymes is similar to that proposed in Figure 2 and is responsible for the transport of various compounds into bacterial cells (9). Because of the clear analogy here, it is suggested that the algal D-lactate-pyruvate system may be involved in transport functions.

At the same time, it should be recognized that whatever the role of the two enzymes in Chlorella, a NAD$^+$-linked lactate

dehydrogenase is apparently not essential for the successful functioning of a number of green algae and of the green tissues of many land plants. Although an L-lactate-pyruvate cycle may be operative in those species of algae and lower land plants having both glycolate oxidase and L-lactate dehydrogenase, it would seem to be lacking in Nitella, Netrium, and most land plants. In green tissues of these species, the capacity to oxidize L-lactate to pyruvate via glycolate oxidase remains, but the ability to reduce pyruvate back to L-lactate has not been detected.

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