

CHAPTER 36

Factors Affecting *Desulfovibrio desulfuricans* Lactate Dehydrogenase

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Lactate dehydrogenase activity in *Desulfovibrio desulfuricans* has a K_m of 36.3×10^{-5} moles at 20 C. The enzyme activity was stable between pH 5.8 and 8.4. The temp of maximum enzyme activity was between 40 and 42 C. The enzyme is particulate in nature but treatment with detergents (1% Triton X-100, 2% sodium cholate or sodium deoxycholate) appear to solubilize it from the membrane. This solubilized enzyme is specific for D(-) lactate. The enzyme is sensitive to sodium cyanide, sodium azide, and EDTA.

INTRODUCTION

Sulfate-reducing bacteria are organisms of economic importance. They cause corrosion of steel and iron pipes and oil well casings. Corrosion of metals occurs by two mechanisms: cathodic depolarization by hydrogenase and anodic attack by H_2S (King and Miller 1971). Booth et al. (1967) found that corrosion was proportional to growth, implying that an adequate carbon and energy source must be available in the environment.

Desulfovibrio sp. are found in stored natural and manufactured gas, both above and underground, producing H_2S which is both corrosive and offensive. In underground storage sites sulfate reducers, together with other organisms, are introduced with the drilling muds. They cause blockages in the storage sand and corrosion of secondary recovery systems (Pankhurst 1968).

Desulfovibrio sp. may be used to modify effluents from acid mine water (Tuttle et al. 1969). A mixed culture system with wood dust cellulose in acid water produces carbon and energy sources which are used by sulfate reducers. The reduction of sulfate yields FeS , with a decrease in the acidity of the water. Sulfate-reducing bacteria also are found in oil deposits. A pseudomonad capable of generating substrates for sulfate reducers also was found (Kuznetzova and Gorlenko 1965).

Substrates used by *Desulfovibrio* sp. as carbon and energy sources are lactate, pyruvate, ethanol, fumarate, malate, choline, etc. The preferred substrate for cultural growth of *Desulfovibrio* is lactate (Postgate and LeGall 1973). In these organisms lactate dehydrogenase (LDH) oxidizes lactate to pyruvate, whereas in most other organisms the opposite transformation occurs. Pyruvate in *Desulfovibrio* sp. is further metabolized to acetate with the generation of 2 ATP's (Senez 1962). The hydrogen atoms generated by the oxidation are used to reduce sulfate to H_2S . A cytochrome C_3 takes up electrons after dehydrogenation of pyruvate and electron transport phosphorylation occurs with the generation of ATP (Decker et al. 1970). Perhaps in the oxidation of lactate a cytochrome C_3 takes up those electrons.

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Barton and Peck (1971) have shown that the LDH in *Desulfovibrio gigas* is membrane bound. Rossmoore et al. (1964) presented evidence that lactate oxidation is controlled by an iron-containing enzyme. The electron acceptor for LDH is neither a NAD nor NADP (Barton and Peck 1971). The possibility of FAD linkage to LDH as reported by Kohn and Kabak (1973) for *Escherichia coli* does exist. The sensitivity to heme inhibitors (Rossmoore et al. 1964) indicates that a cytochrome or other iron-containing electron carrier also could be involved in LDH (Boeri and Tosi 1956).

This presentation deals with the partial isolation and the characterization of some properties of *D. desulfuricans* LDH to better understand its biological role.

MATERIALS AND METHODS

Desulfovibrio desulfuricans (ATCC 7757) was grown at 30 C on Postgate's medium (Postgate 1966) modified by using 0.0001% instead of 0.01% FeSO₄.

Lactate Metabolism: Effect of Lactate Concn, pH, and Temp

Cells were harvested between 48 and 72 h after inoculation and were washed twice in 0.1 M sodium phosphate buffer (pH 7) and resuspended in the same buffer to a transmission density of 5% at 525 nm. This corresponds to 6.4×10^8 bacteria/ml. Lactate reduction in whole cells was determined by the method of Thunberg (Umbreit et al. 1957) using methylene blue ($E = 84,200 \text{ M}^{-1} \text{ cm}^{-1}$) as electron acceptor and monitoring reduction by absorbance decrease at 660 nm.

The effect of lactate concn on lactate reduction was measured in a Bausch and Lomb Spectronic 20 spectrophotometer at 20 C, using 1 ml bacterial suspension, 0.3 ml methylene blue (1:10,000), and 4 ml of 0.1 M sodium phosphate pH 7.0 with appropriate sodium lactate concn (Table 1). The effect of pH on lactate metabolism at a substrate concn of 0.3% sodium lactate in appropriate buffer solution (Table 3) was measured on a Cary 118 spectrophotometer at 20 C. Temperature effect on metabolism was measured between 5 and 57 C, using 0.3% sodium lactate in sodium phosphate buffer pH = 7. A Lauda K-2/RD water bath was attached to a special tube holder in a Cary spectrophotometer to give the temp.

Characterization of LDH

Assay procedure The assay mixture consisted of 4 ml 3.0% sodium lactate in 0.05 M phosphate buffer (pH 7.6), 0.3 ml of 0.01% 2,6-dichloroindophenol (DCIP), and 0.1 or 0.4 ml bacterial extract. Absorbancy decrease was monitored at 600 nm. Enzyme activity is reported in absorbance/ml/min of bacterial extract (see tables). Exceptions to this procedure are noted in the text.

Location. Cells (0.7 g) were washed and resuspended in 0.05 M sodium phosphate buffer (pH 7.7) with 0.5 mM mercaptoethanol and were disrupted in a French pressure cell (18,000 to 24,000 psi). The lysed cells were centrifuged at 8,000 rpm ($7,710 \times g$) in a SR 25 rotor (Servall Type RC-2) for 10 min. Supernatant then was centrifuged at 37,000 rpm ($90,221 \times g$) in a Ti 50 rotor (Servall OTD65) for 90 min. LDH activity was determined for both supernatant fractions.

Removal of LDH from Membrane

Sonication Pressure-ruptured cells were sonicated for various time intervals at 60,000 cycles/min in a Sonic 300 Dismembrator and centrifuged at $90,221 \times g$ for 90 min. LDH activity was determined before and after centrifugation.

Chemical Techniques

Cells were washed in 0.05 M sodium phosphate buffer, pH 7.7, with 1 mM mercaptoethanol, resuspended in the sodium phosphate buffer, and disrupted by two cycles through a French pressure cell (15,000 to 22,000 psi). The disrupted cells were centrifuged at $7,710 \times g$ for 10 min at 4 C. Ammonium sulfate was added to the supernatant (1.7 g ammonium sulfate/10 ml), mixed for 20 min, and centrifuged at $30,900 \times g$ for 30 min at 4 C. The pellet was resuspended in the same buffer.

Sodium Perchlorate (Penefsky and Tzagoloff 1971). Sodium lactate was added to the suspension to yield a final concn of 0.1% solution (w/v). Appropriate amounts of 8 M sodium perchlorate were added to get the desired concn. This was stored anaerobically for 30 min. The samples were centrifuged at $39,100 \times g$ or $90,221 \times g$ for 1 h at 4 C and the LDH activity was determined for the supernatant. Sodium perchlorate was removed from one sample by chromatography on a Sephadex G 25 column.

Triton X-100, Sodium Deoxycholate, and Sodium Cholate. The resuspended pellet from above was divided into 10-ml samples into which appropriate detergent was added to give final concn of 0 to 2%. Some samples were sonicated for 30 sec to facilitate enzyme solubilization. The samples were stored in evacuated Thunberg tubes to prevent oxidation of the enzyme for between 30 and 60 min at 4 C and centrifuged at $30,900 \times g$ for 30 min or $89,463 \times g$ for 60 to 90 min. Supernatant and pellet were assayed for LDH activity.

Effects of Inhibitors on LDH Activity

Cells (0.98 g) were washed and resuspended in 0.1 M phosphate buffer, pH 7.4, with 1 mM mercaptoethanol. The cells were disrupted in a French pressure cell at 20,000 psi. Ten percent Triton X-100 was added to yield a final solution of 0.025%. This was sonicated in an ice bath for 9 min at 80,000 cycles/min. The suspension was centrifuged at $30,900 \times g$ for 20 min and the supernatant was centrifuged further at $90,221 \times g$ for 60 min. The supernatant was divided into 10-ml aliquots in which inhibitors tetrasodium ethylenediamine tetraacetate (EDTA), sodium azide, and sodium cyanide were added separately to determine their effect on LDH activity. Assay mixture consisted of 4 ml of buffered (pH 7.4) 0.3% sodium lactate, 1 ml of enzyme preparation, and 0.3 ml methylene blue.

In another experiment, one which followed the procedure for removal of LDH from the membrane using sodium cholate, the effect of EDTA again was tested. The assay procedure used DCIP instead of methylene blue.

Specificity of LDH

The LDH was tested for specificity using D(-) and L(+) lactate. Analogs of lactate also were tested. These included D,L β -hydroxybutyrate, ethanol, malate, tartrate, and succinate.

The LDH was prepared using the same procedure as described under Chemical Techniques, above. The suspension was centrifuged at $90,221 \times g$ for 90 min. The assay consisted of 0.1 ml enzyme extract, substrate in 0.05 M tris buffer, pH 7.6, and 0.3 ml DCIP.

RESULTS AND DISCUSSION

From the data evaluating the effect of lactate concn on lactate metabolism (Table 1), a plot of $1/\text{substrate}$ vs. $1/\text{velocity}$ gave a K_m for lactate metabolism in whole cells as 3.6×10^{-4} moles and a V_{max} of 1.05×10^{-8} moles/min. The pH and temp optima (Tables 2, 3) indicate a wide stability for this reaction. The chemical composition of the buffers appeared not to affect enzyme activity because of similar LDH rates between pH 5.8 and 8.4 which encompassed three buffer agents. Considering the broad distribution in nature of *D. desulfuricans*, the stability of the lactate reduction system over a wide range of pH and temp suggests that it may play an important role in the energy-requiring metabolism of this species.

After pressure disruption and centrifugation at $95,163 \times g$ for 1 h, examination of the supernatant revealed no enzyme activity, indicating that LDH in *D. desulfuricans*

TABLE 1. *The effect of lactate concn on lactate metabolism of Desulfovibrio desulfuricans ATCC 7757*

Moles Lactate $\times 10^{-5}$	Activity in Moles/Min $\times 10^{-8}$
5.35	0.69
9.8	0.41
20.0	0.35
40.0	0.57
71.3	0.69
91.0	0.72
141.0	0.94
205.0	0.94
496.0	0.94

Reaction in a Bausch and Lomb Spectronic 20 at pH 6.8 and 20 C using sodium lactate.

TABLE 2. *The effect of temp (C) on lactate metabolism of Desulfovibrio desulfuricans ATCC 7757*

Temp (C)	Moles/Min $\times 10^{-8}$
5	0.01
12	0.26
18	1.13
23	2.34
29	2.95
36	4.15
42	6.23
49	5.67
57	4.53

Reaction in a Cary 118 spectrophotometer at pH 7.0 with 0.3% sodium lactate.

TABLE 3. *The effect of pH on lactate metabolism of Desulfovibrio desulfuricans ATCC 7757*

pH	Buffer ^a	Moles/Min × 10 ⁻⁸
5.3	citrate-phosphate	0.38
5.8	citrate-phosphate	2.27
6.85	phosphate	2.19
8.4	tris	2.34
9.0	carbonate-bicarbonate	1.89
9.65	carbonate-bicarbonate	1.13

^a Gomori, G. (1955).

Reaction in a Cary 118 Spectrophotometer at 24 C and 0.3% sodium lactate.

is not a soluble enzyme. Treatment of the centrifuged pellet for as long as 3 min of sonication did not successfully remove enzyme from the pellet. In fact, the longest treatment actually reduced the enzyme activity of the insoluble fraction.

These results indicate that the LDH enzyme was, in fact, part of the membrane complex, as previously reported for *Desulfovibrio gigas* (Barton and Peck 1971). Further attempts to remove bound enzyme from the membrane (Table 4) gave mixed results. Sodium perchlorate, a chaotropic agent, had minimal effect, whereas two detergents, sodium deoxycholate and sodium cholate, proved to be more successful treatments. Removal of sodium perchlorate with Sephadex G 25 did not increase significantly the LDH activity. Sodium perchlorate concn up to 0.83 M did not seriously affect LDH activity.

TABLE 4. *The effect of chemical methods on removal of LDH from membranes of Desulfovibrio desulfuricans ATCC 7757*

Control	LDH Activity as Expressed in Absorbance/10 ml/min				% Removal
	Initial	Supernatant	Resuspended Pellet		
N.T. ^a	11.0	0.19	not done		2
N.T.	15.0	0.005	not done		
Sodium perchlorate/moles					
0.43	5.0	0.5	4.5		10
0.68	5.0	0.25	3.25		5
0.75	17.5	0.6	not done		3
Sodium deoxycholate/%					
0.5	21.0	1.0	16.5		5
2.0	31.0	20.0	2.0		65
Sodium cholate/%					
1.66	20.0	15.5	5.75		78
2.0	41.0	39.0	not done		95

^a N.T. = no treatment.

Reaction in a Cary 118 spectrophotometer at pH 7.0 and 24 C with 0.3% sodium lactate.

Another detergent, Triton X-100, which has been used previously for solubilizing bound enzymes, was used with considerable success in removing LDH from *D. desulfuricans* membranes (Table 5). The lack of LDH activity in the pellets of the control and 0.02% Triton experiments were due to resuspension procedures. Pellets could be resuspended only by sonication for 2 to 3 min, which apparently destroyed activity.

Effects of Inhibitors on LDH Activity

Sodium azide and sodium cyanide decrease LDH activity (Table 6). These results indicate a dependence on some iron-containing moiety for LDH. Rossmore et al. (1964) reported similar results with these inhibitors using whole cells of *D. desulfuricans*. The lack of inhibition with EDTA is difficult to explain considering the apparent need for iron previously demonstrated for this enzyme (Rossmore et al. 1964).

In a later experiment using a different purification procedure (refer to Chemical Techniques, above), the activity of LDH was approx. tenfold higher than shown in Table 6 and the same level of EDTA inhibited LDH activity by approx. 50%. Possibly the earlier experiment somehow contained other cations competing with the coordination of iron by EDTA, or the EDTA was unable to bind with the enzyme due to steric inhibition.

TABLE 5. *Effect of Triton X-100 on removal of LDH from cell membrane of Desulfovibrio desulfuricans ATCC 7757*

% Triton X-100	LDH Activity as Expressed in Absorbance/10 ml/min			% Removal
	Initial	Supernatant	Resuspended Pellet	
0	6.0	0.19	0.38	—
0.02	6.0	0.25	0.5	4
0.05	6.0	0.5	not done	8
0.1	6.0	1.0	not done	17
0.91	11.0	10.5	0	95
1.11	7.0	6.5	0	93
1.4	7.0	3.25	not done	46

Reaction in a Cary 118 spectrophotometer at pH 7.0 and 24 C with 0.3% sodium lactate.

TABLE 6. *Effect of inhibitors on LDH activity of Desulfovibrio desulfuricans ATCC 7757*

Inhibitor	Inhibitor Level $M \times 10^{-3}$	LDH Activity in Absorbance/ml/min	% Reduction
None	none	0.1	
EDTA	1.0	0.09	10
NaCN	0.18	0.065	35
NaCN	0.72	0.03	70
NaN ₃	0.27	0.55	45
NaN ₃	0.9	0.035	65

Reaction in a Cary 118 spectrophotometer at pH 7.0 and 0.3% sodium lactate.

Substrate Specificity for LDH

It is interesting to note that *D. desulfuricans* LDH is stereospecific for D(-) lactate (Table 7). This is in contrast to specificity reported for *D. gigas* (Barton and Peck 1971) in which reduction of L(+) lactate is claimed. Potentially, these two species could occupy noncompeting physiological niches jointly consuming racemic lactate.

Several lactate analogs were used to determine if they would be reduced by the partially purified extract; only β -hydroxybutyrate was. Whether LDH or a β -hydroxybutyrate dehydrogenase reduces this compound can be determined only when LDH is completely purified. In addition, it is hoped that further purification will determine the relationship of this enzyme to other potential electron carriers in the membrane complex.

TABLE 7. Determination of substrate specificity of LDH from *Desulfovibrio desulfuricans* ATCC 7757

Substrate	Moles	Activity in Moles/Min
D(-) lactate $\begin{array}{c} \text{H} \\ \\ \text{O} \\ \\ \text{CH}_3-\text{C}-\text{COOH} \\ \\ \text{H} \end{array}$	2.08×10^{-5}	3.8×10^{-8}
L(+) Lactate $\begin{array}{c} \text{H} \\ \\ \text{CH}_3-\text{C}-\text{COOH} \\ \\ \text{O} \\ \\ \text{H} \end{array}$	2.08×10^{-5}	0
D,L β -hydroxybutyrate $\text{CH}_3\text{CHOHCH}_2\text{COOH}$	1.9×10^{-4}	2.3×10^{-8}
Ethanol $\text{CH}_3\text{CH}_2\text{OH}$	2×10^{-4}	0
Malate $\text{COOHCH}_2\text{CHOHCOOH}$	1.8×10^{-5}	0
Tartrate COOHCHOHCHOHCOOH	1.5×10^{-5}	0
Succinate $\text{COOHCH}_2\text{CH}_2\text{COOH}$	1.9×10^{-5}	0

Reaction in a Cary 118 spectrophotometer at 25 C at pH 7.0 with 0.3% sodium lactate.

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