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Purification and partial characterization of a D(−)-lactate dehydrogenase from *Desulfovibrio desulfuricans* (ATCC 7757)

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SUMMARY

A membrane-bound D(−)-lactate dehydrogenase (LDH), an important enzyme in carbon and energy metabolism in sulfate-reducing bacteria of the genus *Desulfovibrio*, was solubilized from the membrane fraction of *Desulfovibrio desulfuricans* (ATCC 7757). The enzyme was purified 84-fold to a final specific activity of 525 nmol DCPIP-reduced/min/mg protein by ammonium sulfate precipitation, chloroform extraction, gel filtration with Sephadex G-150, and hydrophobic column chromatography with *N*-octylamine Sepharose 4B. The enzyme eluted off a Sephacryl S-300 column as a single peak with a molecular weight of 400000 ± 40000 Da. Denaturing gel electrophoresis showed it to be composed of 5 protein bands. The oxidized and dithionite reduced spectra of LDH resembles the spectra of *c*-type cytochromes found in *Desulfovibrio* species. The addition of lactate to LDH resulted in a partially reduced spectrum. The flavin/cytochrome *c*/non-heme iron content per 400000 Da LDH molecular weight was found to be 1:1.6:4.5. The LDH activity was specific for D(−)-lactate and had a K_m for D(−)-lactate of 4.3×10^{-4} M. The pH optimum was between 6.5 and 8.5.

INTRODUCTION

The metabolism of sulfate-reducing bacteria makes them important agents affecting industrial and environmental processes. Some of their effects may be adverse, such as anaerobic bacterial corrosion of metals, i.e. iron and steel [15]; contamination of injection waters used to displace residual oil in oil wells [16]; spoilage of stored oil and contamination of gas [26,28]; blackening of paper pulp [3]; and pollution of waters, muds and soils, poisoning micro- and macrobiota [30]. However, these bacteria may also have beneficial effects. The controlled reduction of sulfate, utilizing gypsum and organic wastes as carbon and energy sources, could lead to the production of sulfur, an important mineral in modern industry [33]. Sulfate-reducers may also participate in complex fermentations by interspecies hydrogen transfer, leading to the formation of methane [6]. The highly active periplasmic Fe-hydrogenase from the lactate-grown *Desulfovibrio vulgaris* Hildenborough has been proposed as part of an artificially composed biosystem used in the production of hydrogen, an alternative energy source to fossil fuels [1].

One of the more important carbon and energy substrates for sulfate-reducers, particularly *Desulfovibrio* sp., is lactate [20,39]. Lactate is a fermentation product of many bacteria and has been found to be an important electron donor for sulfate reduction in fresh water sediments [7,34]. Lactate in *Desulfovibrio* sp. is oxidized to pyruvate, which is then oxidized to acetate via acetyl CoA. The oxidation of lactate to pyruvate in a species of *Desulfovibrio* (*D. vulgaris* Marburg) has recently been found to require energy [25]. Although lactate is the preferred substrate for growth of *Desulfovibrio* sp. [20], only a few reports have been published [8,23–25,27,35,36] on lactate dehydrogenase (LDH), the initial enzyme in lactate oxidation. Lactate dehydrogenase has been very difficult to study because of its instability, especially when membrane-bound [20]. Consequently very little work has been done on studying this enzyme, its interaction with other enzymes, and electron transfer proteins in the lactate/sulfate metabolism pathway.

In order to gain further understanding of lactate metabolism, the D(−)-LDH from *D. desulfuricans* (ATCC

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7757) was solubilized from the membrane, purified 84-fold, and partially characterized.

MATERIALS AND METHODS

Growth of cells. A 10% *D. desulfuricans* (ATCC 7757) inoculum was grown anaerobically in 40 liters of lactate-sulfate medium [29] for 3 to 5 days at 33 °C. The cells (30 g) were harvested in the late stationary phase by centrifugation (10400 × *g*) for 30 min at 4 °C, washed twice with cold 0.1 M sodium phosphate (pH 7.4) containing 1 mM dithiothreitol, and stored at − 80 °C.

Assay of lactate dehydrogenase. Lactate oxidation was monitored using 2,6-dichlorophenolindophenol (DCPIP) or potassium ferricyanide as electron acceptors. DCPIP reduction was monitored for three minutes at 23 °C under anaerobic conditions using Thunberg cells with a 1 cm light path [37]. The assay mixture contained 4 ml of 6.4×10^{-3} M sodium lactate or 2.6×10^{-3} M lithium lactate in 0.1 M sodium phosphate buffer (pH 7.5), 0.2 ml DCPIP (3.4×10^{-4} M), 0.1 ml of phenazine methosulfate (2.3×10^{-4} M), and between 0.1 and 0.4 ml of the enzyme. The extinction coefficent for DCPIP is 20.6 mM at 610 nm. The other assay system used 0.1 ml of potassium ferricyanide (1.1×10^{-2} M) which has an extinction coefficient of 1.04 mM at 420 nm.

Enzyme activity is expressed as nmol/ml/min, which represents nmol of dye reduced by 1 ml of bacterial extract or enzyme in one minute. Specific activity is expressed as nmol/min/mg protein. Protein was measured colorimetrically using crystalline bovine albumin as a standard [22]. Enzyme-containing detergent was assayed using protein standards containing the equivalent amount of detergent.

Without lactate in the reaction mixture there was no reduction of DCPIP. The assay mixture with lactate and denatured enzyme also did not reduce the electron acceptor.

Molecular weight determination and electrophoresis. Molecular weight (MW) of LDH was determined by Sephacryl S-300 chromatography [31]. Polyacrylamide disc gel electrophoresis was carried out according to the procedure of Davis [9] using a 7% acrylamide gel. Disc electrophoresis containing SDS was performed according to the method of Fairbanks et al. [10]. The separating gel contained 5.6% acrylamide.

Flavin, non-heme iron and c-type cytochrome. Flavin, non-heme iron and *c*-type cytochrome contents were determined for the purest LDH sample. Flavin was removed from the protein by trichloroacetic acid and was extracted from the acid by chloroform [11]. The fluorescence of flavin at 530 nm excited at 450 nm was measured using an Aminco-Bowman spectrophotometer. Non-heme iron extracted by trichloroacetic acid was determined by the *o*-phenanthroline method [17] using an extinction coefficient of 15.2×10^{-3} cm^{-1} M^{-1} at 510 nm. C-type cytochrome spectrum was determined by measuring the dithionite-reduced protein absorbance spectrum difference at 550 nm and 540 nm using an extinction coefficient of 42 cm^{-1} mM^{-1} [2].

Miscellaneous procedures. The *n*-octylamine substituted Sephrose gel was synthesized from Sepharose 4B according to the method described by Wang and Kimura [38].

Sodium lactate (60% solution) was obtained from Fisher Scientific Company while D(−)-lactate (lithium salt), L(+)-lactate (lithium salt) and D,L-β-hydroxybutyrate were obtained from Sigma Chemical Company. All other reagents used were of the highest quality available.

TABLE 1

Purification of D(−)-lactate dehydrogenase from *Desulfovibrio desulfuricans*

Fraction	Protein (mg)	Total activity[a] (nmol DCIP/min)	Specific activity[a] (nmol DCIP/min/mg)	Recovery (%)
Crude extract	3468	21786	6.3	–
Membrane fraction	1680	16854	10.0	77
Cholate and $(NH_4)_2SO_4$	187.7	4951	26.4	23
Chloroform and $(NH_4)_2SO_4$	44.4	3592	80.9	16.5
Sephadex G-150	19.3	2947.8	152.7	14
n-Octylamine Sepharose	2.2	1155.3	525.1	5.5

[a] DCIP reduced.

RESULTS AND DISCUSSION

A membrane-bound D(−)-lactate dehydrogenase from *D. desulfuricans* (ATCC 7757) was solubilized, purified 84-fold, and partially characterized. The purification of membrane-bound LDH involved solubilizing the membrane with detergent and isolating the enzyme by ammonium sulfate fractionation, chloroform extraction and column chromatography. Membrane-bound lactate dehydrogenases are present in *Desulfovibrio* species [25,27,35,36], but have not yet been isolated. Only a partial purification of a soluble LDH from *D. vulgaris* Miyazaki has been achieved [24].

Purification of membrane-bound D. desulfuricans (ATCC 7757) lactate dehydrogenase

All purification procedures were carried out at 4 °C under an atmosphere of nitrogen, to exclude oxygen. The buffer was 0.1 M sodium phosphate (pH 7.8) with 0.8 mM β-mercaptoethanol. Centrifugation was for 30 min at 31000 × *g* unless otherwise stated. A summary of results from a typical purification is presented in Table 1.

Lysis of cells. Frozen cells (30 g) were thawed, suspended in 68 ml of buffer and disrupted by two passages through a French pressure cell at about 21000 psi. The extract was centrifuged at 10000 × *g* for 10 min at 4 °C and the precipitate discarded.

Membrane suspension. Ammonium sulfate (1.76 g/10 ml) was slowly added to the crude extract and mixed for 20 min. The pH of the solution was kept between 7.6 and 7.9 by dropwise addition of 5% ammonium hydroxide. The precipitate was recovered by centrifugation and resuspended in 56 ml of buffer.

Solubilization with sodium cholate and ammonium sulfate fractionation. The membrane suspension was solubilized with sodium cholate to a final concentration of 4%. The suspension was mixed for 4 h and then centrifuged for 60 min. Enzyme activity was found in the supernatant.

The minimal concentrations of other detergents that could solubilize the enzyme were 1% Triton X-100, 2% sodium deoxycholate, 1% Emulgen 913 and 2% cetyltrimethylammonium bromide.

Ammonium sulfate was added to the supernatant and the fraction with LDH activity precipitated at between 25% and 40% salt concentration. The precipitate was suspended in buffer to a final volume of 68 ml.

Chloroform treatment and ammonium sulfate fractionation. Cold chloroform (10 ml) was added to the suspension, mixed for 10 min, and centrifuged. Two ml of saturated ammonium sulfate was slowly added to the light yellow turbid aqueous layer obtained after centrifugation, mixed for 5 min, and then centrifuged. The supernatant was stored under anaerobic conditions for 4 h and then recentrifuged.

Gel filtration with Sephadex G-150. The supernatant (3.7 ml) was passed through a Sephadex G-150 column (1.8 cm × 35 cm) equilibrated with 0.05 M sodium phosphate (pH 7.7). D(−)-LDH activity eluted just after the void volume.

n-Octylamine Sepharose 4B chromatography. The fractions from the Sephadex G-150 column having the highest LDH activity were combined and applied to a *n*-octylamine Sepharose column (1 cm × 8 cm) equilibrated with 0.05 M sodium phosphate buffer (pH 7.7). The column was washed extensively with buffer until the absorbance of the eluant at 280 nm was less than 0.05. The column was then washed with buffer containing 1.6% sodium cholate until protein absorbance at 280 nm was less than 0.3. Some LDH activity was eluted. The column was then washed with buffer containing 5% sodium cholate, and the remaining LDH activity eluted as a single peak. D(−)-LDH activity was purified to a specific activity of 525, about 84-fold with a recovery of 5.5%.

Characterization of lactate dehydrogenase

Molecular weight and purity. The LDH fractions were concentrated and applied to a Sephacryl S-300 column (0.9 cm × 48 cm). The activity eluted as a single peak corresponding to a molecular weight of 400000 ± 40000 Da. Disc gel electrophoresis showed only one major band within the gel. However, denaturing polyacrylamide gel electrophoresis gave five bands. These combinations of proteins may be of physiological significance or a chance combination of hydrophobic proteins. Further work is needed to determine the significance of this complex. This is the purest LDH sample isolated from the membrane of any *Desulfovibrio* species.

Activity. The K_m and specific activity of the purest LDH fraction was 0.43 mM and 525 nmol/min/mg protein, using lithium D(−)-lactate as substrate and DCPIP as electron acceptor. These results are comparable to other less pure LDH enzymes found in other *Desulfovibrio* sp. [24,25,35].

Cytochrome *c* (horse heart), NAD and FAD had no effect on enzyme activity. Phenazine methosulfate (PMS) stimulated activity approximately 20% and helped to maintain linear reaction rates. The K_m of the sodium cholate or Triton X-100 solubilized LDH was 3.8 mM

using lithium D(−)-lactate as substrate and $K_3Fe(CN)_6$ as electron acceptor. The K_m of lactate oxidation using Triton X-100 solubilized LDH with $K_3Fe(CN)_6$ or DCPIP as electron acceptor was comparable. However, lactate was oxidized twice as fast with $K_3Fe(CN)_6$ when compared to DCIP. In partially purified samples (ammonium sulfate precipitated LDH) PMS (0.002 M), $FeSO_4$ (0.019 M) and $MgSO_4$ (0.019 M) slightly stimulated LDH activity (20%) and also helped to maintain a linear reaction rate.

Specificity. The purest LDH fraction oxidized D(−)-lactate but not L(+)-lactate, sodium citrate, sodium tartrate, succinate, malate, or D,L β-hydroxybutyrate. LDH solubilized from the membrane by Triton X-100 or sodium cholate oxidized only D(−)-lactate; L(+)-lactate, ethanol, malate, tartrate, succinate and D,L-β-hydroxybutyrate were not oxidized. Limited substrate specificity has been found with other LDHs from *Desulfovibrio* sp. [24,35]. LDH from *D. desulfuricans* HL 21 was able to oxidize D(−)- and L(+)-lactate, and DL-2-hydroxybutyrate [35] while LDH from *D. vulgaris* oxidized D-lactate and DL-2-hydroxybutyrate [24].

pH. The detergent-solubilized LDH and the purest LDH reduced lactate at all tested pH values, 5.7 to 9.65, with optimum activity at pH 6.7 to 8.8 and 6.5 to 8.5, respectively. This is similar to the pH range reported for L(+)-LDH from *D. desulfuricans* HL 21 [35]. Intact cells

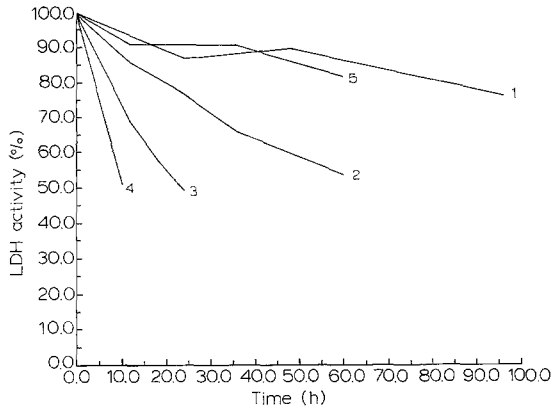

Fig. 1. The stability of LDH with increasing purification: (1) sodium cholate solubilized membranes centrifuged at 3100 × *g* for 30 min; (2) ammonium sulfate precipitated LDH from solubilized membranes; (3) LDH in supernatant from chloroform and ammonium sulfate purification; (4) LDH from Sephadex G-150 column; (5) Purest LDH. LDH activity is reported as percent activity from time zero. The enzyme was stored under anaerobic conditions at 4 °C.

of *D. desulfuricans* (ATCC 7757) oxidized lactate at values of pH, 5.0 to 9.65 [8], with optimum activity between pH 6.7 and 8.5. The ability to reduce lactate over a wide pH range is not surprising because *Desulfovibrio* sp. have been found to grow from about pH 5 to 10, and remain viable at pH 4 to 11.7 [5].

Stability. Sodium cholate solubilized LDH (sp. act. 26.4) was stable with a loss of only 23% activity over a 96 h period (Fig. 1). Similar results were found when the LDH was solubilized by Triton X-100 and Emulgen 913. As LDH was purified it became increasingly unstable with lost activity over time. This was observed after precipitation with ammonium sulfate, extraction with chloroform, and gel filtration on Sephadex G-150 (Fig. 1). Ammonium sulfate precipitation has been reported to decrease yield of LDH activity from *D. vulgaris* Marburg [25]. However, LDH of the final purification step was stable, retaining over 80% activity after 60 h at 4 °C under anaerobic conditions (Fig. 1). The enzyme was probably stable after detergent solubilization from the membrane, and after elution from the *n*-octylamine column by sodium cholate because the detergent kept integral components necessary for activity intact.

During the purification of LDH it was found that the enzyme had higher activity if purification was under anaerobic rather than aerobic conditions. In addition, the purest enzyme was also more stable when stored under anaerobic conditions. Pure LDH stored anaerobically for two weeks at −70 °C exhibited no loss of activity. The instability of LDH under aerobic conditions has been reported for L(+)-LDH from *D. desulfuricans* HL 21 [35].

Visible absorption spectrum. The purest LDH sample is golden in color and its native form exhibits a typical *c*-type cytochrome oxidized visible spectrum with a sharp peak

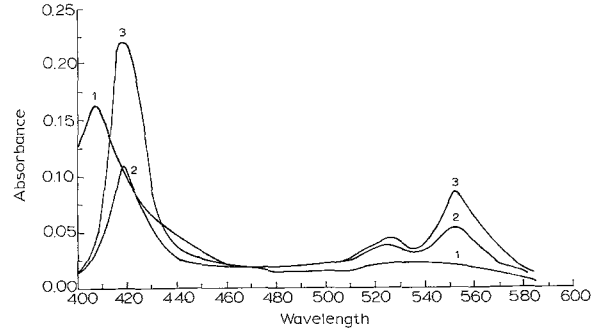

Fig. 2. Electronic absorption spectra of oxidized (1), lactate reduced (2), and dithionate reduced (3) *Desulfovibrio desulfuricans* lactate dehydrogenase (sp. act. 525).

at 410 nm and a broad absorption band around 530 nm. Addition of sodium dithionite results in an absorbance shift of the Soret peak from 410 nm to 418 nm and the appearance of small peaks at 523 nm and 553 nm, with the α peak at 553 nm the larger (Fig. 2). This spectrum corresponds to a reduced *c*-type cytochrome spectrum. The amount of cytochrome was determined to be 4 nmol cytochrome *c* per mg of protein. The addition of lactate to the enzyme under anaerobic conditions resulted in a partially reduced spectrum about 50% of the dithionite reduced spectrum. *c*-Type cytochrome spectra have also been associated with sulfur reductase (tetrahemic cytochrome c_3) [12,18] and nitrite reductase (hexahemic membrane-bound cytochrome *c* [21]) from *Desulfovibrio*. In addition *c*-type cytochromes have been proposed as cofactors to hydrogenase [4,13] and formate reductase [40]. LeGall et al. [19] have proposed that a multi-heme *c*-type cytochrome couples electron transfer between lactate and hydrogenase. A soluble LDH has been found to reduce cytochrome monohemic c_{553} from *D. vulgaris* Miyazaki [24], indicating a possible close association with this cytochrome.

Flavin and non-heme iron. In addition to *c*-type cytochrome, flavin and non-heme iron have been found associated with purest LDH fraction. There are 10 to 12 nmol non-heme iron and 2 to 3 nmol flavin per mg of protein. The flavin/cytochrome *c*/non-heme iron content per MW 400000 Da LDH is 1:1.6:4.5.

It was not determined whether the flavin was FAD or FMN, or had any functional relationship with the LDH. The addition of FAD to the assay mixture for lactate oxidation did not stimulate LDH activity. Flavins have been associated with membrane-bound LDHs from many bacteria [14]. Peck and LeGall [27] have proposed a flavoprotein as an electron acceptor for LDH which would transfer the electrons to a 4 iron center ferredoxin.

Four to five non-heme irons were found in the purest LDH fraction from *D. desulfuricans* ATCC 7757. However, the necessity of non-heme iron for enzyme activity has only been indirectly shown. The purest LDH sample was inhibited 20% to 80% by 1 to 5 mM sodium cyanide and sodium azide, indicating dependence on some metal-containing moiety. Similar results with the same inhibitors have been reported for lactate metabolism using whole cells of *D. desulfuricans* [32]. EDTA has been reported to inhibit detergent solubilized membrane-bound LDH activity from *D. desulfuricans* ATCC 7757 [8], and the stimulation of partially purified LDH activity by $FeSO_4$ and $MgSO_4$ may indicate the necessity of metal for enzyme activity. D(−)-LDH from *D. desulfuricans* (ATCC 27774) requires Zn^{2+} for activity [27]. The soluble LDH from *D. vulgaris* is reported to be a metalloflavoprotein [24].

In conclusion, a membrane-bound D(−)-lactate dehydrogenase from *D. desulfuricans* ATCC 7757 was solubilized by detergents and purified 84-fold to a final specific activity of 525 nmol DCPIP-reduced/min/mg protein. The enzyme activity is fairly stable when it is in detergent. The spectrum of the purest LDH fraction is similar to the spectrum of *c*-type cytochromes found in *Desulfovibrio* sp. The oxidized spectrum is partially reduced by the addition of lactate. Iron and flavin are also found associated with this enzyme fraction but their necessity for enzyme activity has not yet been conclusively established. The purest LDH fraction has a K_m for lactate of 0.43 mM and the pH for highest activity is between 6.5 and 8.5.

The purification of this enzyme should help in gaining a better understanding of lactate metabolism in *Desulfovibrio* species and help to control these organisms in industrial processes.

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