JOURNAL OF BACTERIOLOGY, Dec. 1971, p. 964-972

Vol. 108, No. 3
Printed in U.S.A.

Restoration of Deoxycholate-Disrupted Membrane Oxidases of *Micrococcus lysodeikticus*[1]

R. C. EISENBERG

Department of Biology, Western Michigan University, Kalamazoo, Michigan 49001

Received for publication 28 July 1971

Membrane-associated L-malate and reduced nicotinamide adenine dinucleotide (NADH) oxidase complexes of *Micrococcus lysodeikticus* were inactivated with deoxycholate. Reactivation of NADH oxidase by addition of Mg^{2+} occurred in these detergent-membrane mixtures, but reactivation of L-malate oxidase did not occur in the presence of deoxycholate. Removal of detergent by gel filtration allowed Mg^{2+}-dependent restoration of both L-malate and NADH oxidases. Maximal NADH and L-malate oxidase restoration required 10 min and 40 min, respectively, at 30 mM $MgSO_4$. Maximal restoration of both oxidases required at least 12 mM $MgSO_4$ in an incubation period of 1 hr. Reduced-minus-oxidized difference spectra of Mg^{2+}-restored membrane oxidases showed participation of cytochromes *b*, *c*, and *a* when either L-malate or NADH served as reductant; addition of dithionite did not increase the α- and β-region absorbancy maxima of these hemoproteins when restored membranes were first reduced with the physiological substrates L-malate or NADH. Not all divalent cations tested were equally effective for reactivation of both oxidases. L-Malate oxidase was restored by both Mn^{2+} and Ca^{2+}. NADH oxidase was not activated by Mn^{2+} and only slightly stimulated by Ca^{2+}. Separation of deoxycholate-disrupted membranes (detergent removed) into soluble and particulate fractions showed that both fractions were required for Mg^{2+}-dependent oxidase activities. Electron micrographs indicated conditions of detergent treatment did not destroy the vesicular nature of protoplast ghost membranes.

Reconstitution of deoxycholate-disrupted reduced nicotinamide adenine dinucleotide (NADH) oxidase from *Bacillus megaterium* KM membranes was reported earlier by Eisenberg, Yu, and Wolin (4). Those studies showed that all of the *B. megaterium* KM NADH oxidase complex can be "solubilized" in an inactive form by deoxycholate and these deoxycholate-membrane mixtures can be reaggregated in an active form by dilution in buffer containing Mg^{2+}. A more recent report by Yu and Wolin (13) showed that these deoxycholate-membrane mixtures can be reaggregated, concomitant with restoration of NADH oxidase activity, at *p*H 5.0 in the absence of Mg^{2+}.

This report will show that membrane-associated L-malate and NADH electron transport oxidase complexes of *Micrococcus lysodeikticus* can also be disrupted with deoxycholate under conditions that provide for divalent cation-dependent restoration of these oxidase activities. In contrast to the *B. megaterium* KM deoxycholate-disrupted membranes, only a portion of the *M. lysodeikticus*-disrupted membrane oxidase is "solubilized" by detergent. Furthermore, it will be demonstrated that deoxycholate disruption and Mg^{2+}-dependent restoration of these two oxidase complexes in *M. lysodeikticus* membranes differ. These differences may eventually provide a basis for better understanding of the mechanisms of in vitro bacterial membrane component reaggregation.

[1] Presented in part at the 71st Annual Meeting of the American Society for Microbiology, Minneapolis, Minn., 2-7 May 1971.

MATERIALS AND METHODS

Growth of organism and preparation of membranes. *M. lysodeikticus* (strain 1SU Ad pos) was obtained from the culture collection of W. E. Kloos, Department of Genetics, North Carolina State University, Raleigh. Growth medium contained (per liter): trypticase (Bioquest), 15 g; yeast extract (Bioquest), 1 g; sodium chloride, 5 g; KH_2PO_4, 0.82 g; and $K_2HPO_4 \cdot 3H_2O$, 4.17 g. Glucose (autoclaved separately) was added to 0.05 M; final *p*H was 6.9. Cells were routinely grown aerobically in batch culture (9 liters)

at 32 C in a New Brunswick Microferm fermenter; air flow was 10 liters per min and foaming was controlled with seven drops of antifoam agent (Union Carbide SAG-471). Bacteria were harvested by centrifugation at the end of logarithmic growth and washed twice with a 2.4-liter total volume of 0.01 M tris(hydroxymethyl) aminomethane (Tris)-hydrochloride, *p*H 7.2.

Membranes were prepared by suspending twice-washed cells to one liter with TKM buffer (0.01 M each Tris-hydrochloride, KCl, and $MgCl_2$, *p*H 7.2) and adding 100 to 120 mg of crystalline lysozyme [1 mg of lysozyme per g of cells (wet weight)] with stirring at 23 C. A small quantity of crystalline deoxyribonuclease was added immediately after addition of lysozyme, and the mixture was incubated with continuous stirring for 1 hr. Lysed cell suspensions were centrifuged at 27,000 $\times$ *g* for 30 min, and residues (membranes) were washed three times with 720 ml (total volume) of TKM buffer. The washed membranes were suspended in 150 ml of double-strength TKM buffer containing 30 g of glycerol and 2×10^{-4} M β-mercaptoethanol and then diluted with distilled water to 300 ml. Portions of these membranes (in TKM, 10% w/v glycerol and 10^{-4} M β-mercaptoethanol) were frozen immediately and retained at -60 C until used. These frozen membrane preparations contained 7 to 10 mg of protein per ml (slight variation between cell batches).

Enzyme assays. L-Malate and NADH oxidase activities were estimated by using a Clark oxygen electrode assembly (Oxygraph, model KM, Gilson Medical Electronics, Middleton, Wis.). Assays were routinely made with a 1.5-ml reaction mixture at 32 C. Concentration of dissolved O_2 in distilled water at 32 C was estimated as 0.2 μmole per ml. NADH oxidase activities obtained from this polarographic method were in good agreement with those obtained from spectrophotometric assays at 340 nm assuming an NADH extinction coefficient of 6.2 per mmole per cm. Other conditions of assays are indicated, where appropriate, in the text.

Preliminary experiments showed all the cellular L-malate and NADH oxidase activities to be in the particulate membrane fractions when cells are fractionated as indicated above. The specific activities (micromoles of O_2 consumed per milligram of membrane protein) of both oxidases did not change when membranes were prepared from cells harvested during early-log, late-log, or stationary phase of growth. No significant differences in oxidase activities were found between freshly prepared membranes and those stored at -60 C for 6 months. The stabilization of *B. megaterium* KM NADH oxidase by freezing in Tris buffer containing glycerol was reported earlier by Yu and Wolin (13).

Protein and deoxycholate estimation. Membrane protein was estimated by cold trichloroacetic acid (5% w/v) precipitation, hot alkali extraction (1.0 M NaOH for 15 min in a boiling water bath), and assay of alkali supernatant fluids by the method of Lowry et al. (8). Protein standards (Fraction V bovine serum albumin, Sigma Chemical Co.) were treated identically to membranes. Deoxycholate (Sigma Chemical Co.) was estimated using the colorimetric procedure of Szalkowski and Mader (12).

Electron microscopy. Electron micrographs were obtained with a Siemens Elmiskop 1A electron microscope. Membranes were (i) stained directly with 1% (w/v) phosphotungstic acid, *p*H 5.8, containing 0.5% (w/v) sucrose or (ii) first fixed with osmic acid (1% in phosphate buffer, *p*H 7.4) for 30 min at room temperature and washed with distilled water before staining with phosphotungstic acid.

Miscellaneous. Chemicals employed were of reagent grade quality. Lysozyme (3 $\times$ crystallized) and deoxyribonuclease (1 $\times$ crystallized) were purchased from Sigma Chemical. Difference spectra were obtained with a Coleman-Hitachi model 124 double beam spectrophotometer equipped with an automatic recorder.

RESULTS

Disruption of NADH oxidase, L-malate oxidase, and membrane turbidity by sodium deoxycholate. Inactivation of bacterial membrane functions and disruption of membrane structure by anionic detergents have been described previously (3, 6, 10). Loss of *M. lysodeikticus* membrane oxidase activities and membrane turbidity by increasing concentrations of sodium deoxycholate (DOC) is shown in Fig. 1. Thus, at relatively low DOC concentration (0.6 mg/ml), essentially all the NADH and L-malate oxidase activities were disrupted to the extent that an electron transport couple between reduced substrates (NADH and L-malate) and molecular oxygen was not effective. The influence of DOC concentration from 0 to 0.2 mg/ml had a differential effect on the two oxidase activities. NADH oxidase activity was stimulated at low DOC concentration (0.1 mg/ml), but L-malate oxidase was inhibited by all concentrations of DOC tested. Separate experiments, not shown, gave the same results when protoplast ghost membranes were first sonically treated to give membrane particles before treatment with DOC. Again, membrane particle NADH oxidase was stimulated by low levels of DOC but L-malate oxidase was consistently inhibited by all levels of DOC treatment. These data suggest that stimulation of NADH oxidase by low DOC concentrations is not due solely to simple permeability barriers in these protoplast ghost membrane preparations.

Reactivation of membrane oxidases by magnesium ions. Preliminary experiments demonstrated that the NADH oxidase of these DOC-treated membranes could be restored by addition of Mg^{2+} to assay reaction mixtures similar to those employed for reactivation of *B. megaterium* KM NADH oxidase (4). However, these conditions were not capable of providing for restoration of *M. lysodeikticus* L-malate oxidase activity after DOC inactivation. These observations are summarized by the data in Fig. 2.

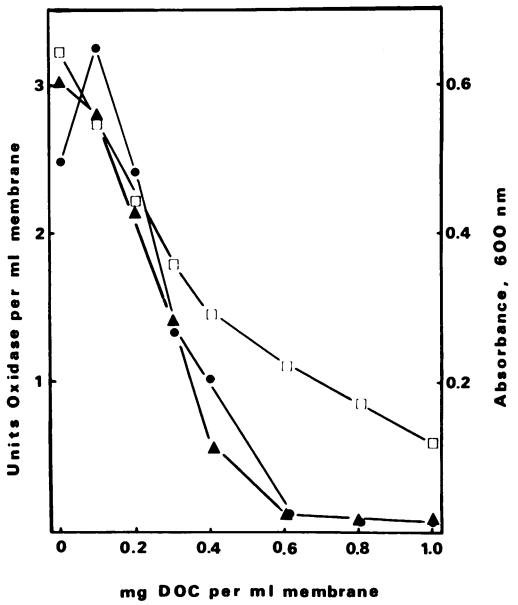

FIG. 1. *Effect of deoxycholate on disruption of membrane oxidase activities and turbidity. Frozen membranes were thawed, washed once with 10 ml of 0.05 M Tris-hydrochloride (pH 7.2; original volume of frozen membranes, 10 ml), and resuspended in 0.05 M Tris-hydrochloride buffer. Membrane suspensions were diluted with buffer to give 1.15 mg of protein/ml, and 3.5 ml of suspension was treated with 3.5 ml of deoxycholate (Na^+; DOC) to give DOC concentrations indicated. Detergent-membrane mixtures were incubated, with stirring, for 15 min at 22 C before assaying for oxidase activities and membrane turbidity. Oxidase assays (1.5 ml) contained 1.4 ml of DOC-membrane mixture and 0.1 ml of substrate [1 μmole of reduced nicotinamide adenine dinucleotide (NADH) or 10 μmoles of L-malate]. One unit of activity is 1 μmole of O_2 consumed per min at 32 C. Symbols: (●) NADH oxidase, (▲) L-malate oxidase, and (□) membrane turbidity (1-cm light path).*

Incubation of intact membranes (mb) with Mg^{2+} ions did not stimulate either NADH or L-malate oxidases (polarographic traces, Fig. 2). Both oxidases were eliminated by suitable DOC treatment, i.e., no O_2 consumption was detected when either NADH or L-malate was added to these detergent-treated membranes (DOC-mb). If, however, DOC-treated membranes were incubated with Mg^{2+}, addition of NADH resulted in a rate of O_2 consumption comparable to the rate observed with intact membranes incubated in the presence or absence of Mg^{2+}. These results suggest that complete inactivation of NADH oxidase by DOC can be achieved and that complete restoration by appropriate Mg^{2+} treatment is possible. In contrast to full restoration of NADH oxidase, L-malate oxidase was not restored by incubation with Mg^{2+}. Addition of L-malate to DOC-mb plus Mg^{2+} (Fig. 2) resulted in no consumption of O_2. Other experiments, utilizing less DOC and maintaining partial L-malate oxidase activity also resulted in little, if any, stimulation of L-malate oxidase activity when Mg^{2+} was added to DOC-membrane mixtures. These observations, together with those from Fig. 1, indicate that the L-malate multienzyme oxidase complex is more sensitive to DOC effects than is the NADH oxidase complex.

One possibility for achieving restoration of DOC-inactivated L-malate oxidase was first to remove DOC from DOC-membrane mixtures and then to test the effect of divalent cation treatment on restoring an L-malate-O_2 electron transport couple. DOC was effectively removed from detergent-treated membranes by passing these mixtures through a column of Sephadex G-25 (0.05 M Tris-hydrochloride, *p*H 7.2) and collecting the yellow-pigmented membrane material in the void volume (established with Blue Dextran 2000). Detergent was recovered from the column by continued elution with Tris-hydrochloride (0.05 M, *p*H 7.2) and accounted for

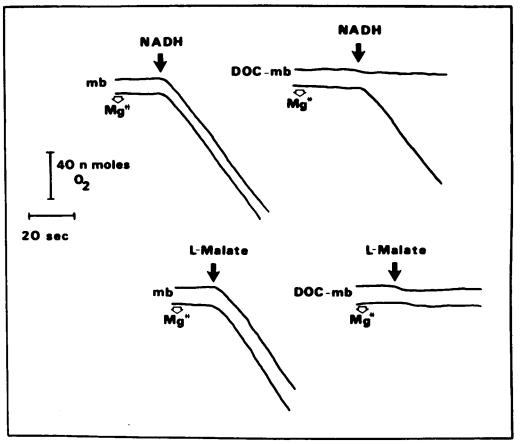

FIG. 2. *Polarographic traces of detergent-treated membrane oxidase activities after Mg^{2+} treatment. Frozen membranes were washed and resuspended as described in the legend to Fig. 1. Washed membranes were diluted to give (i) Tris membrane (mb) containing 0.5 mg of protein/ml and (ii) deoxycholate-membranes (DOC-mb) containing 3 mg of sodium deoxycholate and 0.5 mg of protein/ml of mixture; DOC treatment was 15 min at 22 C. To portions (1.3 ml) of these membrane suspensions was added 0.1 ml of 0.42 M $MgSO_4$ or 0.1 ml of distilled water; these suspensions were then incubated for 1 hr at 32 C before assaying for oxidase activities by addition of 0.1 ml of substrate (1 μmole of NADH or 10 μmoles of L-malate). Complete reaction mixtures (1.5 ml total volume) contained 0.65 mg of protein and, where indicated, 42 μmoles of $MgSO_4$.*

>99% of the DOC present in the initial DOC-membrane mixtures. We consider these "desalted" disrupted membranes collected in the Sephadex G-25 void volume to be free of DOC.

The capacity of these detergent-disrupted "desalted" membranes (disrupted membranes) to be reactivated for L-malate oxidase activity is indicated in Fig. 3. Trace A in Fig. 3 shows that a residual amount of L-malate oxidase was detected when a relatively high membrane protein concentration was used and that this residual activity was not affected by addition of versene to the reaction mixture. Trace B in Fig. 3 indicates that an immediate positive response of L-malate oxidase activity occurred when Mg^{2+} was added to the reaction vessel after addition of substrate. When Mg^{2+} was added ca. 2.3 min (Fig. 3, trace C) before addition of L-malate, an even more rapid rate of O_2 consumption occurred. These data demonstrate that DOC-inactivated L-malate oxidase activity can be restored, in part, by Mg^{2+} if detergent is first removed. The inhibition of Mg^{2+}-stimulated activity by ethylenediaminetetraacetic acid (EDTA) (Fig. 3, traces B and C) suggests the essential role of divalent cation in oxidase activation. It must be pointed out, however, that the function of EDTA as an inhibitor of Mg^{2+}-activated oxidase is not clearly understood at this time. For example, Fig. 3, trace A, shows the rate of O_2 consumption after addition of versene to Mg^{2+}-treated membranes was less than that observed when EDTA was added to disrupted membranes in the absence of Mg^{2+}. Separate experiments (not shown) have demonstrated that (i) EDTA is inhibitory at levels less than stoichiometric with added Mg^{2+} (one-tenth concentration of added cation) and (ii) addition of excess Mg^{2+} to versene-inhibited reaction mixtures does not relieve EDTA inhibition.

Disrupted membranes (no DOC present) were also capable of being activated for NADH oxidase by divalent cation. Data in Fig. 4 show that activation of NADH oxidase by Mg^{2+} was quite similar to the cation-dependent activation of L-malate oxidase. Again, addition of EDTA to Mg^{2+}-activated membrane oxidase resulted in inhibition of both residual and Mg^{2+}-stimulated oxidase activities. Also, addition of Mg^{2+} to disrupted membranes before addition of reductant (Fig. 4, trace C) resulted in more activity than did addition of cation after addition of NADH (Fig. 4, trace B).

Both L-malate and NADH cation-dependent oxidase activities (Fig. 3 and 4) appeared to be time-dependent; more activity was observed when disrupted membranes were incubated with Mg^{2+} before addition of reductant. A more complete account of optimal incubation times and cation concentrations is provided in Fig. 5. The kinetics of oxidase activation at 30 mM $MgSO_4$ is shown in Fig. 5A. Full restoration of NADH oxidase occurred within 10 min, whereas maximal L-malate oxidase stimulation required 40 min under these conditions. Optimal Mg^{2+} concentration, in an incubation period of 1 hr (Fig. 5B), was approximately 12 mM for both NADH and L-malate oxidases. No loss of oxidase activities was observed when these mixtures were retained in the presence of 50 mM $MgSO_4$ for 1 hr at 32 C.

Difference spectra of restored oxidase complexes. *M. lysodeikticus* contains cytochromes of the *a*, *b*, and *c* types (7, 10, 11). Intact membranes of this organism show the participation of cytochromes *a*, *b*, and *c* in O_2 reduction when either NADH or L-malate serves as physiological reductant. The magnesium ion-activated oxidases obtained from disrupted membranes also show participation of these cytochromes when either L-malate or NADH is used as electron donor (Fig. 6). Furthermore, addition of dithionite to

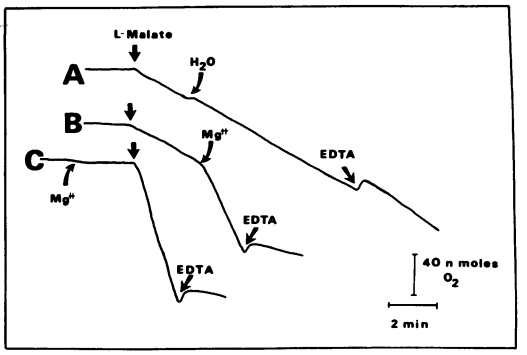

FIG. 3. *Restoration of L-malate oxidase activity after detergent disruption and removal of detergent. Washed membranes were prepared as in (Fig. 1). Membrane suspensions were diluted with sodium deoxycholate to give 10 ml of a membrane-detergent mixture containing 4 mg of protein and 3 mg of sodium deoxycholate per ml (0.05 M Tris-hydrochloride, pH 7.2). This mixture was incubated, with stirring, for 15 min at 22 C, and detergent was removed by gel filtration at 4 C (Sephadex G-25). Disrupted membranes (1.3 ml containing 1.3 mg of protein) were added to the assay reaction mixture vessel (traces A, B, and C) and additions were made as follows: trace A, 0.1 ml of L-malate (10 μmoles), 0.1 ml of distilled water, and 0.2 ml of ethylenediaminetetraacetic acid (EDTA) (46 μmoles, pH 7.2); trace B, 0.1 ml of L-malate (10 μmoles), 0.1 ml of $MgSO_4$ (45 μmoles), and 0.2 ml of EDTA (46 μmoles); trace C, 0.1 ml of $MgSO_4$ (45 μmoles), 0.1 ml of L-malate (10 μmoles), and 0.2 ml of EDTA (46 μmoles). Total volume of all three reaction mixtures after all additions was 1.7 ml. Reaction (trace A) goes to completion after addition of EDTA.*

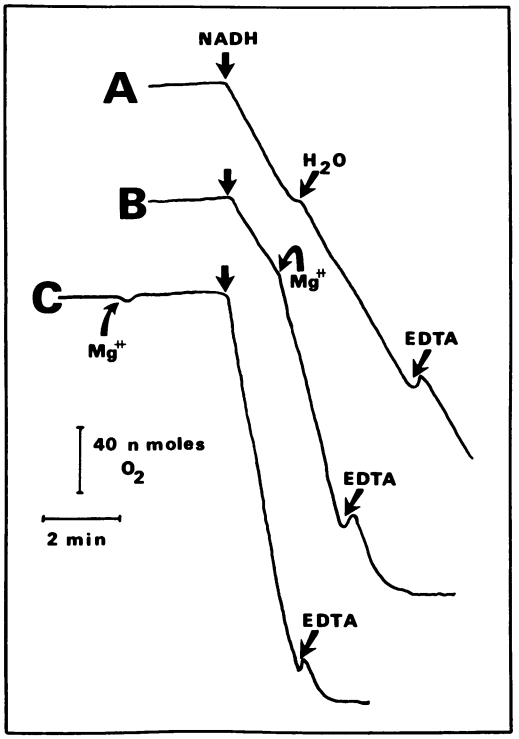

FIG. 4. *Restoration of reduced nicotinamide adenine dinucleotide (NADH) oxidase activity after detergent disruption and removal of detergent. Experimental details were the same as those described in legend to Fig. 3 except that NADH (1 μmole) was added in lieu of* L-*malate. Reaction (trace A) goes to completion after addition of ethylenediaminetetraacetic acid (EDTA).*

NADH- or L-malate-reduced material did not increase the α- and β-region absorbancy maxima of these hemoproteins. These data indicate that reconstituted membrane oxidases, like starting membrane material, involve an electron transport multienzyme complex involving cytochromes of the *a*, *b*, and *c* types.

Comparison of various ions for restoration of disrupted membrane oxidases. Earlier work on divalent cation activation of *B. megaterium* NADH oxidase (4) showed that the alkaline earth cations (barium, calcium, and magnesium) were effective for restoring activity of DOC-treated membranes. Furthermore, the heavier alkaline earth metals were the most effective ($Ba^{2+} > Ca^{2+} > Mg^{2+}$). Data in Table 1 show that monovalent cations have little, if any, effect on stimulating *M. lysodeikticus*-disrupted membrane oxidase activities when tested at ionic strengths comparable to those used for testing divalent cations. Significant differences were found in the capacity of various divalent cations for effecting restoration of L-malate and NADH oxidase activities. L-Malate oxidase was restored by both Mn^{2+} and Ca^{2+} whereas NADH oxidase was unaffected by Mn^{2+} and only slightly enhanced by incubation with Ca^{2+}. The best cation tested, for optimal restoration of both L-malate and NADH oxidase activities, was magnesium ion; no significant differences were observed between the sulfate and chloride salts of Mg^{2+}.

Summary of membrane oxidase disruption and magnesium ion-dependent oxidase reactivation. Data in Table 2 summarize some of the results

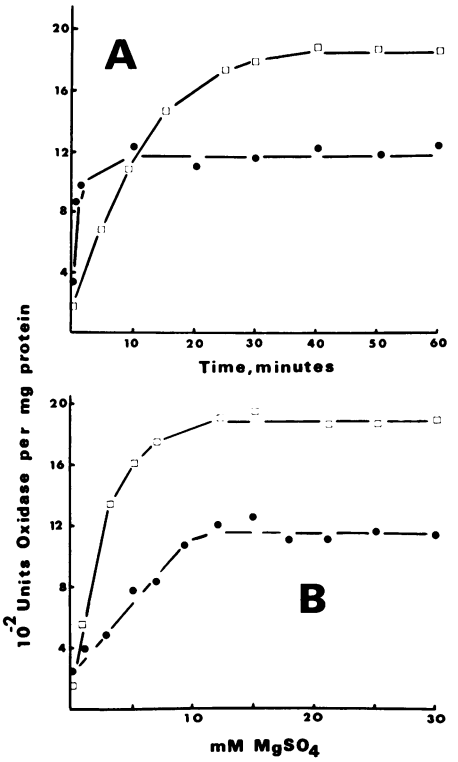

FIG. 5. *Kinetics and Mg^{2+} concentration requirements of disrupted membrane oxidase reactivation. Disrupted membranes (after detergent treatment and removal of detergent) were prepared as described in legend to Fig. 3. Rates of disrupted membrane oxidase activation at 30 mM $MgSO_4$ are shown in A. Disrupted membranes were incubated with Mg^{2+} at 32 C for the times indicated and then assayed for oxidase activities. B shows the amount of oxidase found after 1 hr (32 C) of incubation with Mg^{2+} concentration indicated. Incubation mixtures (A and B) contained 0.5 mg of membrane protein in 1.4 ml of 0.05 M Tris-hydrochloride, pH 7.2. Oxidase assays contained incubation mixtures (1.4 ml) plus 0.1 ml of substrate (1 μmole of reduced nicotinamide and adenosine dinucleotide (NADH) or 10 μmoles of* L-*malate). One unit of oxidase activity is 1 μmole of O_2 consumed per min. Symbols: (□)* L-*malate oxidase and (●) NADH oxidase.*

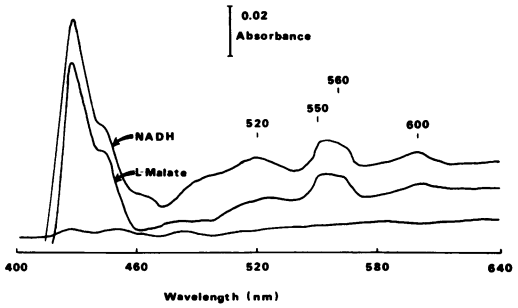

FIG. 6. *Substrate reduced-minus-oxidized difference spectra of Mg^{2+}-restored membrane oxidases. Disrupted membranes (no deoxycholate present) were prepared as described in legend to Fig. 3. Restored membranes were prepared by adding $MgSO_4$ to disrupted membranes (1.07 mg of membrane protein and 30 μmoles of $MgSO_4$ per ml of 0.05 M Tris-hydrochloride, pH 7.2) and incubating 1 hr at 32 C. Difference spectra were obtained by adding 0.2 ml of reduced nicotinamide adenine dinucleotide (NADH; 2 μmoles) or L-malate (20 μmoles) to 2.5 ml of restored membranes and scanning against 2.5 ml of restored membranes diluted with 0.2 ml of distilled water. The lower trace represents an oxidized-minus-oxidized control (2.5 ml of restored membranes in both sample and reference cuvettes).*

presented earlier. Intact membranes lost all of their L-malate oxidase and 80% of their NADH oxidase activities after DOC treatment and removal of DOC by gel filtration (disrupted membranes). All of the initial L-malate oxidase (114%) and 62% of the initial NADH oxidase were recovered when these disrupted membranes were incubated with Mg^{2+} before assaying for oxidase activities. It should be noted that the actual initial activity of bacterial protoplast ghost oxidases is not clearly established (3). This difficulty was indicated in the discussion of effects of low DOC concentrations (Fig. 1). The specific activity (micromoles of O_2 consumed per minute per milligram of protein) of restored oxidases (Table 2) was 0.22 and 0.097 for L-malate and NADH oxidases, respectively.

Separation of soluble and particulate fractions from disrupted membranes and the requirement of both these fractions for Mg^{2+}-dependent oxidase restoration are also presented in Table 2. Supernatant fractions from disrupted membranes, containing over 50% of the disrupted membrane protein, were not capable of being restored for either L-malate or NADH oxidase activities. The particulate fraction, obtained by differential centrifugation and thus partially contaminated with soluble material, contained 16 and 61% of the Mg^{2+}-activatable L-malate and NADH oxidase activities, respectively. Recombination of supernatant and particulate fractions yielded nearly all the initial L-malate oxidase activity, and these fractions clearly acted synergistically. Little synergistic activity for soluble and particulate fractions was observed for restored NADH oxidase activity. Other experiments (not shown) indicated more activity for NADH oxidase by mixing soluble and particulate fractions together with Mg^{2+} than is indicated in Table 2; however, we always found less (ca. 50%) Mg^{2+}-activatable NADH oxidase after centrifugation and recombination of supernatant fluid and pellet fractions than we found when disrupted membranes were treated directly with Mg^{2+}.

Physical structure of disrupted membranes. Electromicrographs of disrupted membranes (membranes treated with DOC and DOC removed by "desalting") are presented in Fig. 7. Staining disrupted membranes with phosphotungtic acid (Fig. 7A) demonstrated that this membrane material consisted, in part, of large membrane structures which could be interpreted as "collapsed bags." This would mean that DOC treatment, under our conditions, did not completely destroy the vesicular nature of these protoplast ghosts. To determine whether these disrupted membranes retain their vesicular character, disrupted membranes were fixed with OsO_4 before staining with phosphotungtic acid (Fig. 7B). These osmic acid-fixed membrane preparations have the appearance of "folded bags" after phosphotungtic acid staining, sub-

TABLE 1. *Effect of various cations on stimulation of disrupted membrane L-malate and NADH oxidases*[a]

Additions to disrupted membranes	O_2 consumed (μmoles per min per mg of protein)	
	L-malate	NADH
Control, no additions	0.011	0.017
NaCl, 0.09 M	0.017	0.023
KCl, 0.09 M	0.011	0.023
LiCl, 0.09 M	0.025	0.020
NH_4Cl, 0.09 M	0.023	0.025
$MnCl_2$, 0.03 M	0.127	0.014
$BaCl_2$, 0.03 M	0.178	0.093
$CaCl_2$, 0.03 M	0.175	0.034
$MgCl_2$, 0.03 M	0.224	0.099
$MgSO_4$, 0.03 M	0.204	0.088

[a] Disrupted membranes (no deoxycholate) were prepared as described in legend to Fig. 3; 0.3 ml of disrupted membranes (0.354 mg of protein) was diluted with 1 ml of 0.05 M Tris-hydrochloride, pH 7.2, and 0.1 ml of salts was added to give concentrations indicated. Mixtures were incubated for 1 hr at 32 C, and oxidase activities were then estimated by addition of 0.1 ml of substrate [10 μmoles of L-malate or 1 μmole of reduced nicotinamide adenine dinucleotide (NADH)].

TABLE 2. *Requirement of soluble and particulate fractions of disrupted membranes for Mg^{2+}-dependent oxidase restoration*

Membrane preparation	Protein conc (mg per ml of membrane preparation)	O_2 consumed[a]	
		L-malate	NADH
Control, intact membranes[b]	0.39	0.074	0.060
Disrupted membranes[c]	0.38	0	0.011
Disrupted membranes plus 30 mM $MgCl_2$[d]	0.38	0.084	0.037
Supernatant fraction of disrupted membranes plus 30 mM $MgCl_2$[e]	0.20	0	0
Particulate fraction of disrupted membranes plus 30 mM $MgCl_2$[f]	0.15	0.014	0.023
Supernatant and particulate fractions plus 30 mM $MgCl_2$[g]	(0.35)	0.072	0.025

[a] All membrane preparations were incubated for 1 hr at 32 C before oxidase activities were estimated. Oxidase assays contained 1.4 ml of membrane preparation and 0.1 ml of substrate [10 μmoles of L-malate or 1 μmole of reduced nicotinamide adenine dinucleotide (NADH).] Values are expressed as micromoles of O_2 consumed per minute per milliliter of membrane preparation.

[b] Frozen membranes were thawed, washed, and suspended in Tris buffer as described in legend to Fig. 1. These washed membranes, without further treatment, were diluted with 0.05 M Tris-hydrochloride, *p*H 7.2, to the protein concentration indicated.

[c] Disrupted membranes (no deoxycholate present) were prepared from washed membranes as described in legend to Fig. 3 and diluted with 0.05 M Tris-hydrochloride buffer, *p*H 7.2, to concentration indicated.

[d] Disrupted membranes were diluted with Tris buffer and $MgCl_2$ to give a suspension containing 0.38 mg of protein/ml and 30 mM $MgCl_2$ all in 0.05 M Tris-hydrochloride) *p*H 7.2.

[e] Disrupted membranes (ca. 1.07 mg of protein/ml) were centrifuged at 50,000 rev per min in a Beckman SW50 rotor for 1 hr and supernatant fluids containing ca. 0.56 mg of protein/ml were diluted with Tris buffer and $MgCl_2$ to give 0.2 mg of protein/ml and 30 mM $MgCl_2$ all in 0.05 M Tris-hydrochloride, Cl, *p*H 7.2.

[f] Insoluble residues from membrane preparation described in footnote *e* were resuspended in Tris buffer to the original volume (resuspended material contained 0.4 mg of protein/ml) and diluted with Tris buffer and $MgCl_2$ to give 0.15 mg of protein/ml in 0.05 M Tris-hydrochloride, *p*H 7.2, at 30 mM $MgCl_2$.

[g] Equal volumes of supernatant fluid (footnote *e*) and resuspended pellet (footnote *f*) were mixed and diluted with Tris-hydrochloride buffer and $MgCl_2$ to give 0.35 mg of protein/ml in 30 mM $MgCl_2$ at 0.05 M Tris-hydrochloride, *p*H 7.2.

stantiating the concept that DOC disruption of protoplast ghost membranes does not destroy the vesicular nature of membrane ghosts. (This is not saying that these disrupted membranes consist of intact vesicles.)

DISCUSSION

M. lysodeikticus contains two membrane-associated electron transport oxidase activities. The NADH oxidase complex consists of a primary dehydrogenase linked via vitamin $K_{2(45)}$ [or something very closely related to $K_{2(45)}$] to cytochromes *b*, *c*, and *a* (5). The NADH dehydrogenase moiety of this complex contains lipid, and has been selectively extracted from the membrane by EDTA (9). *M. lysodeikticus* has been reported to have two L-malate dehydrogenase enzymes (1). The cytoplasmic enzyme requires NAD, but the particulate membrane-bound L-malate dehydrogenase does not require this cofactor (2). Vitamin K_2 is also thought to serve as an intermediary electron transport carrier in this particulate L-malate oxidase complex (6). Thus, NADH and L-malate oxidases may be viewed as two different dehydrogenase enzymes commonly linked to cytochromes *b*, *c*, and *a* via vitamin K_2.

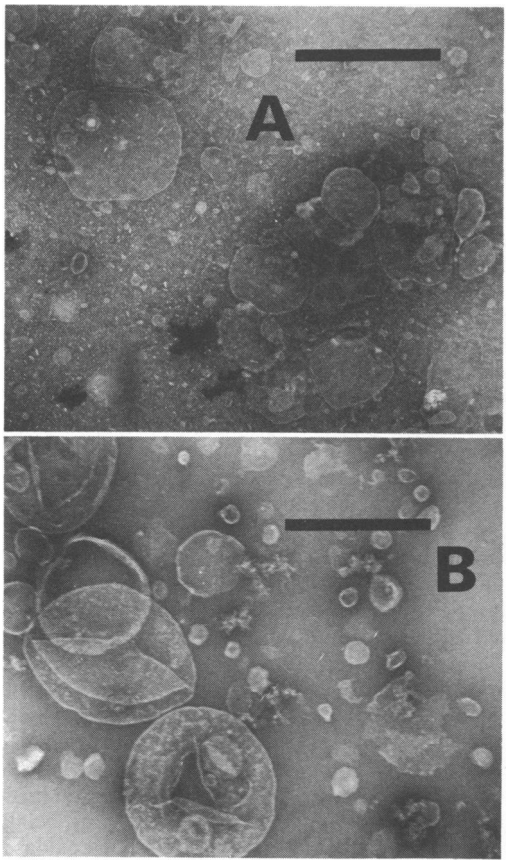

FIG. 7. *Electron micrographs of disrupted membranes. A, disrupted membranes stained with phosphotungstic acid. B, disrupted membranes fixed with osmic acid, centrifuged, suspended in distilled water, and stained with phosphotungstic acid. Marker indicates 1 μmeter.*

Results in this report show that the effects of deoxycholate on these two membrane oxidases differ. When membranes were treated with low concentration of deoxycholate (Fig. 1), the NADH oxidase was stimulated, and further increases in deoxycholate concentration, resulting in inactivation of NADH oxidase, provided for deoxycholate-membrane mixtures that could be restored for NADH oxidase by incubation with Mg^{2+} (Fig. 2). L-Malate oxidase, however, was inhibited by all concentrations of detergent tested, and Mg^{2+}-dependent restoration of L-malate oxidase was not possible when Mg^{2+} was added to disrupted membranes in the presence of detergent. Both detergent-disrupted oxidases could be activated by Mg^{2+} after deoxycholate was removed (Fig. 5). Other differences between L-malate and NADH oxidases were found when disrupted membranes were tested for oxidase restoration by using divalent cations other than Mg^{2+} (Table 1). Thus, Mn^{2+} stimulated L-malate oxidase over 10-fold and had no stimulating effect on NADH oxidase. Ca^{2+} stimulated L-malate oxidase over 15-fold and increased NADH oxidase only twofold. Although information available at this time is not adequate to assess properly the chemical significance of these differences the data demonstrate differences which may serve as good tools for further study of in vitro membrane component reaggregation.

The physiological significance of these restored membrane oxidases is not known. Complete reduction of the hemoproteins (cytochromes *b*, *c*, and *a*) by both substrates, after Mg^{2+} activation, is the best evidence available that these restored oxidases are similar to initial, untreated membrane oxidase activities. Rapid inhibition of restored membrane oxidases by EDTA (Fig. 3 and 4) and the lack of this inhibition in untreated starting membrane material clearly indicate that starting membranes and restored membranes are not identical.

Separation of detergent-disrupted membranes into soluble and particulate fractions and the requirement of both fractions for oxidase activity are best understood on the basis of experiments reported earlier. Detergent treatment, as well as EDTA and alkali treatments, solubilize NADH dehydrogenase [NADH: 2,6-dichlorophenol-indophenol (DCIP) oxidoreductase] activity from protoplast membranes of *B. megaterium* KM (3). Gel'man et al. reported that deoxycholate solubilizes L-malate and NADH dehydrogenases (substrate: DCIP oxidoreductase activities) from *M. lysodeikticus* membranes (6). Salton et al. (10) reported that deoxycholate extraction of these membranes yields lipid-depleted sheets containing cytochromes of the *a*, *b*, and *c* types, although Gel'man (6) more recently reported that this detergent also solubilizes most of these cytochromes. We have confirmed (*unpublished data*) that deoxycholate inactivation of these oxidase activities is due to solubilization of primary dehydrogenase enzymes and that only a small portion (ca. 24%) of cytochrome *b* is solubilized under these conditions. We will show in a future report that restoration of these oxidase activities by Mg^{2+} treatment is due to reconstitution of solubilized dehydrogenases with particular cytochrome-containing vesicles (as indicated in Fig. 7). Data in Table 2 show that recombination of soluble and particulate fractions results in good recovery of Mg^{2+}-activated L-malate oxidase. The reason(s) for poor restoration of NADH oxidase activity by recombination of soluble and particulate fractions is not clearly understood.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant AI 09844-01 from the National Institute of Allergy and Infectious Diseases.

I thank D. A. Buthala for making the electron micrographs and Belden B. Smith for technical assistance.

LITERATURE CITED

1. Cohn, D. V. 1956. The oxidation of malic acid by *Micrococcus lysodeikticus*. J. Biol. Chem. **221**:413–423.
2. Cohn, D. V. 1958. The enzymatic formation of oxalacetic acid by nonpyridine nucleotide malic dehydrogenase of *Micrococcus lysodeikticus*. J. Biol. Chem. **233**:299–304.
3. Eisenberg, R. C., L. Yu, and M. J. Wolin. 1970. Masking of *Bacillus megaterium* KM membrane reduced nicotinamide adenine dinucleotide oxidase and solubilization studies. J. Bacteriol. **102**:161–171.
4. Eisenberg, R. C., L. Yu, and M. J. Wolin. 1970. Divalent cation activation of deoxycholate-solubilized and -inactivated membrane reduced nicotinamide adenine dinucleotide oxidase of *Bacillus megaterium* KM. J. Bacteriol. **102**:172–177.
5. Fujita, M., S. Ishikawa, and N. Shimazono. 1966. Respiratory chain and phosphorylation site of the sonicated membrane fragments of *Micrococcus lysodeikticus*. J. Biochem. **59**:104–114.
6. Gel'man, N. S., G. V. Tikhonova, I. M. Simakova, M. A. Lukoyanova, S. D. Taptykova, and H. M. Mikelsaar. 1970. Fragmentation by detergents of the respiratory chain of *Micrococcus lysodeikticus* membranes. Biochim. Biophys. Acta **223**:321–331.
7. Ishikawa, S., and A. L. Lehninger. 1962. Reconstitution of oxidative phosphorylation in preparations from *Micrococcus lysodeikticus*. J. Biol. Chem. **237**:2401–2408.
8. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
9. Nachbar, M. S., and M. R. J. Salton. 1970. Characteristics of a lipid-rich NADH dehydrogenase containing particulate fraction obtained from *Micrococcus lysodeikticus* membranes. Biochim. Biophys. Acta **223**:309–320.
10. Salton, M. R. J., J. H. Freer, and D. J. Ellar. 1968. Electron transport components localized in a lipid-depleted sheet isolated from *Micrococcus lysodeikticus* membranes by deoxycholate extraction. Biochem. Biophys. Res. Commun. **33**:909–915

11. Smith, L. 1954. Bacterial cytochromes. Difference spectra. Arch. Biochem. Biophys. **50**:299–314.
12. Szalkowski, C. R., and W. J. Mader. 1952. Colorimetric determination of desoxycholic acid in ox bile. Anal. Chem. **24**:1602–1604.
13. Yu, L., and M. J. Wolin. 1970. Factors affecting deoxycholate inactivation and Mg^{++} reactivation of *Bacillus megaterium* KM membrane nicotinamide adenine dinucleotide (reduced form) oxidase. J. Bacteriol. **103**:467–474.