ELECTRON TRANSPORT COMPONENTS LOCALIZED IN A LIPID-DEPLETED SHEET ISOLATED FROM MICROCOCCUS LYSODEIKTICUS MEMBRANES BY DEOXYCHOLATE EXTRACTION

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SUMMARY: Extraction of isolated membranes from Micrococcus lysodeikticus with 1% deoxycholate in 0.05M tris buffer yields an insoluble residue which accounts for 15% of the dry weight of the membrane. This fraction is composed largely of protein and contains 3-5% residual lipid compared to 23-26% for the initial membrane lipid and consequently has a higher density than the membranes on gradient centrifugation. These lipid-depleted residues are in the form of a membranous sheet when examined in the electron microscope. Cytochromes a, b and c and succinic dehydrogenase activity were localized almost exclusively in this deoxycholate insoluble fraction of the membrane.

Investigations in our laboratory have been directed towards the isolation and characterization of specific functional markers from the membranes of Micrococcus lysodeikticus in order to gain some insight into the molecular architecture and functioning of this membrane system. A Ca^{2+} activated adenosinetriphosphatase has been isolated and purified by selective release from the membranes of M. lysodeikticus (Munoz, et al., 1968a) and the enzymatic activity has been found to be associated with particles of about 100 Å diameter possessing a central subunit surrounded by 6 additional subunits (Munoz, et al., 1968b). Although suitable fractionation procedures were developed for the selective separation of cytochromes from most of the M. lysodeikticus membrane lipids, carotenoids and from about two thirds of

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the total membrane protein (Salton, et al., 1967), these fractions showed no structural organization and were completely amorphous when viewed in the electron microscope. In this paper we wish to report the separation and localization of the cytochromes and succinic dehydrogenase in a lipid-depleted sheet isolated from membranes of M. lysodeikticus by extraction with deoxycholate (DOC).

Membranes from M. lysodeikticus isolated as described previously (Salton and Freer, 1965) were washed 6 times with 0.05M tris buffer, pH 7.5, according to the 'standard' procedure outlined by Salton (1967). Freshly washed membrane pellets were then taken up in 0.05M tris buffer containing 1% sodium deoxycholate (w/v). The DOC-treated suspensions were then centrifuged at 0-2°C for 50 min. at 30,000 xg and the yellow supernatant was carefully removed from the red-brown pellet. The DOC-insoluble residues were resuspended in 1% DOC in tris and subjected to successive washes on the centrifuge (50 min. at 30,000 xg) until the supernatant fluids were devoid of yellow carotenoid. In a typical experiment, membrane suspensions containing 5-10 mg protein/ml required 3-5 successive extractions with 1% DOC in tris to remove most of the carotenoid and phospholipids. Analysis of the supernatant fluids for the release of membrane lipid proved difficult because of the presence of the DOC and the solubility of the latter in lipid solvents. This difficulty was circumvented by the use of ^{32}P-labelled membranes as reported in previous studies (Salton, 1967; Salton, et al., 1967) since most of the radioactivity can be accounted for by the phospholipids. The release of protein determined by the method of Lowry, et al. (1951), phospholipid as ^{32}P-labelled material, and carotenoids measured by absorbance at 475 mμ, was followed for 6 successive extractions of membranes with 1% DOC in 0.05M tris buffer (pH 7.5) and the results are illus-

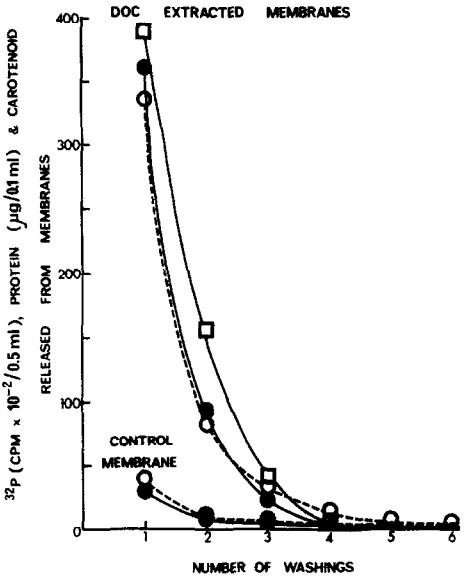

Fig. 1 Release of ^{32}P-labelled lipid (O), protein (O) and carotenoid (□), absorbance at 475 mμ x 10^2, 1 cm cuvette) from M. lysodeikticus membranes subjected to 6 successive washes with 1% DOC in 0.05 M tris, pH 7.5 (DOC extracted membranes) and untreated membranes washed x 6 with 0.05 M tris (control membrane). Determinations were performed on supernatant washes after centrifugation for 50 min. at 30,000 xg at 0°C. Initial control and DOC extracted membrane suspensions contained 4.9 mg protein/ml.

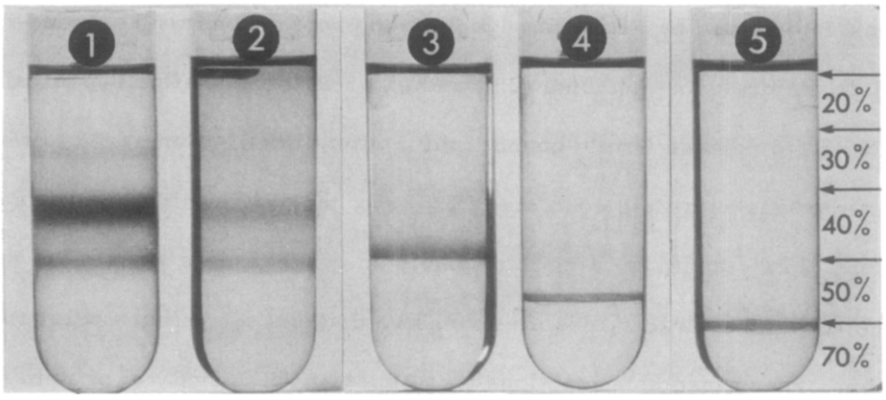

Fig. 2 Centrifugation of M. lysodeikticus membrane fractions on discontinuous gradients of 70%, 50%, 40% 30% and 20% sucrose in 0.05 M tris buffer (pH 7.5) at 24,000 rpm for 2 hours at 5°C in SW 25 rotor. 1, control membrane; 2, membrane in 1% DOC-tris layered on gradient; 3, insoluble residue after 1 wash in 1% DOC-tris; 4, insoluble residue after 2 washes in 1% DOC-tris; 5, DOC-insoluble residue, 6 washes. Fractions in 1 ml tris layered on top of gradients. Interfaces between sucrose solutions indicated at right of gradient 5.

trated in Fig. 1. Under these conditions of extraction, 95% of the ^{32}P-labelled lipid was removed and over 95% of the carotenoid. The continued washing of the control membranes for another 6 washes released only small amounts of protein and lipid (Fig. 1); absorbance due to carotenoid release was <0.1 at 475 mμ.

Dissociation of the membranes by DOC resulted in marked changes in the behavior of the components upon sucrose density gradient centrifugation as shown in Fig. 2. Residues of intermediate density to that observed for the final, x6 washed, DOC-insoluble fraction were obtained after 1 & 2 extractions. The higher density of the residues would be in accord with the removal of membrane lipids.

The DOC-insoluble residues accounted for 10.8-15.4% of the initial membrane protein and determinations of dry weights of the residues, after extensive dialysis against distilled water showed that they constituted 15% of the membrane dry weight (mean of 4 determinations). Residual lipid was determined by extracting lyophilized preparations with acetone-methanol (7:2), chloroform-methanol (2:1) and ether extraction after hydrolysis with 2N HCl to release firmly bound lipid (Salton, 1953). Compared to whole membranes with lipid contents of 23-26%, the DOC residues contained 3-5% 'total lipid', and this value is probably an overestimate since DOC was detected in the extracts, despite extensive dialysis of residues prior to extraction. These insoluble fractions thus appear to be largely of protein nature, with small contents of residual lipid.

PROPERTIES OF THE DOC-INSOLUBLE RESIDUES: The DOC-insoluble residues from M. lysodeikticus membranes prepared by 6 extractions with DOC as described above were examined in the electron microscope after

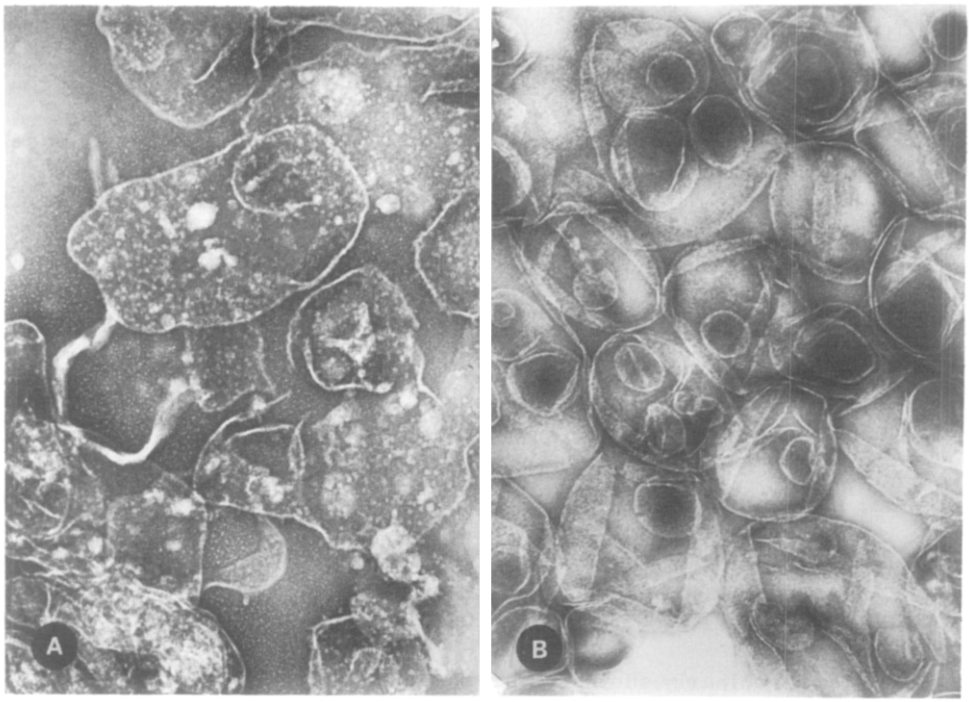

Fig. 3 Electron micrographs of M. lysodeikticus membrane fractions negatively stained with 2% ammonium molybdate. A, control membrane after 6 washes with 0.05 M tris; B, DOC-insoluble residues after 6 successive washes with 1% DOC in 0.05 M tris. A and B, x 86,000.

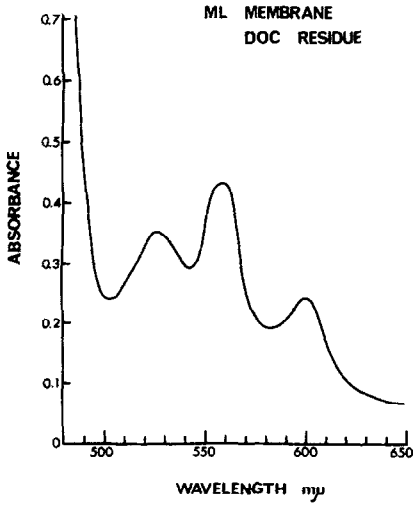

Fig. 4 Absorption spectrum of the DOC-insoluble residue isolated from M. lysodeikticus membranes. Following solubilization in 0.05 M tris buffer (pH 7.5) containing 0.5% SDS, the fraction was reduced with crystals of $Na_2S_2O_4$ and the spectrum recorded immediately in 1 cm cuvette. The protein concentration was 5.0 mg/ml.

negative staining with 2.0% ammonium molybdate. The appearance of the untreated, washed membranes and the corresponding DOC-insoluble fraction is shown respectively in Figs. 3A and 3B. Very clean membranous sheets result from the DOC extraction as seen in Fig. 3B, with few of the ATPase particles (Munoz, et al., 1968b) remaining attached after this treatment. The collapsed, folded appearance of the residues suggests that these lipid-depleted sheets are rather more rigid than the original membranes. DOC-insoluble fractions of identical appearance have also been obtained from Sarcina lutea and Bacillus subtilis membranes.

The pigmentation of the DOC-insoluble residues was suggestive of the presence of cytochromes and the spectrum of this fraction from M. lysodeikticus, solubilized with SDS (sodium dodecyl sulfate) and reduced with $Na_2S_2O_4$ (Fig. 4) clearly shows the presence of cytochromes a, b and c in this lipid-depleted fraction. That the cytochromes are almost exclusively localized in this membranous sheet was confirmed by the detection of only trace amounts of material giving a slight peak at 560 mμ (absorbance less than 0.03) when reduced spectra of the DOC-soluble fractions were examined either before or after removal of the DOC by dialysis or extraction of the lipid with n-butanol.

DOC-insoluble residues were examined for succinic dehydrogenase activity by the spectrophotometric method of Ells (1959) which utilizes 2, 6-dichlorophenolindophenol and phenazine methosulfate. All of the residues from M. lysodeikticus membranes so far tested have shown considerable activity (e. g. absorbance change at 600 mμ exceeding 0.4/min. / 100 μg preparation) and moreover, the activity in these fractions was insignificant in the absence of succinate and phenazine methosulfate. Succinic dehydrogenase activity in this particulate form appears to be quite stable at

0-4°C for 1-2 weeks. Both the method of Ells (1959) and a modification of the tetranitro-blue tetrazolium method of Sedar and Burde (1965) showed that most of the succinic dehydrogenase activity was in the DOC-insoluble residue with less than 10% in the DOC-soluble fractions. DOC-insoluble residues from S. lutea and B. subtilis also contained succinic dehydrogenase activity.

The isolation of the cytochromes and succinic dehydrogenase in a well-defined structural entity from the cell membranes of M. lysodeikticus should enable us to fully characterize some of the components of the electron transfer chain in this organism and localize them specifically in the intact cell membrane structure.

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