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SOLUBILIZATION OF BACTERIAL MEMBRANE PROTEINS USING ALKYL GLUCOSIDES AND DIOCTANOYL PHOSPHATIDYLCHOLINE

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SUMMARY

The non-ionic detergent octyl glucoside solubilizes a substantial amount of *Streptococcus faecalis* membrane protein without loss of the monitored enzyme activities. A secondary detergent, dioctanoyl phosphatidylcholine, appears to increase the yield of solubilized material. In addition, the effect of ionic strength indicates that it may be possible to selectively extract groups of membrane proteins by their characteristic solubility at different ionic strengths.

The solubilized membrane-associated enzymes, ATPase and NADH dehydrogenase, enter polyacrylamide gels as distinct species. Electrophoretic studies suggest that there are two membrane-associated ATPases in the *Streptococcus faecalis*, one which dissociates from the membrane in the absence of Mg^{2+} ions and the other which remains particulate until solubilized by detergents.

Octyl glucoside can be easily removed from a solution containing solubilized proteins and lipid by dialysis.

INTRODUCTION

The isolation and purification of membrane proteins and the study of their function in many instances has become a problem in the art of membrane protein solubilization by detergents. In particular, polyoxyethylene ether type non-ionic detergents have been widely used for solubilization and subsequent isolation of membrane proteins [1]. However, while these detergents are apparently mild and thus useful in isolating functional membrane proteins, they are extremely difficult to separate from the isolated proteins. This problem is due to the low critical micelle concentration (CMC) of the alkyl and alkyl-aryl polyoxyethylene ether detergents (e.g., Brij 36-T, Lubrol PX and WX, and Triton X-100) and possible high affinities that these detergents may have for membrane proteins.

Abbreviations: C_8-glucoside, octyl glucoside; C_{10}-Glu, decyl glucoside; diC_8-phosphatidylcholine, dioctanoyl phosphatidylcholine; DCCD, dicyclohexylcarbodiimide; CMC, critical micelle concentration.

To overcome the problem of detergent removal, we have examined the properties of two easily dialyzable non-ionic and zwitterionic detergents. The following criteria were considered in comparing these detergents with several commonly used non-ionic detergents: i) the amount of intrinsic membrane protein solubilized, ii) the retention of function by solubilized protein, and iii) the ease of separation of the detergent from the solubilized protein.

We report here studies on the solubilization of the membrane proteins of *Streptococcus faecalis* using octyl and decyl glucosides and dioctanoyl phosphatidylcholine. In particular, we examined the solubilization of a membrane-associated NADH dehydrogenase, the residual ATPase remaining after removal of most of the ATPase by aqueous washing [2], a dicyclohexylcarbodiimidesensitive ATPase, and total protein.

MATERIALS AND METHODS

Acetobromo-α-D-glucose, dicyclohexylcarbodiimide (DCCD) and Triton X-100 were purchased from the Sigma Chemical Company. Octanol and decanol were purchased from Fisher Scientific. Brij 36-T was a generous gift of Dr M. Montal, Centro de Investigacion y de Estudios Avanzados del Instituto Politecnico Nacional, Mexico. Dioctanoyl phosphatidylcholine was kindly provided by Dr B. Sears, University of Virginia School of Medicine.

Synthesis of octyl and decyl glucosides

Both alkyl glucosides were synthesized following the procedure of Noller and Rockwell [3] with the following modifications. i) The synthesis of tetra-acetylalkylglucosides was allowed to proceed overnight. ii) Instead of steam distillation to remove excess alcohol, the tetra-acetylalkylglucosides were precipitated by the addition of cold petroleum ether. After diethylether was removed under reduced pressure, cold petroleum ether was added to the tetra-acetyl-C_8-glucoside dissolved in octanol. The mixture became slightly cloudy after addition of 125 ml of petroleum ether and was then placed at -10 °C for three days. The supernatant was then decanted and the insoluble material dissolved in methanol. The solvent was removed under reduced pressure. The residue was then redissolved in methanol (or ethanol) and crystallized as described previously [3]. iii) The deacylation of the tetra-acetylalkylglucosides was allowed to proceed for 2 h. The alkyl glucosides were crystallized from acetone by addition of petroleum ether [4].

Preparation of S. faecalis membranes

Membranes depleted of ATPase activity (depleted membranes) were prepared according to Abrams and Baron [5] substituting 2 mM Tris/0.96 mM maleate, pH 7.0, for 1 mM Tris/Cl, pH 7.5 in the final washes. Membranes were also prepared which retained all the ATPase activity. These were washed identically as above except 1 mM $MgCl_2$ was included to prevent the release of any ATPase from the membrane [2].

Enzyme activities and protein determination

ATPase activity was assayed as previously described [6]. NADH dehydroge-

nase activity was determined by following the reduction of 2,6-dichlorophenolindophenol at 600 nm, 25 °C [7]. The assay system contained 67 mM Tris/32 mM maleate, pH 7.0, 40 μM NADH and 40 μM 2,6-dichlorophenolindophenol. Activity was monitored continuously on a Gilford 2400 Spectrophotometer. One unit of NADH dehydrogenase is that amount of enzyme which oxidizes 1 μmol NADH/min at pH 7.0, 25 °C. Protein was determined by the method of Lowry et al. [8].

Polyacrylamide gel electrophoresis

Electrophoresis was carried out in a Bio-Rad Model 150 gel electrophoresis Cell. The gels contained 4 % (w/v) acylamide, 0.2 % *N*, *N'*-methylene bisacrylamide (w/v), 30 mM C_8-glucoside, 20 mM Tris/Cl, pH 9.0 and 1 mM $MgCl_2$ if indicated in the legend. Polymerization was accomplished by the addition of 0.07 % *N*, *N*, *N'*, *N'*-tetramethylethylenediamine (v/v) and 0.07 % ammonium persulfate (w/v). The running buffer was 2 mM Tris/maleate, pH 8.0 and contained 1 mM $MgCl_2$ if indicated in the figure legends. Gels stained for enzyme activities were first soaked for at least 4 h at 4 °C in 2 mM Tris/maleate, pH 7.0, with 1 mM $MgCl_2$ if required, in order to lower the concentration of C_8-glucoside to 25 mM or less. Determination of ATPase activity in the gels was carried out as previously described [9]. NADH dehydrogenase activity was assayed as described above, except that *p*-nitroblue tetrazolium, 0.2 mg/ml, was substituted for 2, 6-dichlorophenolindophenol. Gels were also stained for protein with Coomassie blue.

RESULTS AND DISCUSSION

Stability of NADH dehydrogenase in various detergents

Before attempting to solubilize *S. faecalis* membrane proteins with any of the detergents, conditions were determined in which NADH dehydrogenase activity was stable. Depleted membranes were mixed with five different detergents over a range of concentrations and then stored at 0–4° (Table I). At zero (within 30–120 min of mixing), 2 and 4 days, aliquots of the mixtures were assayed for NADH dehydrogenase activity. Each detergent tested, with the exception of Brij 36-T, showed a particular concentration above which enzyme activity was extensively and irreversibly lost: C_8-glucoside at greater than 30 mM, C_{10}-glucoside at greater than 2 mM, diC_8-phosphatidylcholine at greater than 0.38 mM (determined in other experiments) and Triton X-100 at greater than 0.16 mM. The samples stored in all concentrations of Brij 36-T showed only small losses of NADH dehydrogenase activity. The results described in Table I indicate that long term stability of NADH dehydrogenase activity requires that the concentration of each detergent be maintained below a critical denaturing concentration.

Solubilization of membrane proteins by various detergents

Solubilization of depleted membrane proteins were examined using each of the five detergents tested above (Table II). When NADH dehydrogenase, residual ATPase and protein remained in the supernatant after centrifugation at $10^5 \times g$ for 1 h, they were considered to be solubilized. Note that highly washed depleted membranes will not release these enzymes or much additional protein in further aqueous washes. In the cases where the level of detergent used for solubilization would have caused

TABLE I

STABILITY OF MEMBRANE-ASSOCIATED NADH DEHYDROGENASE IN VARIOUS DETERGENTS

Depleted membranes, at a final concentration of 89 μg protein per ml, were mixed with the indicated detergents in 63 mM Tris/maleate, pH 7.0, and stored at 0–4 °C. Duplicate 0.1 ml aliquots were assayed for NADH dehydrogenase activity as described in the Materials and Methods section.

Detergent	[Detergent] (mM)	(%)	μmol NADH oxidized/min		
			Day 0	2	4
None	—	—	1.24	1.30	1.28
C_8-glucoside	10.0	0.29	1.16	1.09	0.96
	20.0	0.58	1.39	1.28	1.21
	30.0	0.88	1.62	1.60	1.58
	50.0	1.46	0.59	0.13	0.08
C_{10}-glucoside	1.0	0.032	1.22	1.01	1.00
	2.0	0.064	1.65	1.69	1.79
	4.0	0.13	1.66	0.33	0.36
	8.0	0.26	1.27	0.14	0.10
diC_8-phosphatidylcholine	0.95	0.05	1.89	0.56	0.18
	1.9	0.1	1.90	0.18	0.05
	3.8	0.2	2.02	0.17	0.04
	9.5	0.5	0.82	0.16	0.05
Brij 36-T	5.0	0.31	1.87	1.97	1.69
	10.0	0.63	2.04	2.30	1.88
	20.0	1.25	2.04	2.25	1.74
Triton X-100	0.08	0.05	1.28	1.40	1.30
	0.16	0.1	1.27	1.43	1.16
	0.32	0.2	1.18	0.80	0.41
	0.80	0.5	1.68	0.39	0.13

loss of NADH dehydrogenase activity over an extended period of time, the supernatants were diluted to a concentration of detergent in which the enzyme was stable (see Table I). The result indicates that C_8-glucoside, diC_8-phosphatidylcholine and Brij 36-T were the best solubilizing agents for NADH dehydrogenase. In addition, diC_8-phosphatidylcholine solubilized almost twice as much residual ATPase as C_8-glucoside. Triton X-100 at 0.8 mM was a slightly poorer solubilizing agent by comparison, but caused considerable denaturation of NADH dehydrogenase. On the basis of the above result one might conclude that C_8-glucoside, diC_8-phosphatidylcholine and Brij 36-T would be satisfactory detergents for solubilization of membrane proteins. However, diC_8-phosphatidylcholine and Brij 36-T have certain disadvantages not associated with C_8-glucoside. First, solubilization with diC_8-phosphatidylcholine requires a subsequent dilution to a detergent concentration of 0.38 mM in order to prevent loss of NADH dehydrogenase activity. This concentration is the CMC for diC_8-phosphatidylcholine (Baron and Thompson, unpublished results). When the proteins and enzymes were concentrated in a collodion bag apparatus, diC_8-phosphatidylcholine was also concentrated to an extent that caused 50 % loss of NADH dehydrogenase activity. This problem may be overcome

TABLE II

SOLUBILIZATION OF DEPLETED MEMBRANE PROTEINS BY VARIOUS DETERGENTS

Depleted membranes, at a final concentration of 2 mg protein/ml, were mixed with the indicated detergents with either 67 mM Tris/maleate, pH 7.0 (TM), or 10 mM potassium phosphate, pH 7.0 (K_3PO_4). The mixtures were centrifuged at $10^5 \times g$ for 60 min. The supernatants (S1) were diluted with buffer, if necessary, to prevent any further loss of NADH dehydrogenase activity (C_{10}-glucoside to 2 mM, diC_8-phosphatidylcholine to 0.38 mM and Triton X-100 to 0.08 mM). The pellets were resuspended and extracted a second time. The supernatants (S2) were then diluted as described above for the first extract, and the pellets (P) suspended in buffer without any detergent. Duplicate aliquots were assayed for NADH dehydrogenase, ATPase and protein.

Detergent	[Detergent] (mM)	Buffer	Percent distribution								
			NADH Dehydrogenase				ATPase	Protein			
			S1	S2	P	Total enzyme recovered	S1+S2	P	S1	S2	P
C_8-glucoside	30.0	TM	83	7	10	128	—	—	24	5	71
	30.0	KP	80	14	8	147	38	62	30	6	64
C_{10}-glucoside	4.0	TM	4	1	95	83	—	—	17	3	80
	2.4	KP	3	15	82	96	12	88	17	3	78
diC_8-phosphatidylcholine	1.9	TM	89	9	2	140	—	—	32	16	52
	0.95	KP	51	43	6	162	68	32	24	13	64
Brij 36-T	5.0	TM	68	11	21	109	—	—	29	12	59
	10.0	KP	71	13	16	145	—	—	29	5	65
Triton X-100	0.8	TM	50	13	37	59	—	—	36	15	49
	0.4	KP	33	11	56	88	—	—	36	6	62

by controlling the rate at which the protein is concentrated so that it would be compatable with the rate of dialysis of the detergent. Second, Brij 36-T, an oxyethylene ether detergent, is difficult to remove by dialysis because the concentration of monomer is only about 0.1 of the total detergent concentration (CMC is about 0.5–1 mM). A similar disadvantage is encountered with Triton X-100 which has a CMC of 0.2–0.3 mM. These disadvantages are not encountered with C_8-glucoside. Not only is enzyme activity preserved, but since this detergent has a CMC of 25 mM [10], it is easily dialyzable (Fig. 1).

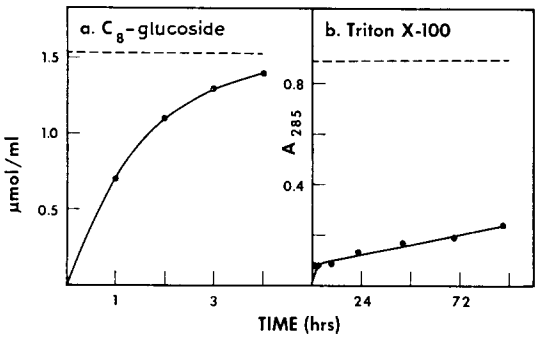

Fig. 1. Dialysis of C_8-glucoside and Triton X-100. (a) 13.5 ml of a solution containing 3.6 mg of solubilized depleted membrane protein, 21 mg of *S. faecalis* lipid, 1.1 µmol diC_8-phosphatidylcholine, and 405 µmol of C_8-glucoside buffered with 2 mM Tris/maleate, pH 7.0, was dialyzed against approx. 250 ml of 50 mM Tris/Cl, pH 7.5, 10 mM $MgCl_2$ and 100 mM KCl. C_8-glucoside appearing outside the dialysis bag was assayed as described previously [11]. (b) 13.5 ml of a solution containing 1.6 mM (1 %) Triton X-100 in 2 mM Tris/maleate, pH 7.0 was dialyzed against the same solution as in (a). Triton X-100 appearing outside the dialysis bag was monitored at 285 nm. Union Carbide no. 8 dialysis tubing was used in both (a) and (b). The dashed horizontal lines represent the concentration of detergent at equilibrium.

Effect on extraction of combinations of detergent and ionic strength

Optimum conditions for the solubilization of depleted membrane proteins were obtained with 30 mM C_8-glucoside and a small amount of diC_8-phosphatidylcholine (0.38 mM) in a low ionic strength buffer. This level of diC_8-phosphatidylcholine did not cause denaturation on stogate. Two extractions under these conditions resulted in the following solubilizations: 99 % (range 98–99) of NADH dehydrogenase, 95 % (range 94–97) of residual ATPase and 69 % (range 60–85) of total protein. As shown in Table III, a single extraction of depleted membranes at a higher ionic strength resulted in a diminished solubilization of ATPase and protein, but did not alter the amount of NADH dehydrogenase solubilized.

Membranes obtained from protoplasts of *S. faecalis* by osmotic shock contain a membrane-associated ATPase which can be inhibited by DCCD (particulate DCCD-sensitive ATPase) [6]. ATPase released from these membranes by aqueous washing in the absence of Mg^{2+} is no longer sensitive to this inhibitor. The site of DCCD inhibition remains in the depleted membranes after release of the enzyme [12]. Membranes which retained all of their ATPase activity (i.e., washed in the presence of 1 mM $MgCl_2$ as described in the Materials and Methods section) were extracted with C_8-glucoside and diC_8-phosphatidylcholine, and a supernatant containing 53 %

TABLE III

EFFECT OF COMBINED DETERGENT EXTRACTION AND IONIC STRENGTH ON SOLUBILIZATION OF MEMBRANE PROTEINS

Depleted and non-depleted membranes, at a final concentration of 2 mg protein/ml, were mixed with diC_8-phosphatidylcholine, 0.38 mM in 2 mM Tris/maleate, pH 7.0, 0.1 mM dithiothreitol and KCl and $MgCl_2$ as indicated and incubated for 30 min at 0–4 °C. C_8-glucoside was then added at a level of 30 mM and the mixtures incubated an additional 30 min at 0–4 °C. The mixtures were then centrifuged at $10^5 \times g$ for 1 h. The supernatants (S) were removed and the pellets (P) suspended in 2 mM Tris/maleate, pH 7.0. Duplicate aliquots were assayed for NADH dehydrogenase, ATPase and protein.

Detergent + Salts	Membranes	Percent distribution					
		NADH dehydrogenase		ATPase		Protein	
		S	P	S	P	S	P
1. C_8-glucoside + diC_8-phosphatidylcholine	Depleted	84	16	80	20	59	41
2. C_8-glucoside + diC_8-phosphatidylcholine + 50 mM KCl	Depleted	86	14	48	52	25	75
3. C_8-glucoside	Depleted	70	30	75	25	51	49
4. C_8-glucoside + diC_8-phosphatidylcholine + 1 mM $MgCl_2$	Non-depleted	79	21	53[a]	47	39	61

[a] Detergent solubilized DCCD-sensitive ATPase

of the total ATPase activity was obtained. This solubilized enzyme was inhibited 75 % by 10^{-4} M DCCD. In the following sections it will be referred to as solubilized DCCD-sensitive ATPase. This should not be confused with residual ATPase which is apparently also sensitive to DCCD but which cannot be released by aqueous washes in the absence of Mg^{2+}.

Thus, extraction of *S. faecalis* membranes with C_8-glucoside and diC_8-phosphatidylcholine in a buffer of low ionic strength solubilized several tightly bound membrane enzymes (NADH dehydrogenase, DCCD-sensitive ATPase and residual ATPase) and numerous other membrane proteins of unknown function. The membrane proteins solubilized by this procedure may possibly be resolved by conventional procedures such as gel chromatography or preparative polyamylamide-gel electrophoresis. However, care should be taken not to use solutions with ionic strengths as large as those employed in ion exchange chromatography because aggregation of the detergent solubilized proteins may result.

Gel electrophoresis of solubilized proteins

Although the detergent extracted membrane enzymes did not sediment at $10^5 \times g$ for 1 h, the question of whether these enzymes exist as distinct species or as small membrane fragments remained to be answered. To resolve this point, gel electrophoresis of the detergent solubilized proteins were carried out in the presence of

30 mM C_8-glucoside. The gels were stained for ATPase and NADH dehydrogenase activities and for protein. The results shown in Fig. 2 compare the electrophoretic mobilities of detergent solubilized DCCD-sensitive ATPase, detergent solubilized residual ATPase, and aqueous solubilized ATPase. Mg^{2+} ions were present in the gels to prevent dissociation of the DCCD-sensitive ATPase complex with subsequent loss of DCCD sensitivity. It is apparent in Fig. 2a that the detergent solubilized

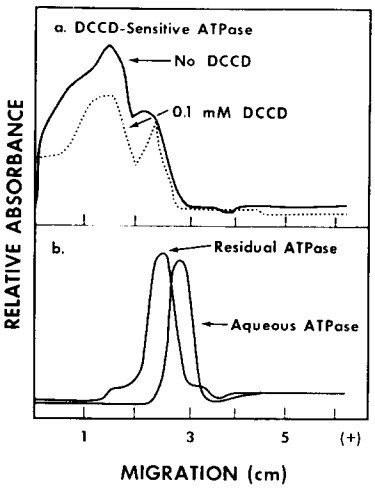

Fig. 2. Electrophoresis of solubilized ATPases. Gels, containing 1 mM $MgCl_2$, were prepared as described in the Materials and Methods section. The different ATPase preparations were layered on different gels: (a) 0.006 units of detergent solubilized DCCD-sensitive ATPase (see Table III), and (b) 0.002 units of C_8-glucoside:diC_8-phosphatidylcholine solubilized residual ATPase (see Table III) and 0.005 units of aqueous solubilized ATPase. The gels were run at 200 V, 1.4 mA/tube for 2 h at approx. 10 °C. ATPase activity was determined as described in the Materials and Methods section with an incubation at 37 °C for 60 min. In (a), ATPase activity was carried out in the presence and absence of DCCD as indicated. The gels were scanned on an E-C Apparatus Densitometer.

DCCD-sensitive ATPase has two bands of activity, one slow moving and the other faster moving with a mobility equal to that of residual ATPase (Fig. 2b). The aqueous solubilized ATPase, presumably the dissociated form of the slower moving enzyme in Fig. 2a, has a slightly faster mobility than detergent solubilized residual ATPase. This difference in mobility occurred in fresh enzyme preparations and was apparently not due to aging. In time, prearpations of aqueous solubilized ATPase show a faster mobility than do fresh preparations (Baron and Abrams, unpublished results). These results suggest that the residual ATPase is a different enzyme than the one released by washing the membranes in the absence of Mg^{2+}.

NADH dehydrogenase activity entered polyacrylamide gels as a single electrophoretic species (Fig. 3). Proteins stained with Coomassie blue (Fig. 3) exhibited some distinct bands, but also showed some areas of diffuse staining. A diffuse area in Fig. 3 (4b) is the position where ATPase activity was present (Fig. 2a). Since the enzyme activity appears to be resolved to a greater extent than the Coomassie blue stained proteins, it may be possible that proteins in this region show considerable degree of overlap.

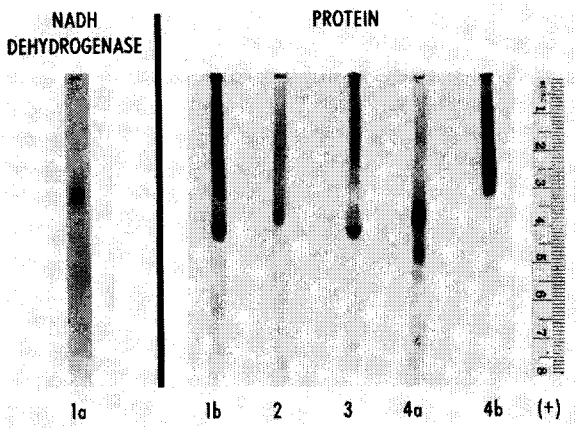

Fig. 3. Electrophoresis of NADH dehydrogenase and detergent solubilized protein. Gels, containing no $MgCl_2$ (except for 4b which was run as in Fig. 2), were prepared and stained as described in the Materials and Methods section. The gels were run at 200 V, 1.4 mA/tube for 2 h at approx. 10 °C. The samples corresponding to the supernatants in Table III were 1a and b, 29 μg of C_8-glucoside: diC_8-phosphatidylcholine solubilized depleted membranes; 2–12 μg of C_8-glucoside:diC_8-phosphatidylcholine: KCl solubilized depleted membranes; 3–25 μg of C_8-glucoside solubilized depleted membranes; 4a and b 24 μg of C_8-glucoside:diC_8-phosphatidylcholine:$MgCl_2$ solubilized non-depleted membranes.

The result above indicate that the *S. faecalis* membrane enzymes and proteins solubilized by C_8-glucoside with a small amount of diC_8-phosphatidylcholine appeared to be separate species as judged by their electrophoretic properties on polyacrylamide gels. In the future, purification of some of these solubilized proteins will be attempted. Then it may be possible to reconstitute some functional membrane systems as has been done using the detergents cholate and deoxycholate for reconstitution of oxidative phosphorylation [13–15], adenine nucleotide transport [16], and calcium transport [17, 18].

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