Biochimica et Biophysica Acta 821 (1985) 97–105
Elsevier

BBA 72791

Solubilization and reconstitution of cholinephosphotransferase from sarcoplasmic reticulum: stabilization of solubilized enzyme by diacylglycerol and glycerol

Rosemary Cornell * and David H. MacLennan

Banting and Best Department of Medical Research, University of Toronto, 112 College Street, Toronto, Ontario M5G 1L6 (Canada)

(Received May 10th, 1985)

Key words: Cholinephosphotransferase; Diacylglycerol; Glycerol; Enzyme solubilization; Reconstitution; (Sarcoplasmic reticulum)

Cholinephosphotransferase (CDPcholine: 1,2-diacylglycerol cholinephosphotransferase, EC 2.7.8.2), which catalyzes the terminal step in phosphatidylcholine synthesis via the CDPcholine pathway, is present in sarcoplasmic reticulum from rabbit skeletal muscle (Cornell, R. and MacLennan, D.H. (1985) Biochim. Biophys. Acta 835, 567–576). The conditions for solubilization and reconstitution of this enzyme were investigated as a preliminary step towards its eventual purification. The activity was not released by treatment of membranes with 1 M KCl, but was solubilized after dissolution of membranes with detergents. Cholinephosphotransferase was inactivated by cholate, deoxycholate, Triton X-100, octylglucoside, Tween-20 or SDS at concentrations which solubilize the membrane. However, the activity could be fully recovered after reconstituting the membrane by adding excess lipid (soybean) and removing detergent by gel filtration, dialysis or by absorption to Bio-Beads. When the membrane was solubilized with octylglucoside or cholate at weight ratios of detergent: membrane protein of at least 10, the activity was irreversibly lost unless stabilizers were added with detergent. The substrate diacylglycerol and glycerol were effective stabilizers.

Introduction

Cholinephosphotransferase, the enzyme which catalyzes the terminal step in the synthesis of phosphatidylcholine from CDPcholine and diacylglycerol, is an integral membrane protein of the endoplasmic reticulum [1,2] with its active site facing the cytoplasm [3,4]. Interest in this enzyme has focussed on its intracellular location [1,2,5], its distinction from ethanolaminephosphotransferase [6–9], its regulation of the acyl composition of phosphatidylcholines [6,9–13] and the physiological role of its back reaction [14–16]. Cholinephosphotransferase activity is present in skeletal muscle sarcoplasmic reticulum [17,18]. Recently we have shown that it is present in highly purified calcium phosphate-loaded sarcoplasmic reticulum [19].

The sarcoplasmic reticulum, with its comparatively simple protein composition was considered as a source for the solubilization and isolation of this protein. Sensitivity of cholinephosphotransferase to detergents [6,7,20] and to organic solvents [6] has previously been shown. Kanoh and Ohno [7] solubilized the enzyme from rat liver microsomes and purified it 4-fold with 50% yield by a two-step sonication in the presence of 20% glycerol

* To whom correspondence should be addressed at (present address): Department of Biochemistry, University of British Columbia, Faculty of Medicine, Vancouver, B.C., V6T 1W5, Canada.

Abbreviations: CPT, CDPcholine: 1,2-diacylglycerol cholinephosphotransferase; PMSF, phenylmethylsulfonyl fluoride; SM, sphingomyelin.

and 4–5 mM deoxycholate. Since the activity eluted in the void volume of a Sepharose 4B column, it likely consisted of large mixed micelles containing multiple proteins [7,21]. Treatment of this preparation with triton X-100 and further attempts at purification resulted in loss of activity [7]. This problem of inactivation by detergents must be overcome before the enzyme can be purified.

In this paper we describe the reconstitution of cholinephosphotransferase activity from sarcoplasmic reticulum membranes after solubilization and inactivation by a variety of detergents. We have found that the substrate diacylglycerol, and glycerol (at concentrations of at least 20%) are effective stabilizers of the enzyme against irreversible inactivation at high detergent: membrane protein ratios.

Materials and Methods

Materials. [^{14}C]CDPcholine (40–60 mCi/mmol) was obtained from New England Nuclear (Boston, MA). Sodium [^{14}C]cholate (50 mCi/mmol) was obtained from Amersham (Arlington Heights, IL). Asolectin was obtained from Associated Concentrates (Woodside, NY). 1,2-Diolein, CDPcholine, Tween-20, Triton X-100, deoxycholic and cholic acids, SDS and phospholipase C (Type V) were obtained from Sigma (St. Louis, MO). Octylglucoside was obtained from Calbiochem (San Diego, CA). Sephadex G-25 and Sepharose 4B were obtained from Pharmacia (Uppsala, Sweden). Bio-Beads SM-2 were obtained from Bio-Rad (Richmond, CA).

Preparation of sarcoplasmic reticulum and its subfractions. Sarcoplasmic reticulum from adult rabbit skeletal muscle was prepared according to the procedure of MacLennan [22] as modified by Campbell and MacLennan [23]. Preparations were stored at −70°C. To prepare salt washed sarcoplasmic reticulum, solid KCl was added to a 10 mg/ml suspension to a final concentration of 0.6 M. After 20 min at 0°C, the suspension was centrifuged at 150 000 × *g*, for 30 min at 4°C. The pellet was resuspended in Buffer A (10 mM Tris-HCl (pH 8.0)/1 mM histidine/0.25 M sucrose/0.1 mM PMSF). An intrinsic membrane protein fraction (R_2) was prepared from sarcoplasmic reticulum as described by MacLennan [22]. Fractions were stored at −70°C.

Cholinephosphotransferase assay. Cholinephosphotransferase was assayed as described using final concentrations of 1.6 mM 1,2-diolein suspended in asolectin (0.75 mg/ml) and Tween-20 (75 μg/ml) [19]. The reaction was linear for 30 min with up to 25 μg protein. The K_m for CDPcholine was 60 μM, and for the diacylglycerol was 0.36 mM in R_2. Cholinephosphotransferase activity in R_2 was stable to at least five freeze-thaw cycles (less than 20% decrease in specific activity).

Solubilization. All procedures were carried out at 0–4°C. Fraction R_2 was suspended in Buffer A containing variables such as glycerol, CDPcholine, KCl. Detergents were then added from stocks and the fractions were vortexed immediately. The final protein concentration was 10 mg/ml. Cholate and deoxycholate solubilizations were carried out in the presence of 1 M KCl. In some cases lipids were first dissolved in the detergent and then added to the sarcoplasmic reticulum preparation. To facilitate the dissolution of diacylglycerol in cholate, it was mixed with a vortexed suspension of asolectin, cholate, and 1 M KCl before addition to samples. After addition of the solubilization medium, samples were incubated for 20 min at 0°C before centrifugation at 150 000 × *g* for 45 min, or 200 000 × *g* for 30 min.

Turbidity measurements. Sarcoplasmic reticulum vesicles were added to a cuvette containing cholinephosphotransferase assay buffer (50 mM Tris-HCl (pH 8.0)/1 mM EDTA) at a concentration of 0.25 mg/ml. The decrease in absorbance at 600 nm was monitored with successive increments of detergent. The absorbance change due to detergent addition was accounted for by adding detergent to buffer containing no sample protein. Measurements were made at room temperature.

Reconstitution methods. All procedures were carried out at 0–4°C.

(a) Dialysis [24]. Asolectin was added from a 10% sonicated suspension in H_2O (15 min in sonicator bath) to membranes solubilized in octylglucoside, cholate or deoxycholate. The concentration of asolectin was 1–4-times the detergent concentration. The samples were dialyzed against at least 100 volumes of 10 mM Tris-HCl (pH 8.0), 0.125 M sucrose, 0.1 M NaCl and 0.1 mM PMSF. In some experiments Bio-Beads were added to the

dialysis buffer to adsorb detergents (1.5–2.5 g beads/g detergent), and in some cases glycerol (20%) was substituted for sucrose in the dialysis buffer. Dialyzed samples were assayed directly, or a membrane pellet was collected and assayed after centrifugation at 150 000 × *g* for 30 min and resuspension in Buffer A.

(b) Sephadex G-25 gel filtration [25]. When samples were solubilized at a detergent: protein ratio greater than 10 a gel filtration step preceded dialysis. Asolectin was added, as described above, to cholate or deoxycholate solubilized membranes. The solution was applied to a Sephadex G-25 column (bed volume at least five times the volume of sample) and eluted with Buffer A. The turbid void volume was collected. This fraction was then dialyzed as described above. Reconstituted membrane vesicles were collected by centrifugation as described above.

(c) Bio-Beads SM-2 [26]. Asolectin was added as described above to Triton X-100 solubilized membranes. Bio-Beads were added at a ratio of approx. 0.2 g per mg detergent and the sample was incubated at 0–5°C with stirring for 1–3 h. The sample was separated from Bio-Beads either by pasteur pipet transfer or by elution through a 10 ml column. Protein absorbed to Bio-Beads was eluted by washing several times with Buffer A. Reconstituted samples were assayed directly or after concentration of membranes by centrifugation (see above).

Miscellaneous. Large quantities of diacylglycerol were required for stabilization of enzyme. Diacylglycerol was prepared from asolectin by phospholipase C digestion [27], using 2 units/ml phospholipase C Type V. The reaction was carried out for at least 16 h at 32°C. The solvent was evaporated and the dried lipid film was extracted with acetone. The composition of the acetone-soluble fraction was checked by TLC in petroleum ether/diethyl ether/acetic acid (70 : 30 : 1, v/v). Triacylglycerol was present as a minor contaminant (1–3%). The yield of diacylglycerol from the original phospholipid was at least 60%. The diacylglycerol content of asolectin was determined using thin-layer chromatography [28] and quantitated by the method of Marsh and Weinstein [29]. Deoxycholic acid and cholic acid were recrystallized from ethanol solution [22] and converted to potassium salts. The impure detergents were more potent inhibitors of cholinephosphotransferase activity. Protein was determined by the method of Lowry et al. [30]. When the lipid : protein ratio of reconstituted vesicles exceeded 4, the lipids contributed substantially to the sample absorbance. To account for the absorbance due to lipid, the lipid phosphorus content of the sample was measured [31] and the correlating absorbance from a phospholipid (asolectin) standard curve was subtracted from the total sample absorbance.

Results and Discussion

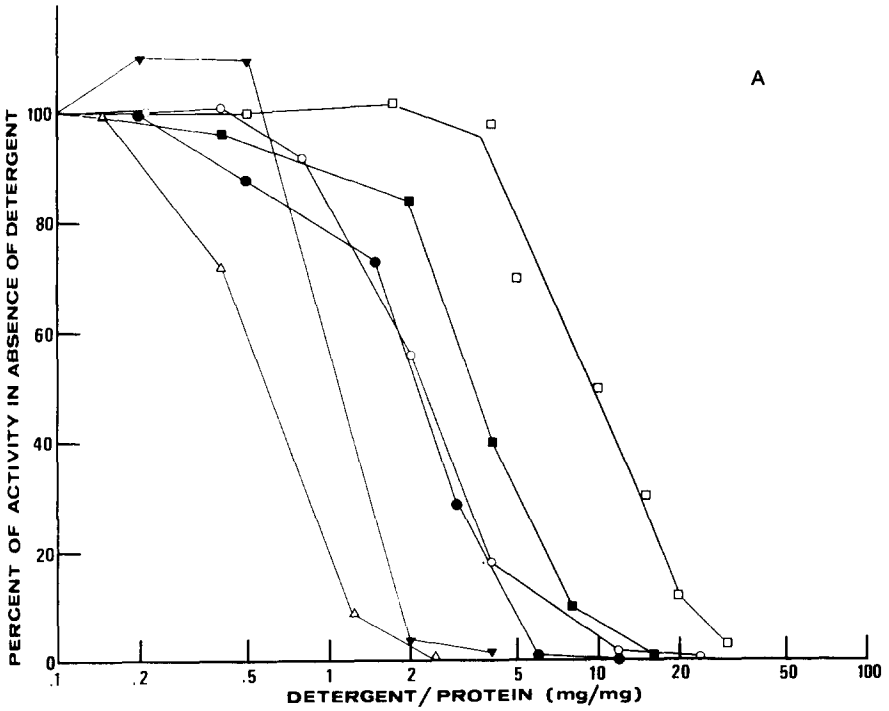
Interaction of cholinephosphotransferase with the sarcoplasmic reticulum membrane

When sarcoplasmic reticulum was washed with 0.6 M KCl to remove extrinsic proteins, cholinephosphotransferase activity remained bound to the membrane (Table I). The activity was not released from the membrane after treatment with a low concentration of deoxycholate (0.1 mg/mg protein) plus 1 M KCl (Table I). The same treatment released calsequestrin, the major extrinsic protein located on the luminal side of the vesicle [32], while leaving the Ca^{2+}-ATPase intact in the membrane. Other intrinsic proteins such as cytochrome oxidase [33] and $(Na^+ + K^+)$-ATPase [34] also resisted extraction by sub-solubilizing levels of detergent. This treatment resulted in a 1.6-fold purification of cholinephosphotransferase.

Sensitivity of cholinephosphotransferase to detergents

It has been shown that very low concentrations (less than 100 μM) of Tween-20 or lysophosphatidylcholine stimulate cholinephosphotransferase activity [20,35]. This stimulation probably involves detergent-diacylglycerol interactions which facilitate utilization of substrate by the enzyme [35]. We tested the effects of detergents on cholinephosphotransferase activity by preincubating the enzyme preparation with various amounts of detergents * for five minutes prior to the addition of di-

* Detergent concentrations throughout this paper have been expressed as the weight ratio of total detergent : membrane protein (approximately equal to the detergent : membrane lipid) as this is the best measure of the solubilizing power of a detergent [36,37].


A
PERCENT OF ACTIVITY IN ABSENCE OF DETERGENT
100
80
60
40
20
0
.1
.2
.5
1
2
5
10
20
50
100
DETERGENT/ PROTEIN (mg/mg)

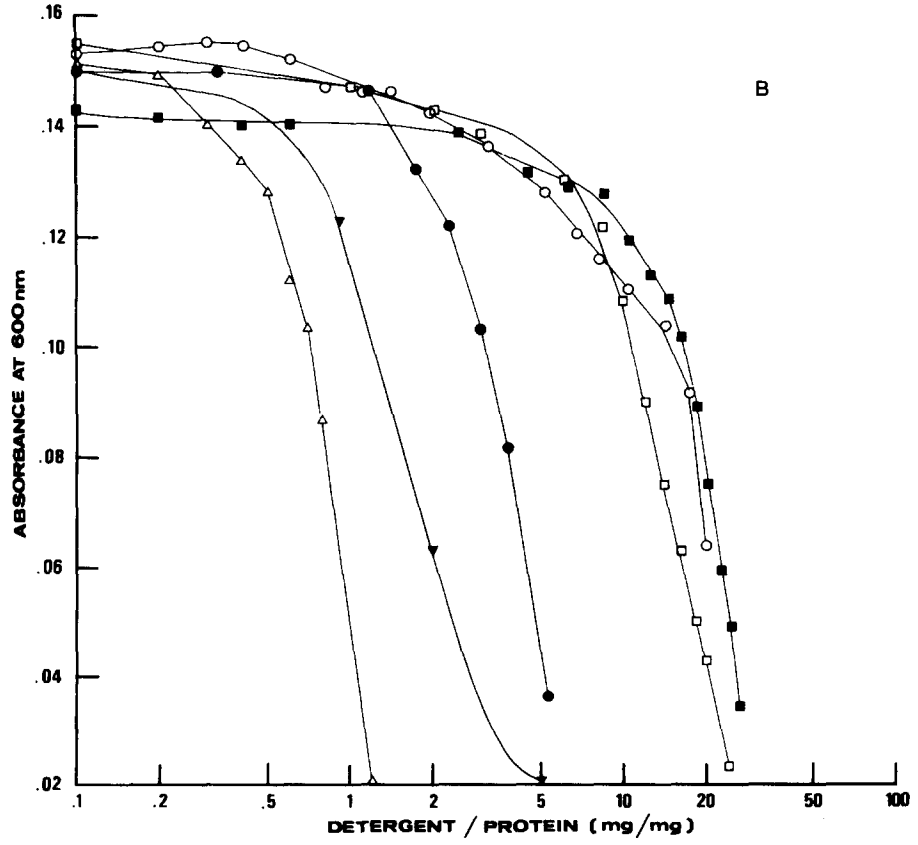

B
ABSORBANCE AT 600nm
.16
.14
.12
.10
.08
.06
.04
.02
.1
.2
.5
1
2
5
10
20
50
100
DETERGENT / PROTEIN (mg/mg)

TABLE I

CHOLINEPHOSPHOTRANSFERASE ACTIVITY AFTER TREATMENT OF SARCOPLASMIC RETICULUM WITH KCl AND DEOXYCHOLATE

Sarcoplasmic reticulum (10 mg/ml in Buffer A) was treated with KCl and deoxycholate (DOC) at the indicated concentrations at 0°C for 20 min. The suspensions were centrifuged at 140000 × g for 30 min. Pellets were resuspended in Buffer A. Proteins are normalized to 100 mg of the original preparation.

N	Extraction medium	Fraction	Protein (mg)	Specific activity (nmol/min/mg)	Units (nmol/min)
3	None	original	100	2.88 ± 0.64	288
2	0.6 M KCl	Pellet	88 ± 3	3.22 ± 0.03	284 ± 8
		Sup	12 ± 3	0.04 ± 0.01	0.4 ± 0.02
4	1 M KCl + 0.1 mg/ml DOC	Pellet (R_2)	71 ± 3	4.80 ± 0.55	339 ± 33
		Sup	29 ± 3	0.24 ± 0.08	8 ± 3

acylglycerol. In this way, the effect of detergent on the membrane was not influenced by the lipid substrate.

The criteria we used to judge solubility were optical clarity and lack of sedimentation after 30 min centrifugation at 200000 × g_{av}. The bulk of protein and cholinephosphotransferase activity (62% and 93% of that applied, respectively) after solubilization with cholate at a detergent : protein ratio of 2 eluted in the void volume of a Bio-Rad A-0.5M column. However, both protein and cholinephosphotransferase activity were retained on Sepharose 4B, eluting as a broad band with a peak at twice the void volume indicating a heterogeneous size distribution ($(0.2–2)\cdot 10^6$) of mixed micelles which were large enough to contain several protein monomers.

We observed a slight activation of cholinephosphotransferase activity at presolubilizing concentrations of Triton X-100 (Fig. 1A). All six detergents tested inactivated the enzyme ($\geq$ 97% inhibition) at concentrations which solubilized the membrane (Figs. 1A and B). The inhibitory effect of detergents on cholinephosphotransferase activity at solubilizing concentrations was primarily due to the disruption of the lipid environment during solubilization. Inactivation curves coincided with solubilization curves for all detergents except Tween-20 and octylglucoside. In the case of these latter detergents, inactivation occurred at concentrations well below the range required for solubilization, suggesting inactivation by direct binding of detergent to the enzyme or by perturbation of lipid-enzyme interactions prior to solubilization. In addition, the presence of detergent in the assay could also prevent the utilization of the exogenous lipid substrate (diacylglycerol in a phospholipid suspension) by the enzyme.

Reconstitution of cholinephosphotransferase at low detergent : protein ratios (< 3)

To recover cholinephosphotransferase activity after detergent solubilization we tried various methods of detergent removal including Sephadex G-25 gel filtration [25], dilution [24], dialysis and Bio-Beads [26]. Dialysis proved to be the most practical method for removal of detergents other than Triton when working with a large number of samples. Biobeads were used to reconstitute Triton X-100-solubilized membranes [26] since the low critical micelle concentration of Triton makes it very difficult to remove by dialysis.

Some representative reconstitution experiments are shown in Table II. At the detergent concentra-

Fig. 1. (A) Sensitivity of cholinephosphotransferase activity to detergents. Detergents were added to R_2 (25 μg in 0.1 ml cholinephosphotransferase assay buffer). After 5 min at 21°C, diolein was added. After an additional 5 min at 37°C, the reaction was initiated with [^{14}C]CDPcholine. The diolein was added from a stock containing Tween-20 such that all assays contained 75 μg/ml Tween-20 in addition to the indicated detergent. Data are averages of two separate experiments. □, Cholate; ■, octylglucoside; ●, deoxycholate; ▼, Triton X-100; ○, Tween-20, △, SDS. (B) Membrane solubilization by detergents. See Materials and Methods for turbidity measurements. Solubilization conditions were identical in (A) and (B).

TABLE II

RECONSTITUTION OF CHOLINEPHOSPHOTRANSFERASE ACTIVITY AFTER DETERGENT SOLUBILIZATION

Sarcoplasmic reticulum (R_2) was suspended in buffer A at 10 mg protein/ml and solubilized at the indicated detergent: protein ratio. CDPcholine was added to some of the solubilization media at a concentration of 0.8 mM. Asolectin was added to the transparent supernatants recovered after centrifugation at 150000 × *g* for 45 min and the detergent was removed by dialysis for 24 h, except for Triton X-100 which was removed by mixing with Bio-Beads for 3 h (see Materials and Methods). With the exception of Triton reconstitution, the reconstituted activities were determined on membranes concentrated by centrifugation at 150000 × *g* for 30 min. Specific activities are in nmol/min per mg protein.

Detergent	Detergent: protein (mg/mg)	CDP-choline	Asolectin: protein (mg/mg)	Specific activities		
				presolubilized	solubilized	reconstituted
Octylglucoside	1.5	+	0	3.6	0.2	1.7
		+	5	3.6	0.2	3.3
		−	5	4.2	0.13	1.8
Cholate	2.0	+	8	4.4	0.04	2.5
Triton X-100	2.0	+	2	3.7	0.01	4.2
Deoxycholate	0.5	+	0	3.6	0.2	3.6
		+	2	3.6	0.2	3.6
		−	2	3.6	0.17	1.5

tions used, at least 80% of the membrane protein was solubilized * and the solubilized CPT was more than 90% inactivated. The presence of the substrate CDP-choline during solubilization was found to increase the recovery of activity (Table II). It was not necessary to include CDPcholine in the subsequent dialysis step. The concentration of CDPcholine for maximum recovery was 0.4 mM, or approximately 6-times the K_m value (60 μM in sarcoplasmic reticulum). The rate of removal of CDPcholine during dialysis paralleled the rate of cholate removal as monitored with [^{14}C]CDPcholine and [^{14}C]cholate (data not shown). The dialysis time required for recovery of cholinephosphotransferase activity (approx. 40% recovery) after cholate solubilization was not longer than 8 h at which time 85% of the cholate had been removed. Further dialysis removed up to 98% of the detergent but did not improve the recovery of activity. Reconstitution after deoxycholate solubilization also required approx. 8 h dialysis; reconstitution from octylglucoside solution required approx. 20 h dialysis. It was possible to recover 100% of the activity after reconstitution from octylglucoside, deoxycholate, or Triton solution, and 50–60% of the activity from cholate solution at detergent: protein ratios ≤ 2.

The reconstituted samples could be concentrated by centrifugation at 150000 × *g* for 30 min. The smaller vesicles which remained in the supernatant were in all cases as active as those that pelleted. The solubilized enzyme was temperature sensitive in that warming to 37°C or even room temperature for only a few minutes decreased its recovery after reconstitution. If the soluble enzyme were frozen, activity could not be regained. The reconstituted enzyme, however, was stable throughout at least one freeze-thaw cycle.

The membranes which reformed upon removal of detergent by dialysis, gel filtration, Bio-Beads or upon dilution were not necessarily identical to the presolubilized membranes with regard to size or to the orientation and associations of proteins [24,38–40]. Moreover, in most cases, excess asolectin was added prior to detergent removal;

* The ratios of detergent to membrane protein required for solubilization in Table II do not correspond in all cases to the ratios in Fig. 1B because this value is influenced by the membrane concentration [25]. The membrane concentration for experiments shown in Table II was 40-times higher than that used in Fig. 1B. In dilute membrane suspension solubilization did not occur below the critical micelle concentration. At a membrane concentration of 10 mg protein/ml, the critical micelle concentration is not relevant to the solubilization process since the detergent avidly partitions into the membrane, thus maintaining a low free monomer concentration.

TABLE III

EFFECT OF OCTYLGLUCOSIDE CONCENTRATION ON RECOVERY OF CHOLINEPHOSPHOTRANSFERASE ACTIVITY

Sarcoplasmic reticulum fraction R_2 (10 mg/ml) in buffer A containing 0.8 mM CDPcholine was solubilized with octylglucoside (15 mg/ml) and centrifuged at $150000 \times g$ for 45 min. The clear supernatant was diluted with buffer A containing 15 mg/ml octylglucoside to the indicated detergent: protein ratios; or buffer A containing 15 mg/ml octylglucoside plus 10 mg/ml of a diacylglycerol/phospholipid (DG/PL, 1:4) mixture. Samples were reconstituted by dilution and dialysis. Activities (nmol/min per mg) were determined in reconstituted membranes concentrated by centrifugation at $150000 \times g$ for 30 min. This experiment was repeated twice with similar results.

Octylglucoside: protein ratio (mg/mg)	Lipid addition (DG/PL, 1:4)	Specific activity
0	−	3.40
5	−	2.10
10	−	2.15
20	−	0.45
30	−	0.20
30	+	1.95

thus, the lipid composition of the reconstituted vesicles was different from the original membranes. (The phospholipid composition of asolectin is: PC/PE/PA/PI, 39:23:5:20, the remaining 13% being unidentified [41]; the phospholipid composition of sarcoplasmic reticulum is: PC/PE/SM/PS/PI, 74:13.5:3:3:7 and neutral lipids (cholesterol) comprise 8% [42].) These changes in the reconstituted vesicles did not lower the specific activity, however. Inclusion of asolectin actually improved recovery of activity after octylglucoside dissolution (Table II), probably by acting as a detergent 'sponge', i.e. by partitioning the detergent away from the enzyme.

Protection of cholinephosphotransferase at high detergent: protein ratios

We found that fractionation schemes involving dilution of sample in buffer containing fixed concentrations of detergent resulted in very poor recovery of enzyme activity after detergent removal. Table III illustrates the effects of increasing the octylglucoside: protein ratio in the solubilization buffer on subsequent recovery of activity. In this experiment, the detergent concentration remained fixed and the protein concentration was varied by dilution in buffer containing detergent. At detergent: protein ratios less than 10, approximately two-thirds of the original activity was recovered. At higher detergent levels, the recovery was drastically reduced. At a detergent: protein ratio of 30, only 6% of the original activity was recovered. However, if, during solubilization, a mixture of diacylglycerol and phospholipid were present at a detergent: lipid ratio of 1.5, two-thirds of the original activity was recovered. It appeared that the enzyme was inactivated irreversibly at high detergent: protein ratios unless a lipid stabilizer was present.

The ability of various lipids and glycerol to stabilize cholinephosphotransferase in the presence of high detergent: protein ratios was investigated in more detail (Table IV). When samples were dissolved at a cholate: protein ratio of 2.0, only one-fourth of the presolubilized activity was recovered after reconstitution. Inclusion of asolectin during solubilization improved recovery to about 50%. The diacylglycerol content of asolectin was determined to be less than 1% by weight. Complete recovery was achieved if a diacylglycerol/asolectin (1:3, w/w) mixture or glycerol ($\geq 20\%$) was present during solubilization. The protective effect of diacylglycerol and glycerol was apparent even in the solubilized samples prior to reconstitution. The specific activity of the solubilized sample containing 40% glycerol and diacylglycerol/phospholipid (1:3) was 10-times higher than that of sample solubilized without glycerol or lipid present. The detergent was diluted 5-fold in the assay in each case so that the detergent concentration in the assay was constant.

When samples were dissolved at a cholate: protein ratio of 20, only 3% of the presolubilized activity was recovered if no stabilizers were added (Table IV). The solubilized enzyme retained some activity only if diacylglycerol was present. Diacylglycerol was also necessary for complete recovery of activity in the reconstituted membranes. Soy phospholipids (diacylglycerol-free) were ineffective as stabilizers. Egg PC/PE (2:1) mixture and muscle total phospholipids were also ineffective (data not shown). 40% glycerol offered greater protection than 20% glycerol. Glycerol (20%) was also included in the dialysis step. Diacylglycerol or

TABLE IV

PROTECTION OF CHOLINEPHOSPHOTRANSFERASE FROM IRREVERSIBLE DETERGENT INACTIVATION BY LIPIDS AND GLYCEROL

Membranes (R_2) were solubilized with cholate in buffer A containing 0.4 mM CDPcholine and the components indicated. Lipid components were present at a lipid : cholate ratio of 1. After centrifugation at 200000 × *g* for 30 min, samples were reconstituted by addition of asolectin (total lipid : protein = 5) followed by dialysis for 18 h. In samples solubilized at cholate : protein = 20, a Sephadex G-25 gel filtration step preceded the dialysis. 90% of the cholate was removed after gel filtration and more than 97% after dialysis. n.d., not determined.

Solubilization conditions	Percent of presolubilized specific activity [a]			
	Cholate/protein = 2 [b]		Cholate/protein = 20 [c]	
	Soluble	Reconstituted	Soluble	Reconstituted
no additions	1.1	24	0.0	3.3
20% glycerol	3.8	105	0.0	27.0
40% glycerol	13.4	104	1.0	44.0
soy PL	n.d.	n.d.	0.0	9.2
soy PL + 40% glycerol	n.d.	n.d.	1.1	39.0
soy total lipid	1.3	54	1.1	37.0
soy DG/PL (1 : 3)	10.4	109	7.0	92.0
soy DG/PL (1 : 3) + 40% glycerol	11.5	109	8.3	99.0

[a] The presolubilized specific activities for five separate experiments ranged from 2.6 to 6.3 nmol/min per mg. Data are averages of two or more determinations for each condition.

[b] [protein] = 4 mg/ml; [cholate] = 8 mg/ml.

[c] [protein] = 1 mg/ml; [cholate] = 20 mg/ml.

glycerol were effective as stabilizers only if present during solubilization. If added after the membrane was dispersed by detergent, there was no improvement in the recovery of activity.

Irreversible inactivation by detergents has been observed with other membrane proteins. Inclusion of phospholipid in the solubilization medium prevents inactivation of the sodium channel from rat brain [43] and several sugar transport systems from *Escherichia coli* [44,45] by stabilizing the native conformation of the proteins. The presence of cholesterol in addition to phospholipid enhances the ion influx response of solubilized acetylcholine receptors [46]. Enzyme substrates (or receptor agonists) have also been used to protect the active sites of solubilized membrane enzymes against denaturation [47–50].

Protection of solubilized cholinephosphotransferase at high level of detergents was achieved only with diacylglycerol and, to a lesser extent, glycerol. CDPcholine was a stabilizer of cholinephosphotransferase only at low detergent concentrations. The requirement for diacylglycerol at high detergent concentrations suggests that there are two levels of detergent inactivation: a reversible inactivation which involves removal of the lipid environment surrounding the enzyme, and an irreversible inactivation that involves denaturation of the active site upon binding of detergent. The latter occurs only at high detergent concentrations. Diacylglycerol may protect cholinephosphotransferase from irreversible inactivation because the strength of its interaction with cholinephosphotransferase exceeds that of the detergent. Thus when both diacylglycerol and detergent are present at the same concentration, the lipid substrate successfully competes for the active site. Glycerol has been widely used to stabilize enzymes. It preserves protein native conformation by raising the barrier against unfolding and exposure of hydrophobic segments to the solvent [50]. Thus glycerol may protect cholinephosphotransferase from detergents such as cholate by preventing their penetration into the active site.

Tests of these proposed mechanisms for substrate and glycerol protection of cholinephosphotransferase from detergents await binding studies utilizing purified enzyme. The discovery that

glycerol and diacylglycerol stabilize the enzyme in the presence of detergent should greatly increase the chances for purification of this enzyme. The purification of this enzyme along with other enzymes involved in the synthesis of phospholipids would enable the construction of an in vitro defined phospholipid generating system with which to probe the regulation of PC synthesis and the mechanism of membrane assembly.

Acknowledgements

We thank Dr. Tom Madden for helpful discussions of this work and Dr. Dennis Vance for reading the manuscript. This work was supported by grants from The Medical Research Council of Canada and The Muscular Dystrophy Association of Canada. R.C. was a postdoctoral fellow of The Muscular Dystrophy Association of Canada.

References

1 Wilgram, G.F. and Kennedy, E.P. (1963) J. Biol. Chem. 238, 2615–2619
2 Van Golde, L.M.G., Fleischer, B. and Fleischer, S. (1971) Biochim. Biophys. Acta 249, 318–330
3 Vance, D.E., Choy, P.C., Farren, B., Lim, P. and Schneider, W. (1977) Nature 270, 268–269
4 Coleman, R. and Bell, R.M. (1978) J. Cell Biol. 76, 245–253
5 Baker, R. and Chang, H.Y. (1982) Canad. J. Biochem. 60, 724–733
6 Coleman, R. and Bell, R.M. (1977) J. Biol. Chem. 252, 3050–3056
7 Kanoh, H. and Ohno, K. (1976) Eur. J. Biochem. 66, 201–210
8 Radominska-Pyrek, A., Pilarska, M. and Zimniak, P. (1978) Biochem. Biophys. Research Commun. 85, 1074–1081
9 Morimoto, K. and Kanoh, H. (1978) J. Biol. Chem. 253, 5056–5060
10 De Kruijff, B., Van Golde, L.M.G. and Van Deenan, L.L.M. (1970) Biochim. Biophys. Acta 210, 425–435
11 Kanoh, H. and Ohno, K. (1975) Biochim. Biophys. Acta 380, 199–207
12 Holub, B.J. (1978) J. Biol. Chem. 253, 691–696
13 Ide, H. and Weinhold, P. (1982) J. Biol. Chem. 257, 14926–14931
14 Kanoh, H. and Ohno, K. (1973) Biochim. Biophys. Acta 306, 203–217
15 Goracci, G., Francescangeli, E., Horrocks, L. and Porcellati, G. (1981) Biochim. Biophys. Acta 664, 373–379
16 Van Heusden, G.P.H. and Van Den Bosch, H. (1982) Biochim. Biophys. Acta 711, 361–368
17 Pennington, R. and Worsfold, W. (1969) Biochim. Biophys. Acta 176, 774–782
18 Sarzala, M.G. and Pilarska, M. (1976) Biochim. Biophys. Acta 441, 81–92
19 Cornell, R. and MacLennan, D.H. (1985) Biochim. Biophys. Acta 835, 567–576
20 Parthasarathy, S. and Baumann, W. (1979) Biochem. Biophys. Res. Commun. 91, 637–642
21 Shankland, W. (1970) Chem. Phys. Lipids 4, 109–130
22 MacLennan, D.H. (1970) J. Biol. Chem. 245, 4508–4518
23 Campbell, K.P. and MacLennan, D.H. (1981) J. Biol. Chem. 256, 4626–4632
24 Racker, E., Violand, B., O'Neal, S., Alfonso, M. and Telford, J. (1979) Arch. Biochem. Biophys. 198, 470–477
25 Helenius, A. and Simons, K. (1971) Biochemistry 10, 2542–2547
26 Holoway, P.W. (1973) Anal. Biochem. 53, 304–308
27 Myher, J. and Kuksis, A. (1979) Canad. J. Biochem. 57, 117–124
28 Freeman, C. and West, D. (1966) J. Lipid Res. 7, 324–326
29 Marsh, J. and Weinstein, D. (1966) J. Lipid Res. 7, 574–576
30 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
31 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468
32 MacLennan, D.H. and Wong, P.T.S. (1971) Proc. Natl. Acad. Sci. USA 68, 1231–1235
33 Kuboyama, M., Yong, F.L. and King, T.E. (1972) J. Biol. Chem. 247, 6375–6383
34 Jørgensen, P.L. (1970) Biochim. Biophys. Acta 356, 36–52
35 Arthur, G., Tam, S.W. and Choy, P.C. (1984) Can. J. Biochem. 62, 1059–1063
36 Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29–79
37 Lichtenberg, D., Robson, R. and Dennis, E.A. (1983) Biochim. Biophys. Acta 737, 285–304
38 Meisner, G. and Fleischer, S. (1974) J. Biol. Chem. 249, 302–309
39 Brunner, J., Hauser, H. and Semenza, G. (1978) J. Biol. Chem. 253, 7538–7546
40 Carrol, R.C. and Racker, E. (1977) J. Biol. Chem. 252, 6981–6990
41 Chapman, G.W. (1980) J. Am. Oil Chem. 57, 299–304
42 Zubrzycka, E., Michalak, M., Kosk-Kosicka, D. and Sarzala, M.G. (1979) Eur. J. Biochem. 93, 113–121
43 Catterall, W.A., Morrow, C.S. and Hartshorne, R.P. (1979) J. Biol. Chem. 254, 11379–11387
44 Henderson, P., Kagawa, Y. and Hirata, H. (1983) Biochim. Biophys. Acta 732, 204–209
45 Newman, M.J. and Wilson, T.H. (1980) J. Biol. Chem. 255, 10583–10586
46 Ochoa, E.L.M., Dalziel, A.W. and McNamee, M.G. (1983) Biochim. Biophys. Acta 727, 151–162
47 King, T.E. (1963) J. Biol. Chem. 238, 4037–4051
48 Moller, J.V., Lind, K.E. and Anderson, A.P. (1980) J. Biol. Chem. 255, 1912–1920
49 Vogel, H.J. and Bridger, W.A. (1981) J. Biol. Chem. 256, 11702–11707
50 Nedivi, E. and Schramm, M. (1984) J. Biol. Chem. 259, 5803–5808
51 Gekko, K. and Timasheff, S.N. (1981) Biochemistry 20, 4677–4686