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THE ROLE OF PHOSPHOLIPIDS IN THE MODULATION OF ENZYME ACTIVITIES IN THE CHROMAFFIN GRANULE MEMBRANE

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Summary

(1) 93% of the protein of chromaffin granule membranes can be solubilized by 1.3% (w/v) sodium cholate. The solubilized material can be substantially delipidated by ammonium sulphate precipitation. After three such cycles less than 2% of the endogenous phospholipids remain.

(2) The chromaffin granule membrane Mg^{2+}-ATPase depends on the presence of phospholipids for retention of its full activity. Soybean and extracted chromaffin granule phospholipids fully reactivate the delipidated enzyme provided only one delipidation step is used.

(3) Successive ammonium sulphate precipitation steps result in a delipidated, and deactivated ATPase preparation which can be only partially reactivated on re-addition of phospholipids.

(4) The phospholipid specificity for reactivation of the Mg^{2+}-ATPase is broad. Although acidic phospholipids allow higher activities than neutral phospholipids, the main requirement appears to be the hydrophobic environment provided by the phospholipid hydrocarbon chains.

(5) Correlations between changes in slope in the Arrhenius plot of the Mg^{2+}-ATPase, and phase transitions in the phospholipid used for reactivation suggest that the 'fluidity' of the hydrocarbon chains modulates the activity of the enzyme.

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Abbreviations: ATPase, adenosine triphosphatase (EC 3.6.1.3); dopamine β-hydroxylase, 3,4-dihydroxyphenylethylamine, ascorbate:O_2 oxidoreductase (β-hydroxylating) (EC 1.14.17.1); NADH oxidase, NADH: ferricyanide oxidoreductase (EC 1.6.99.3); HEPES, *N*-2-hydroxymethylpiperazine-*N'*-2-ethanesulphonic acid.

(6) The chromaffin granule membrane dopamine β-hydroxylase activity does not depend on the presence of a phospholipid.

(7) The chromaffin granule membrane NADH : ferricyanide oxidoreductase activity is increased on delipidation. This increase is reversed when phospholipids are added back to the delipidated preparation.

Introduction

Complete understanding of the function of the cell requires a knowledge of membrane structure. The interactions between membrane components play a fundamental role in the regulation, and ordering, of many physiological processes, and their study might help in understanding the functional complexity of membrane systems. Of particular interest is the nature of lipid-protein interactions and the way in which lipids regulate the activity of membrane-bound enzymes. Techniques recently developed for the delipidation of such enzymes include treatments with phospholipase [1—3], detergent [2—5] or organic solvent [6—8]. Delipidation often results in deactivation of the enzyme and it is the subsequent re-activation by addition of chemically defined lipids that provides information on the lipid specificity of membrane-bound enzymes. Such studies have lead to the concept of a 'boundary lipid' layer or lipid annulus [9,10] surrounding the enzyme in the membrane, and necessary not only for the full expression of enzyme activity but also for stabilization of the tertiary structure of the protein [11,12].

The membrane of the adrenomedullary storage vesicles (chromaffin granules) has been analysed in several studies (for reviews see Refs. 13—15), and contains a number of enzyme activities. The Mg^{2+}-dependent ATPase has been shown to be an inwardly directed, electrogenic proton pump [16—21] and has been implicated in the mechanism of uptake of catecholamines [22—25] and nucleotides [26]. Apps and Glover [27] have used dichloromethane extraction and glycerol density gradient centrifugation to isolate and purify the enzyme which has a molecular weight of approximately 400 000. These studies have recently been extended by Apps and Schatz [28] who have shown that, although sharing a number of common properties, the chromaffin granule ATPase and the mitochondrial ATPase are, nevertheless, distinct enzymes.

The membrane-bound ATPase shows a non-linear Arrhenius temperature versus activity relationship [29] and the change in slope has been correlated with phase changes in the membrane as detected by fluorescent [29] and spin-labelled [30] probes. It appears that the motional properties of the membrane phospholipids have a direct influence on the activity of this enzyme.

The NADH: (acceptor) oxidoreductase activity was originally attributed to a flavoprotein, cytochrome *b*-561, and one of the enzymes of catecholamine metabolism, dopamine β-hydroxylase, as a possible terminal acceptor [17,31, 32]. This interpretation has recently been questioned [33], and the functional significance of cytochrome *b*-561 and the NADH: (acceptor) oxidoreductase activity in the granule membrane remains to be determined.

Understanding the molecular mechanism of neurotransmitter release from the chromaffin granule requires a complete description of the interactions in

the granule membrane and, in particular, those between the lipids and proteins. Current ideas suggest that the release mechanism involves fusion of the granule and the plasma membrane (exocytosis) [34] and it is likely that the granule membrane plays a fundamental role in this process. In this paper we extend our previous work [35] on the reconstitution of membrane-bound Mg^{2+}-ATPase, and present the results of studies aimed at explaining the nature of lipid-protein interactions in the chromaffin granule membrane. The results of such studies may enable a more complete description to be made of the role of the membrane in granule function and, in particular, in the molecular mechanism of exocytosis.

Experimental procedure

Materials

Sodium cholate was obtained from the Sigma Chemical Co. and was recrystallized twice from 70% aqueous ethanol after treatment with activated charcoal. Chromaffin granule phospholipids were extracted from the membranes by the method of Bligh and Dyer [36]. Soybean phospholipids were obtained from the Sigma Chemical Co. and purified as described [37]. Phosphatidylinositol, phosphatidylcholine, sphingomyelin and lysophosphatidylcholine were obtained from Lipid Products; phosphatidylethanolamine, phosphatidylserine and dipalmitoyl phosphatidylcholine from Koch-Light Laboratories and dimyristoyl phosphatidylcholine from the Sigma Chemical Co. Dipalmitoleoyl phosphatidylcholine was the kind gift of Dr. B. de Kruijff (University of Utrecht). Phospholipids were stored as stock solutions in chloroform or chloroform/methanol (1 : 1 v/v) under nitrogen at −20°C and were checked for purity by thin-layer chromatography before use. All other chemicals were of the highest purity available commercially. Water was doubly distilled in an all glass apparatus, and other solvents were distilled before use.

Methods

Purified chromaffin granule membranes were prepared from the adrenal glands of freshly slaughtered cattle by differential centrifugation as described previously [38]. The final membrane pellet was resuspended in a medium containing 10 mM Hepes, 10% (w/v) glycerol, 1 mM magnesium sulphate, 0.5 mM EDTA and 3 mM β-mercaptoethanol (pH 8.0) (membrane buffer) when assaying for ATPase activity, or 10 mM Hepes (pH 8.0) when assaying for NADH oxidase or dopamine β-hydroxylase activities.

The membranes were solubilized by dropwise addition of a 10% (w/v) sodium cholate, pH 8.0, (final concentration 1.3% w/v) to the membrane suspension made 10% saturated with ammonium sulphate. The final cholate: protein ratio was in the range 5—7 (w/w). After stirring at 4°C for 20 min, the preparation was centrifuged at 100 000 × g_{av} for 1 h in the 6 · 14 ml Ti rotor of an MSE Prepspin ultracentrifuge. The supernatant (cholate extract) was decanted from the pellet and kept at 4°C until use.

The solubilized preparation was delipidated by dropwise addition of a saturated solution of ammonium sulphate (pH 8.0) to a final saturation of 45%. After 10 minutes stirring at 4°C, the suspension was centrifuged at 27 000 × g_{av} for 20 min. The pale yellow supernatant (labelled 45S) was decanted from the

red pellet (labelled 45P) which was dissolved in either membrane buffer containing 1.3% (w/v) sodium cholate at pH 8.0 or 10 mM Hepes containing 1.3% (w/v) sodium cholate at pH 8.0. Complete solubilization was obtained by gentle mixing with a glass rod.

Reconstitution was carried out by addition of the 45P pellet to a sonicated solution of phospholipids (approx. 10 mg/ml) in either membrane buffer or 10 mM Hepes. In both cases the buffer contained 1.3% (w/v) sodium cholate at pH 8.0. This solution was dialysed at 4°C against a 100-fold excess of membrane buffer or 10 mM Hepes (pH 7.0) with five changes over a period of 36 h. At the end of this period, samples were removed for assay of enzyme activity, protein and lipid phosphorus.

Unless otherwise stated in the text, ATPase activity was assayed at pH 6.6 and 30°C using an ATP-regenerating system coupled to NADH oxidation, the absorbance change at 340 nm being monitored continuously [39].

NADH oxidase activity was assayed at 25°C. The reaction medium contained 10 mM Tris sulphate, 1 mM potassium ferricyanide and 20—100 μg of membrane protein in a total volume of 3.0 ml. The reaction was initiated by addition of 60 μM NADH and the absorbance change at 340 nm measured continuously.

Dopamine β-hydroxylase activity was assayed at 37°C according to the method of Nagatsu and Udenfriend [40], using tyramine as substrate. In contrast to the findings of Helle et al. [41], it was found necessary to include the column step in the procedure.

Protein was assayed by the Biuret reaction [42] and lipid phosphorus according to the method of McClare [43] after perchloric acid digestion.

Fluorescence polarization-temperature scans were made on the apparatus described by Bashford et al. [44] using diphenylhexatriene as the probe. The probe (final concentration 1 μM) was added as a concentrated stock solution in tetrahydrofuran, and was allowed to bind for 1 h prior to running the scan.

Results

Phospholipid dependence of the Mg^{2+}-ATPase

Sodium cholate at 1.3% (w/v) solubilized 93% (range 89—97% for 18 determinations) of the protein of the chromaffin granule membrane (Table I). The solubilized membrane protein was substantially delipidated by addition of ammonium sulphate to a final saturation of 45%, followed by centrifugation of the precipitated protein. This procedure gave a preparation with a lipid-to-protein ratio of 0.22 μmol/mg (range 0.12—0.35 for 12 preparations), representing removal of 83—94% of the endogenous phospholipids. Parallel with this level of phospholipid depletion, the ATPase activity was reduced by approximately 75% (Table I), although the activity obtained for the delipidated preparations was critically dependent on the level of phospholipid depletion.

Addition of extracted chromaffin granule phospholipids to the pellet (45P) restored the ATPase activity. The maximal activity (223 nmol P_i released/min per mg protein), was obtained at a lipid-to-protein ratio of 1.0 μmol/mg (Fig. 1a). It is clear from Fig. 1 that the activity of the ATPase depends on the presence of phospholipid.

TABLE I

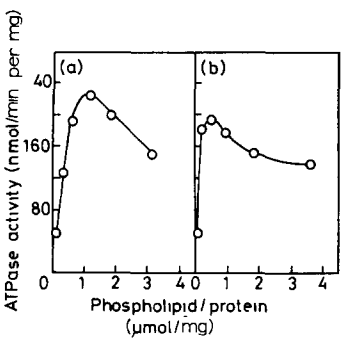
THE EFFECT OF SOLUBILIZATION AND LIPID DEPLETION ON THE Mg^{2+}-ATPase ACTIVITY OF THE CHROMAFFIN GRANULE MEMBRANE

Chromaffin granule membranes were solubilized and delipidated by ammonium sulphate precipitation as described in Experimental procedure. After extensive dialysis to remove ammonium sulphate and sodium cholate, samples were removed for assay of protein, lipid phosphorus and ATPase activity. Figures for ATPase activity represent the mean ± S.D. for four determinations, results obtained for a typical experiment.

	% Protein, mean (and range)	Lipid/protein (μmol/mg), mean (and range)	ATPase activity (nmol P_i released/min per mg protein)
Membranes	'100'	2.06 (1.54—2.52)	182.5 ± 6.3
Cholate extract	93 (89—97)	2.26 (1.77—2.84)	202.1 ± 27.3 *
45P	57 (50—65)	0.22 (0.12—0.35)	47.6 ± 2.3 **
45S	36 (28—43)	5.02 (3.63—7.42)	—

* Before dialysis activity was 126.8 ± 20.1.

** Activity depends critically on lipid-to-protein ratio.



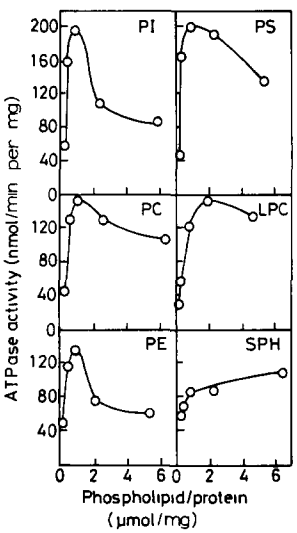


Fig. 1. Reactivation of the chromaffin granule membrane Mg^{2+}-ATPase by chromaffin granule or soybean phospholipids. Chromaffin granule membranes were solubilized and delipidated by ammonium sulphate precipitation as described in Experimental procedure. Extracted chromaffin granule (a) or purified soybean phospholipids (b) were added to the delipidated preparations and the samples were extensively dialysed to remove sodium cholate and ammonium sulphate. At the end of the dialysis period, samples were removed for assay of protein, lipid phosphorus and ATPase activity. Each point represents the mean for at least four determinations.

Fig. 2. Reactivation of the chromaffin granule membrane Mg^{2+}-ATPase by phospholipids. Chromaffin granule membranes were solubilized and delipidated by ammonium sulphate precipitation as described in Experimental procedure. Phospholipids were added to the delipidated preparation and the samples were extensively dialysed to remove sodium cholate and ammonium sulphate. At the end of the dialysis period, samples were removed for assay of protein, lipid phosphorus and ATPase activity. Each point represents the mean for at least four determinations. Abbreviations: PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; SPH, sphingomyelin.

Further depletion of phospholipid by repeating the ammonium sulphate precipitation step resulted in a preparation with an ATPase activity of only 6 nmol P_i released/min per mg protein at a lipid-to-protein ratio of 0.02 μmol/mg. On addition of extracted chromaffin granule phospholipids to this preparation, maximal activities of only 40 nmol P_i released/min per mg protein were obtained. Thus, extensive delipidation results in a deactivation which is only partially reversible.

The activity of the delipidated preparation was also restored by addition of purified soybean phospholipids (Fig. 1b), although in this case, the maximal activity, obtained at a lipid-to-protein ratio of 0.6 μmol/mg, was less than that observed when chromaffin granule phospholipids were used. In the absence of the protein fraction 45P, neither chromaffin granule nor soybean phospholipid preparations, treated in the same way, showed any ATPase activity.

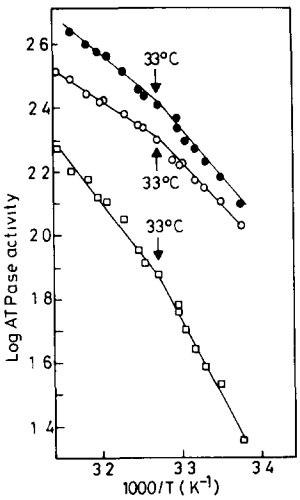
Fig. 2 shows the results of experiments in which the reactivation of the ATPase was carried out by addition of pure phospholipids of a particular class. The curves for the activities against lipid protein ratios all have similar shapes, with the exception of that for sphingomyelin. Peak ATPase activity is obtained at a lipid-to-protein ratio of 1.0 μmol/mg except for sphingomyelin and lysophosphatidylcholine, in common with that found for chromaffin granule phospholipid (Fig. 1a). The curve for lysophosphatidylcholine has a peak activity at a lipid-to-protein ratio of 2.0 μmol/mg. This difference may be connected with this phospholipid having only one fatty acid chain per molecule. While all of the phospholipids used reactivated the delipidated ATPase, reactivation with the acidic phospholipids phosphatidylserine and phosphatidylinositol resulted in significantly higher peak activities than those obtained using the neutral phospholipids.

The effect of temperature on the Mg^{2+}-ATPase activity

The Arrhenius plot for the activity of the chromaffin granule Mg^{2+}-ATPase is non-linear (Fig. 3). There is a change in slope at approx. 33°C, with activation energies of 30.8 kJ/mol and 51.1 kJ/mol above and below this temperature, respectively (Table II). The temperature at which the change in slope occurs has previously been correlated with phase changes in the membrane as detected by fluorescence [29] and spin-labelled [30] probes.

The Arrhenius plot for the delipidated preparation is also non-linear, although the activation energies both above and below the break temperature are increased (Fig. 3 and Table II). Similarly, the change in slope was maintained, and both activation energies were decreased when extracted chromaffin granule phospholipids were added back to the delipidated preparation.

In order to investigate the role of phospholipid fatty acid chain 'fluidity' in modulating the activity of the ATPase, Arrhenius plots were constructed for the enzyme reactivated with three synthetic phosphatidylcholines: dimyristoyl, dipalmitoyl and dipalmitoleoyl phosphatidylcholines. These phospholipids exhibit gel-to-liquid-crystalline phase transitions at temperatures of 23, 41.5 and −36°C, respectively [45]. As shown in Fig. 4, the Arrhenius plot obtained when the ATPase was reactivated with dipalmitoleoyl phosphatidylcholine was linear over the entire range of temperatures studied. The plots obtained for dimyristoyl phosphatidyl- and dipalmitoyl phosphatidylcholine-reactivated



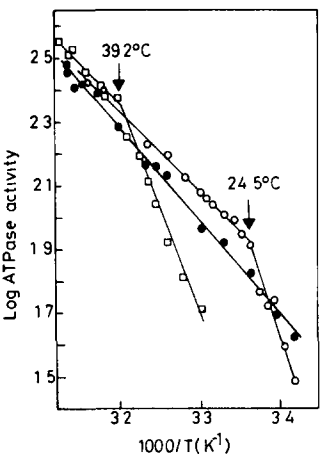


Fig. 3. The temperature-dependence of the chromaffin granule membrane ATPase following lipid modification. 45P was reconstituted with (●) or without (□) extracted chromaffin granule phospholipids as described in Experimental procedure. After extensive dialysis, samples were removed for assay of ATPase activity, protein and lipid phosphorus. ○, Control (dialysed) membranes. Lipid-to-protein ratios were: ○, 2.0; ●, 2.2 and □, 0.18 μmol/mg protein. Each point represents the mean for at least two determinations.

Fig. 4. The temperature-dependence of the chromaffin granule membrane ATPase following lipid modification. 45P was reconstituted with: □, dipalmitoyl phosphatidylcholine; ○, dimyristoyl phosphatidylcholine or ●, dipalmitoleoyl phosphatidylcholine as described in Experimental procedure. After extensive dialysis, samples were removed for assay of ATPase activity, protein and lipid phosphorus. Lipid-to-protein ratios were 5.9 (□), 6.3 (○) and 4.2 (●) μmol/mg protein. Each point represents the mean for at least two determinations.

ATPase, however, were non-linear, with changes in slope at 24.5 and 39.2°C, respectively. These temperatures are very close to the observed gel-to-liquid-crystalline phase-transition temperatures of the pure phospholipids.

The phase behaviour of the reconstituted systems was investigated by mea-

TABLE II

THE EFFECT OF LIPID MODIFICATION ON THE ACTIVATION ENERGY OF THE CHROMAFFIN GRANULE MEMBRANE ATPase

Activation energies were obtained from the slopes of the temperature-activity plots shown in Figs. 3 and 4. T_k is the temperature at which the change in slope occurs. $E_a > T_k$ and $E_a < T_k$ represent the activation energies (kJ/mol) above and below the break temperature, respectively. Abbreviations: CG, chromaffin granule; DPePC, dipalmitoleoyl phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; DMPC, dimyristoyl phosphatidylcholine.

	T_k (°C)	$E_a > T_k$ (kJ/mol)	$E_a < T_k$ (kJ/mol)
Membranes	33	30.8	51.1
Delipidated membranes	33	62.2	93.0
Membranes reconstituted with:			
CG lipids	33	38.7	57.4
DPePC	—	56.1	
DPPC	39.2	50.1	126.1
DMPC	24.5	47.5	142.8

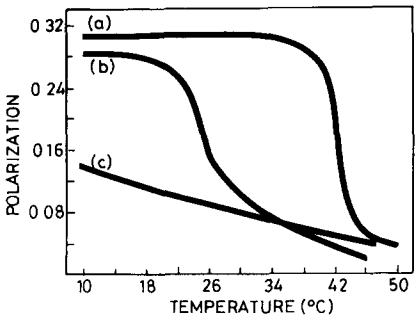


Fig. 5. Fluorescence polarization-temperature scans for diphenylhexatriene in 45P reconstituted with. a, dipalmitoyl phosphatidylcholine; b, dimyristoyl phosphatidylcholine and c, dipalmitoleoyl phosphatidylcholine. 45P fraction was reconstituted with synthetic phosphatidylcholines as described in Experimental procedure. After extensive dialysis, samples were removed and diluted into 10 mM Hepes, pH 7.0. Diphenylhexatriene was added as a concentrated stock solution in tetrahydrofuran to 1 μM final concentration and allowed to bind for 1 h prior to running the scan. The excitation wave length was 360 nm, and emission was monitored above 440 nm using cut-off filters. Protein concentration was 0.1 mg/ml and lipid-to-protein ratios were: a, 5.9; b, 6.3 and c, 4.2 μmol/mg protein.

suring the fluorescence polarization as a function of temperature using diphenylhexatriene as probe. The results obtained for the 45P fraction reconstituted with dipalmitoyl phosphatidylcholine or dimyristoyl phosphatidylcholine demonstrate that the lipid component undergoes gel-to-liquid-crystalline phase transitions at temperatures of 42 and 24°C, respectively (Fig. 5). In contrast, no phase transition is observed when the system is reconstituted with dipalmitoleoyl phosphatidylcholine, and the polarization-temperature plot is characteristic of the probe in a fluid lipid environment.

The effect of phospholipid depletion on the NADH oxidase activity

Initial experiments suggested that the NADH: ferricyanide oxidoreductase activity of the chromaffin granule membrane was not dependent on the phospholipid component of the membrane. The ammonium sulphate fractionation step was, therefore, carried out three times, in order to achieve a more extensive delipidation. The third step resulted in a protein preparation with less than 2% of the endogenous phospholipids remaining. Even at this level of phospholipid depletion, the NADH oxidase activity was not significantly reduced (Table III). Indeed, when compared to that of the solubilized membranes, there was a marked stimulation in activity. On reconstitution with chromaffin granule phospholipids, the activity of the NADH oxidase decreased (Table III), suggesting that the enzyme activity is restricted by phospholipids.

In the absence of added protein, chromaffin granule phospholipids had no detectable NADH oxidase activity.

The effect of phospholipid depletion on the dopamine β-hydroxylase activity

The results of a study on the phospholipid dependence of the dopamine β-hydroxylase activity of the chromaffin granule membrane are shown in Table III. Depletion of 98% of the endogenous membrane phospholipids did not significantly change the activity of this enzyme compared to that of the solubilized preparation. Similarly, reconstitution with extracted chromaffin granule

TABLE III

THE EFFECTS OF LIPID MODIFICATION ON THE NADH OXIDASE AND DOPAMINE β-HYDROXYLASE ACTIVITIES OF THE CHROMAFFIN GRANULE MEMBRANE

Chromaffin granule membranes were solubilized, delipidated and reconstituted as described in Experimental procedure. After extensive dialysis, samples were removed for assay of enzyme activity, protein and lipid phosphorus. Values for enzyme activities represent the mean ± S.D. for at least four determinations. CG, chromaffin granule.

	Lipid/protein (μmol/mg)	Enzyme activity (μmol/min per mg protein)	
		NADH oxidase	Dopamine β-hydroxylase
Membranes	1.86	0.21 ± 0.01	0.6 ± 0.1
Cholate extract	1.87	0.35 ± 0.02	1.1 ± 0.2
45P			
1st fractionation	0.22	0.46 ± 0.01	1.1 ± 0.2
2nd fractionation	0.09	0.44 ± 0.03	1.1 ± 0.3
3rd fractionation	0.04	0.39 ± 0.01	1.3 ± 0.1
45P, 3rd fractionation + CG lipids	4.49	0.27 ± 0.01	1.3 ± 0.1
	3.83	0.31 ± 0.02	1.2 ± 0.2
	2.02	0.32 ± 0.00	1.3 ± 0.3
	0.83	0.27 ± 0.01	1.3 ± 0.3
	0.51	0.29 ± 0.01	1.5 ± 0.1

phospholipids had no effect on the enzyme activity. It may, therefore, be concluded that the activity of the dopamine β-hydroxylase does not depend on a specific interaction of the enzyme with phospholipid.

In the absence of protein, chromaffin granule phospholipids showed no dopamine β-hydroxylase activity.

Discussion

The results presented in this paper show that the proton-translocating Mg^{2+}-ATPase of the chromaffin granule membrane can be solubilized and reconstituted in an active form using sodium cholate. Depleting the membrane of phospholipids by solubilization and ammonium sulphate precipitation results in a preparation with a substantially decreased ATPase activity. The activity is recovered on reconstitution with either chromaffin granule or soybean phospholipids. Substantial delipidation, however, results in a deactivation which is only partially reversible, and suggests that the presence of phospholipids is not only required for the Mg^{2+}-ATPase activity, but also for maintaining the stability of the enzyme. Similar observations have been made for the proton-translocating ATPases of mitochondria [37,46,47] and the thermophilic bacterium PS3 [48], and it is likely that phospholipid-dependent ATPase activity is a general feature for membrane-bound enzyme complexes of this type.

The concept of a 'boundary' layer of lipid [9,10] surrounding membrane proteins, and which may be necessary for the full expression of enzyme activity [12], has been advanced in recent years. The irreversible deactivation of the chromaffin granule membrane ATPase by extensive delipidation may also be

related to the existence of this boundary layer, the removal of which results in deactivation of the enzyme. Similar irreversible deactivation on substantial delipidation has also been demonstrated for the Ca^{2+}-ATPase of sarcoplasmic reticulum [12] and the Mg^{2+}-ATPase of *Acholeplasma laidlawii* [49].

Several previous studies have shown that the re-activation of delipidated and deactivated membrane enzymes is sometimes dependent on the presence of certain headgroups in the lipids used for reconstitution [11,49—51]. In contrast, other studies have demonstrated that the lipid specificity for reactivation is broad [8,52—55]. In some cases detergents can replace phospholipids in the reactivating role [53,56]. The situation is further complicated by conflicting observations on the same enzyme (see, e.g., Ref. 50).

The chromaffin granule membrane Mg^{2+}-ATPase belongs to the group of integral membrane proteins requiring phospholipid for activity. The activity depends mainly on the hydrophobic environment provided by the lipid hydrocarbon chains rather than any specific headgroup interaction. Thus, although the acidic phospholipids phosphatidylserine and phosphatidylinositol activate the enzyme to a greater extent than the neutral phospholipids tested, both groups of lipids supported substantially higher activities than in the delipidated preparation. It should, however, be remembered that the hydrocarbon chains of the lipids used for reactivation are heterogeneous. Strictly, comparisons of the effectiveness of lipid headgroups in the reactivation role should be made with lipids containing identical hydrocarbon chains. This has been done in the case of the Ca^{2+}-ATPase of sarcoplasmic reticulum by using a series of dioleoyl phospholipids [57] and for the $(Na^+ + K^+)$-ATPase by using a series of phospolipids derived from a single natural source by enzymic modification [58].

The relationship between activity and lipid/protein ratios obtained in this study shows the importance of testing reactivation over a wide range of lipid-to-protein ratios. Misleading conclusions concerning the phospholipid specificity of reactivation may be obtained if the activity is tested at only one lipid-to-protein ratio.

The relationships between the physical state of membrane lipids and the activities of membrane-bound enzymes have been often analysed [3,49,59—63]. Changes in the activation energies of such enzymes were sometimes found to depend on the fatty acid composition of the membrane lipids, and correlations have been made between the 'fluidity' of the lipid hydrocarbon chains, and enzyme activities [3,49,51,59—63]. Kimelberg and Papahadjopoulos [61] have introduced the term 'viscotropic regulation' to describe the influence of fatty acyl chain fluidity on enzyme function. The ATPase of the chromaffin granule membrane exhibits such viscotropic regulation as shown by the correlations between changes in slope in the Arrhenius activity-temperature relationships, and the physical state of the membrane lipids.

The maintenance of the change in slope at 33°C in the delipidated ATPase preparation is also of interest. Marsh et al. [30] have shown that a covalently bound iodoacetamide spin label reveals a transition, at 32° C, within the protein component of the membrane, or its immediate lipid environment. The change in slope in the Arrhenius plot for the delipidated preparation may, therefore, be connected with a possible transition or phase change in the boundary lipid surrounding the ATPase. Such a transition has been observed in the cytochro-

me *P*-450-cytochrome *P*-450 reductase system in liver microsomes [64]. Alternatively, the change in slope could be due to a conformational change in the enzyme itself. This, however, would appear to be unlikely since the change in slope at 33°C is absent when the ATPase is reconstituted with some synthetic phosphatidylcholines (Fig. 4).

In contrast to the observations on the chromaffin granule membrane ATPase, the membrane-bound dopamine β-hydroxylase does not appear to require the presence of phospholipids. Although this conclusion was also reached by a previous study [65] using phospholipase degradation to deplete the membrane of phospholipid, these authors did not quantify the extent of phospholipid hydrolysis, and we have shown that phospholipases can only hydrolyse up to 50% of the phospholipid in the chromaffin granule membrane [40]. The observations made in this study indicate that the membrane-bound form of this enzyme may be classed in the group of peripheral membrane proteins, which, being bound only to the membrane surface, tend to have activities which are not dependent on the phospholipid component. Certainly, it might be expected that an enzyme existing actively in both a soluble and a membrane-bound form [66], and identical in amino acid composition, electrophoretic mobility, and immunochemical properties [67], would have an activity which does not require the presence of phospholipid. Bjerrum et al. [68] have shown that the membrane-bound form of this enzyme is amphiphilic, and can be converted to the soluble, hydrophilic form by the action of thermolysin or chymotrypsin. They have suggested that small hydrophobic 'tail' anchors the enzyme in the membrane, and that the action of the proteases is to remove this portion of the molecule. This interpretation correlates with the conclusions on the peripheral nature of the enzyme given above. The hydrophobic 'tail' is not required for expression of enzyme activity.

The NADH oxidase activity of the chromaffin granule membrane appears to be negatively modulated by phospholipids. Thus, removal of 98% of the membrane phospholipids increases enzyme activity; an effect which is reversed when phospholipids are added back to the delipidated preparation. Similar increases in activity were obtained for the NADH oxidase and *p*-nitrophenylphosphatase of the *A. laidlawii* membrane after phospholipase digestion [49]. Although the precise physiological advantages of this arrangement are not clear, it may be that subtle changes induced in the membrane in vivo result in an enzyme activity that is internally regulated to meet varying demand. Alternatively, enzymes regulated in this way may require a hydrophilic environment in which to express their full activity, and their positioning in the membrane may simply be related to their metabolic role. Thus, for example, the membrane location of the chromaffin granule NADH oxidase may be necessary for efficient interaction with an endogenous membrane-bound electron acceptor. In this connection, it is notable that although the NADH oxidase of *A. laidlawii* has been shown to be a membrane-bound enzyme [69,70] it does not exhibit viscotropic regulation [62].

Finally, it should be noted that although the NADH oxidase of microsomal membranes exhibits a phospholipid requirement when cytochrome *c* is used as electron acceptor [71], no such requirement is observed using ferricyanide. This finding may indicate that phospholipid is required for the transfer of elec-

trons only when lipid-soluble electron acceptors are involved, and supports the suggestion that the membrane location of the NADH oxidase activity is a direct requirement of its metabolic role.

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