RECONSTITUTION OF THE Mg^{2+}-ATPase OF THE CHROMAFFIN GRANULE MEMBRANE

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1. Introduction

It has been demonstrated that the Mg^{2+}-dependent ATPase of the adrenomedullary storage vesicles (chromaffin granules) is an inwardly directed electrogenic proton pump [1–6]. The active accumulation of catecholamines is dependent on ATP-linked proton translocation [7] and it appears that both the pH gradient [8,9] and the electrochemical potential [10,11] can drive catecholamine uptake [12]. The proton translocating ATPase is also responsible for active ATP transport [13]. The similarity of the enzyme to the mitochondrial ATPase [14] together with the possibility that neurotransmitter storage vesicles also contain comparable ATPases [15,16] imply that a study of this system does not only help in elucidating the mechanism of neurotransmitter storage but may also lead to a general understanding of how ATP hydrolysis generates a proton gradient.

Any detailed molecular analysis of the reaction requires simplification of the system and in this paper we report experiments designed to examine the possibility of reconstituting coupled ATPase activity. Such reconstitution experiments provide the first step for examining the lipid specificity of the process as well as for the functional characterization of the individual protein components in the chromaffin granule membrane. The experiments described rely on the 'cholate dialysis' technique developed [17].

Abbreviations: ATPase, adenosine triphosphatase; S-13, *N*-(3-tert-butyl 5-chlorosalicilyl)-2-chloro-4-nitroanilide; Hepes, *N*-2-hydroxy ethyl piperazine *N'*-2-ethane sulphonic acid

2. Materials and methods

Sodium cholate (Sigma Chemical Co.) was recrystallized twice from 70% aqueous ethanol after treatment with activated charcoal. Chromaffin granule phospholipids were obtained from membranes by the extraction method in [18]. Soybean phospholipids were obtained from Sigma Chemical Co. and purified as in [17]. S-13 was the generous gift of Dr B. Chance. Phospholipids were stored as stock solutions in chloroform under nitrogen at −20°C. Water was twice distilled from an all glass apparatus and other solvents used were distilled.

Purified chromaffin granule membranes were prepared from the adrenal glands of freshly slaughtered cattle as in [19]. The membrane pellet was suspended in a medium containing 10 mM Hepes, 10% (w/v) glycerol, 1 mM magnesium sulphate, 0.5 mM EDTA, 3 mM β-mercaptoethanol (membrane buffer) at pH 8.0 and kept at 4°C until use. Under these conditions the membranes lose ~ 10% of the Mg^{2+}-ATPase activity over a period of 3 days, although dialysis of the membranes against membrane buffer at 4°C resulted in no loss of activity over this period.

Solubilization was carried out by dropwise addition of a 10% (w/v) solution of sodium cholate (pH 8.0) (final conc. 1.5%, w/v) to the membrane suspension made 10% saturated in ammonium sulphate. The final cholate : protein ratio was in the range 5–7 (w/w). After stirring at 4°C for 20 min the suspension was centrifuged at 100 000 × g_{av} in an MSE Prepspin centrifuge for 1 h. The supernatant (cholate extract) was decanted from the pellet and kept at 4°C until use.

Delipidation was carried out by dropwise addition

of a saturated ammonium sulphate solution to the required final saturation. After 10 min stirring at 4°C, the suspension was centrifuged at 27 000 × g_{av} for 20 min. The pale yellow supernatant was decanted from the red pellet which was dissolved in membrane buffer containing 1.5% (w/v) sodium cholate at pH 8.0 by gentle mixing with a glass rod.

The pellet obtained at 45% saturation is designated 45P and the supernatant from this fractionation 45S. When a two stage fractionation procedure was employed, the pellets obtained at 33% saturation and 45% saturation are designated 33P and 33–45P, respectively.

Reconstitution was carried out by addition of the appropriate protein fraction to a sonicated solution of phospholipids (~ 10 mg/ml) in membrane buffer containing 1.5% (w/v) sodium cholate at pH 8.0. This solution was dialysed at 4°C against a 100-fold excess of membrane buffer at pH 7.0 with 5 changes over 36 h. At the end of this period, samples were removed for assay of ATPase activity, protein, and lipid phosphorus.

ATPase activity was assayed at 30°C and pH 6.6 using an ATP regeneration system coupled to NADH oxidation, ΔA_{340} being monitored continuously [20]. Protein and lipid phosphorus were assayed as in [19].

3. Results

The treatment of chromaffin granule membranes with 1.5% (w/v) sodium cholate solubilizes 94% of the membrane protein (table 1). Ammonium sulphate (45% final saturation) precipitates protein (45P pellet) which is relatively poor in phospholipids compared to the membrane and the supernatant (table 1). The precipitation removes ~ 90% of the phospholipids. The 45P fraction has a low ATPase activity (table 1) in contrast to the preparation where 50% of the lipid is removed by phospholipase digestion [19] when only a small effect on the ATPase activity is observed (R.M.B., G.K.R., Shennan, unpublished observations).

Addition of chromaffin granule lipids to the 45P fraction reactivates the ATPase (fig.1a), showing maximal activity at a lipid : protein ratio of ~1.0 μmol/mg. At lipid : protein ratios > 1.5 μmol/mg, the ATPase activity is stimulated by the addition of the uncoupler S-13 (fig.1a). Dicyclohexyl-carbodiimide, which is thought to block proton

Table 1
Solubilization and delipidation of the chromaffin granule membrane Mg^{2+}-ATPase

	% Protein (range)	Lipid : Protein (μmol/mg) (range)	ATPase activity[a] (nmol P_i released .min^{-1} .mg $protein^{-1}$) (mean ± SD)
Membranes	'100'	2.05 (1.54–2.52)	182.5 ± 6.3
Cholate extract	94 (93–97)	2.15 (1.77–2.46)	126.8 ± 20.1[b]
45P	54 (48–58)	0.22 (0.14–0.33)	47.6 ± 2.3[c]
45S	43 (36–46)	4.58 (3.63–5.64)	–

[a] Results obtained for a typical experiment
[b] Activity (assayed before dialysis) depends on cholate : protein ratio. After dialysis, activity increases to 202.1 ± 27.3 nmol/mg
[c] Activity depends critically on lipid : protein ratio

Chromaffin granule membranes were solubilized and delipidated by ammonium sulphate precipitation as in section 2. ATPase activities were assayed after extensive dialysis to remove cholate

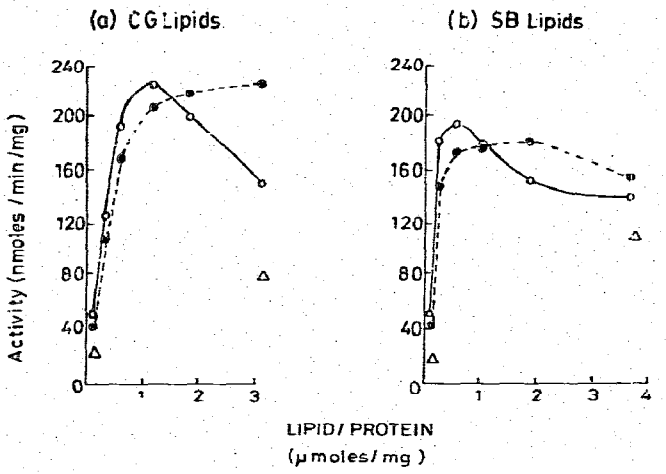

Fig.1. Reconstitution of the chromaffin granule membrane Mg^{2+}-ATPase. 45P fraction was reconstituted with (a) extracted chromaffin granule phospholipids or (b) soybean phospholipids as in section 2. After extensive dialysis, samples were removed for assay of ATPase activity, protein and lipid phosphorus. (○) No additions; (●) + 1 μM S-13; (△) + dicyclohexyl-carbodiimide (1 mg/mg protein).

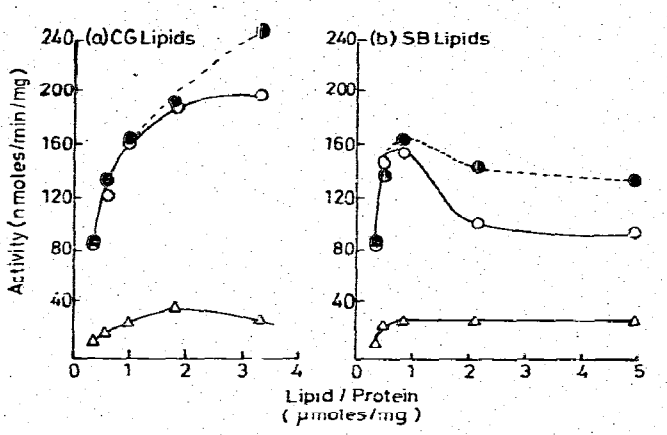

Fig.2. Reconstitution of the chromaffin granule membrane Mg^{2+}-ATPase. 33P fraction was reconstituted with (a) extracted chromaffin granule phospholipids or (b) soybean phospholipids as in section 2. After extensive dialysis, samples were removed for assay of ATPase activity, protein and lipid phosphorus. (○) No additions; (●) + 1 μM S-13; (△) + dicyclohexyl-carbodiimide (1 mg/mg protein).

conducting channels [21,22] and inhibit both mitochondrial and chromaffin granule ATPase [2], inhibits the reconstituted system by ~ 50% (fig.1a).

Further delipidation of the 45P fraction by repeating the ammonium sulphate precipitation step resulted in a lipid : protein ratio of 0.02 μmol/mg and an ATPase activity of only 6 nmol P_i released $.min^{-1}.mg\ protein^{-1}$. However, on readdition of chromaffin granule phospholipids to this preparation, maximal activities of only 40 nmol P_i release $.min^{-1}.mg\ protein^{-1}$ were obtained, and it appears that the delipidation procedure results in a partially irreversible deactivation of the enzyme.

Soybean phospholipids also reactivate the ATPase (fig.1b), although in this case the maximal activity, obtained at a lipid : protein ratio of 0.6 μmol/mg is less than that with chromaffin granule lipids. Again activity is stimulated by addition of S-13, and inhibited by addition of dicyclohexyl-carbodiimide, although both effects are smaller than those observed when the system is reconstituted with chromaffin granule lipids.

When the fractionation is carried out in two stages, to yield a pellet 33P (at 33% ammonium sulphate saturation), and a pellet 33–45P (at 45% ammonium sulphate saturation) both fractions can be delipidated and reconstituted to give active preparations (fig.2,3). The 33P fraction is more sensitive to both dicyclohexyl-carbodiimide and S-13 than is the 33–45P fraction. Indeed, when the 33–45P fraction is reconstituted with soybean phospholipids, the ATPase activity is neither significantly stimulated by addition

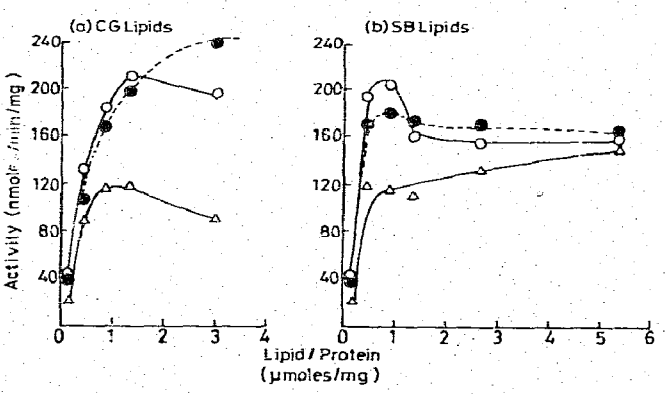

Fig.3. Reconstitution of the chromaffin granule membrane Mg^{2+}-ATPase. 33–45P fraction was reconstituted with (a) extracted chromaffin granule phospholipids or (b) soybean phospholipids as in section 2. After extensive dialysis, samples were removed for assay of ATPase activity, protein and lipid phosphorus. (○) No additions; (●) + 1 μM S-13; (△) + dicyclohexyl-carbodiimide (1 mg/mg protein).

of S-13 nor, at high lipid : protein ratios, inhibited by dicyclohexyl-carbodiimide, although when reconstituted with chromaffin granule phospholipids this fraction still shows residual sensitivity to both these compounds.

4. Discussion

We have shown that the proton translocating ATPase of chromaffin granules can be solubilized in an active form by sodium cholate. The activity lost by removing the lipids is recovered on reconstitution with both chromaffin granule and soybean phospholipids. (Substantial delipidation results in a deactivation which is only partially reversible.) Both preparations are stimulated by uncouplers above a certain lipid : protein ratio, in common with the native membrane. We believe this stimulation is dependent on the formation of 'closed vesicles', which is also responsible for the decrease in ATPase activity at high lipid : protein ratios. We have examined some of our preparations by electron microscopy and the results are consistent with this interpretation. Dicyclohexyl-carbodiimide, which is thought to act in mitochondria by blocking the proton conducting channel [21,22] inhibits the reconstituted chromaffin granule ATPase as it does in the native membrane [2].

The two stage fractionation reported here results in the separation of one fraction (33P) which is relatively more sensitive in the reconstituted system to uncouplers and dicyclohexyl-carbodiimide than the other fraction (33–45P). This is particularly evident when activity is reconstituted with soybean phospholipids. The significance of these observations is best considered in the light of the proton translocating ATPases of mitochondria, chloroplasts and bacteria, which are, of course, involved in ATP synthesis. In these systems a proteolipid which binds dicyclohexyl-carbodiimide has been found [23–25]. This component can be extracted by chloroform–methanol mixtures [23,24] and has been implicated in the process of proton translocation. We suggest, therefore, that by analogy, the proton translocating ATPase of chromaffin granules contains a similar protein component which is more abundant in the 33P than in the 33–45P fraction. If the chloroform–methanol extraction (used for the preparation of chromaffin granule lipids) also contains this protein we can rationalize the observation that reconstitution with this extract re-establishes the sensitivity to dicyclohexyl-carbodiimide while that with soybean lipids does not. It also follows that the enhancement of the ATPase rates by uncouplers should only be observed when this proton conducting proteolipid is present. The results shown are entirely consistent with this.

In summary, coupled activity of the chromaffin granule ATPase can be reconstituted and the results suggest that a search for the proton conducting protein component is both feasible and logical.

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