A NEW PROCEDURE FOR THE RECONSTITUTION OF BIOLOGICALLY ACTIVE PHOSPHOLIPID VESICLES*

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SUMMARY

A simple and rapid procedure is described for the reconstitution of biologically active phospholipid vesicles without the use of detergents. It consists of suspending dried phospholipids in salt solution containing membrane proteins and exposing the mixture to sonic oscillation. The following systems have been reconstituted by this procedure: A rutamycin and uncoupler-sensitive P_i-ATP exchange, cytochrome oxidase vesicles exhibiting respiratory control, a proton pump made with bacterial rhodopsin and a Ca^{++} pump made with an ATPase from sarcoplasmic reticulum.

A procedure developed previously for the reconstitution of biologically active phospholipid vesicles consisted of solubilization of hydrophobic proteins in cholate or deoxycholate and recombination with mixtures of phospholipids that had been exposed to sonic oscillation in the presence of 2% sodium cholate. After removal of cholate by dialysis (or in some instances by passage through a Sephadex column) vesicular structures with biological activity were formed. By this procedure we reconstituted the $^{32}P_i$-ATP exchange and the mitochondrial proton pump (1,2), the third site of oxidative phosphorylation (3), cytochrome oxidase vesicles with respiratory control (4,5), the Ca^{++} pump of sarcoplasmic reticulum (6), the first site of oxidative phosphorylation (7) and a proton pump with bacterial rhodopsin (8). With a Na^+, K^+-ATPase and lipids from kidney, vesicles have been reconstituted which catalyzed a rutamycin-sensitive uptake

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Abbreviations used: F_1, coupling factor 1 (ATPase)
OSCP, oligomycin-sensitivity conferring protein

of sodium (9). However, this method failed when some of the components were sensitive to either the detergent or to prolonged dialysis as was encountered with the second site of oxidative phosphorylation. In this paper a simpler and more rapid procedure is described which has been successfully applied to several systems.

MATERIALS AND METHODS

Phospholipids, the oligomycin-sensitive ATPase complex (hydrophobic fractions 25-50 P) and coupling factors were prepared as described previously (1,2,7). Cytochrome oxidase was prepared according to Yonetani (10). Preparations of bacterial rhodopsin were generously supplied by Dr. W. Stoeckenius, University of California. Egg lecithin and cardiolipin were purchased from General Biochemical Inc., Chagrin Falls, Ohio. A crude mixture of lipids from Halobacterium cutirubrum was a gift from Dr. J. K. Lanyi, Ames Research Center, California.

Method of reconstitution of "instant" vesicles -- Phospholipids, usually stored under nitrogen in chloroform-methanol (4:1), were dried in small test tubes under a stream of nitrogen, dissolved in a small volume of ether to remove residual chloroform and dried again under nitrogen. To the dry preparation a solution containing 0.15 M KCl and various membrane proteins used for reconstitution were added. The final concentration of the phospholipid suspension was 25 umoles per ml and that of the proteins between 0.5 and 2 mg/ml. In some experiments reconstitution was performed with 0.25 M sucrose - 10 mM Tris buffer, pH 8.0 or with phosphate buffer. The mixture was exposed to sonic oscillation by immersion of the test tube in a Sonblaster model G201 (Narda Ultrasonic Corp., N.Y.) 90 watts, 90 Kc at 1.3 A with tank NT201 for various time periods as specified in the legends of the figures and tables.

Assays -- $^{32}P_i$-ATP exchange (1), cytochrome oxidase (5), proton uptake and associated ATP formation (8) were measured as described in the references.

RESULTS AND DISCUSSION

Reconstitution of mitochondrial $^{32}P_i$-ATP exchange -- As shown in Table I a

TABLE I

Reconstitution of $^{32}P_i$-ATP Exchange

Soybean phospholipids (5 µmoles) that had been treated with acetone (1) and dissolved in chloroform-methanol (4:1) were dried as described under Methods and suspended in 0.3 ml of 0.15 M KCl or 0.25 M sucrose -10 mM Tris Cl, pH 8, containing 620 µg of hydrophobic protein 25-50 P (1). After sonication for the indicated time periods, 50 µl of the clarified mixture was incubated at 23° for 15 min with 40 µg of F_1 and 42 ug of OSCP and then assayed for $^{32}P_i$-ATP exchange. The uncoupler 1799 (2×10^{-5} M) or rutamycin 4 ug/ml) were added where indicated.

	Sonication		$^{32}P_i$-ATP exchange
	Time in minutes	Medium	nmoles/min/mg/protein
Complete system	3	KCl	42.3
" "	6	"	72.4
" "	9	"	73.0
" "	3	sucrose-Tris	72.4
" "	6	" "	70.8
" "	9	" "	74.6
Complete system plus 1799	9	KCl	11.4
Complete system plus rutamycin	9	"	1.8
Complete system without F_1 and OSCP	9	"	8.7

rapid $^{32}P_i$-ATP exchange was catalyzed by the vesicles reconstituted with the oligomycin-sensitive ATPase complex by the new method. There was not much difference between vesicles prepared in the presence of 0.15 M KCl or of 0.25 M buffered sucrose. The P_i-ATP exchange was inhibited by the uncoupler 1799 or the energy transfer inhibitor rutamycin and was dependent on the addition of the coupling factors F_1 and OSCP. As mentioned previously (11) the dependency on coupling factors varied with the preparations of hydrophobic proteins. Only a few minutes of sonication was sufficient to obtain maximal activity. A pronounced clearing of the turbid phospholipid suspension was associated with the appearance of activity.

TABLE II

Effect of Salt Concentration on Proton Pump

The vesicles were reconstituted as described in the legend of Fig. 1 except that 5 mg of crude Halobacteria lipids were used in the second series of experiments. Assays were carried out in the presence of the indicated salts with no buffers added. The pH of the assay was adjusted to 6.2 and after each experiment the response of the pH meter was standardized by addition of 2.5 and 5 nmoles of HCl.

Phospholipid source	Salt in assay	ng Atoms H^+/mg protein
Soybean	0.15 M KCl	245
	0.15 M NaCl	276
	1.0 M KCl	476
	1.0 M NaCl	520
	4.0 M KCl	591
	4.0 M NaCl	693
Halobacteria	0.15 M NaCl	80
	1.0 M NaCl	119
	4.0 M KCl	395
	4.0 M NaCl	393

Reconstitution of cytochrome oxidase vesicles with respiratory control -- "Instant" cytochrome oxidase vesicles were prepared by suspending 20 mg of crude soybean phospholipid that had been treated with acetone (1) in 0.5 ml of 50 mM KP_i buffer, pH 7.5. After addition of about 250 ug of cytochrome oxidase, the suspension was sonicated for 5 min as described under Methods. The response of the resulting vesicles to uncouplers and ionophores was similar to that of vesicles prepared by the cholate-dialysis procedure (4,5). Addition of an uncoupler or of nigericin together with valinomycin plus K^+ resulted in a four to five fold stimulation of ferrocytochrome c oxidation.

Reconstitution of a proton pump with bacterial rhodopsin -- Phospholipid vesicles reconstituted with bacterial rhodopsin by the cholate-dialysis procedure catalyze a light-driven uptake of protons (8). An equilibrium of proton uptake and release was reached depending on the light intensity. At saturating light

TABLE III

ATP Generation by Light-driven Proton Pump

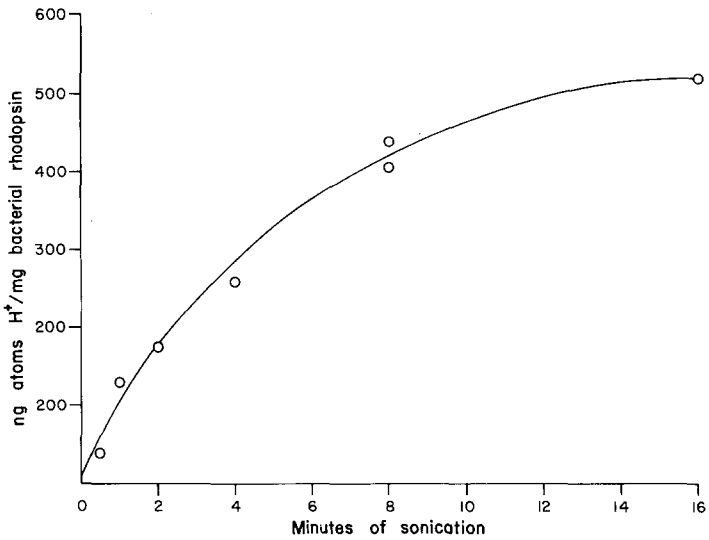
Soybean phospholipids (5 umoles) were dried as described under Methods and suspended in 0.3 ml of 0.15 M KCl or 0.25 M sucrose - 10 mM Tris-Cl, pH 8.0, containing 160 ug of bacterial rhodopsin and 920 ug of hydrophobic protein 25 - 50 P (1). After sonication for the indicated time periods, 50 ul samples were incubated with coupling factors as described in the legend of Table I and assayed for ATP formation in the light as described previously (8).

Reconstitution additions	Time of sonication minutes	ATP formation nmoles/min/mg rhodopsin
KCl	5	62.9
Sucrose-Tris	5	55.5
KCl	10	44.4
Sucrose-Tris	10	48.1

intensity (2×10^6 erg/cm^2/sec) the extent of proton uptake was about 100-200ng atom H^+/mg rhodopsin. Fig. 1 shows that the extent of H^+ uptake by vesicles reconstituted by the new procedure increased with prolonged sonication. After 16 min the rate was 2 to 4 times higher than that of vesicles reconstituted by the cholate-dialysis procedure. When measured at 10^4 erg/cm^2/sec an optimal response was obtained at 563 nm corresponding to the peak of absorption of the reconstituted vesicles. At 630 nm or at 460 nm proton uptake was less than 20% of maximal.

The amount, the source and composition of the phospholipids affected the extent of proton uptake. Suitable mixtures of soybean phospholipids (e.g. phosphatidylcholine and phosphatidylethanolamine were as effective as the crude mixtures. Pure soybean phosphatidylcholine was rather ineffective when used alone, but egg phosphatidylcholine gave vesicles with about 30% of the activity observed with mixtures.

Table II shows the effect of salts on the assay of vesicles prepared with either soybean phospholipids or a crude lipid mixture of Halobacterium cutirubrum. High concentrations of either KCl or NaCl greatly increased the extent



Legend to Fig. 1

Crude soybean phospholipids (5 umoles) dried as described in the legend of Table I and suspended in 0.3 ml of 0.15 M KCl containing 120 ug of bacterial rhodopsin were sonicated for the indicated time periods as described under Methods. Samples (20 ul) were assayed for light-driven proton uptake in 1 ml of 0.15 M KCl after adjustment of the pH to 6.2 (8).

of proton uptake with either vesicles but the effect was particularly striking at the very high salt concentration when lipids from the Halobacteria were used.

Incorporation of the oligomycin-sensitive ATPase complex isolated from bovine mitochondria yielded vesicles that catalyzed light-driven ATP formation. As can be seen from Table III there was not much difference in the rate of ATP formation when the vesicles were prepared with either buffered sucrose or 0.15 M KCl. The rate was similar after 5 to 10 minutes of sonication and considerably higher than with vesicles prepared by the cholate dialysis procedure. This is in line with the observation that the latter show a lower extent of proton uptake (80 to 200 ng atoms H^+ per mg protein compared to 500). These experiments were performed in the presence of a large excess of hexokinase to eliminate the $^{32}P_i$-ATP exchange catalyzed by the oligomycin-sensitive ATPase complex. It can be seen that control vesicles incubated in the dark under identical conditions, were completely inactive.

Reconstitution of vesicles catalyzing sodium uptake -- We have also encountered some failures with this new method. Preliminary attempts to reconstitute site I of oxidative phosphorylation have not yet been successful. Experiments on the reconstitution of an ATP-driven Na^+ pump with inverted vesicles reconstituted with kidney ATPase are in progress but have been variable particularly with respect to a dependency on ATP and Mg^{++}. However, pronounced stimulation of Na^+ uptake by two lipid fractions has been observed (9). The reconstitution method described in this paper should facilitate the assays of these fractions during purification.

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