between these two species may provide a framework for understanding this phenomenon.

Received for publication 4 May 1981.

REFERENCES

1. Liu, S. S., H. L. Tsai, T. M. Tsou, and F. H. Gao. 1976. Comparative studies on the energy-transducing ATPase complex of biological membranes. II. Reconstitution of hybrid submitochondrial membrane vesicle from the soluble ATPase of rat liver mitochondria and the inner mitochondrial membrane of human primary liver carcinoma, with an analysis of genetic control in carcinogenesis. *Acta Biochem. Biophys. Sin.* 8:307–317.
2. Shatz, G., H. S. Penefsky, and E. Racker. 1967. Partial resolution of the enzymes catalyzing oxidative phosphorylation. XIV. Interaction of purified mitochondrial ATPase from Baker's yeast with submitochondrial particles from beef heart. *J. Biol. Chem.* 242:2552.
3. Abrams, A., and J. B. Smith. 1974. Bacterial membrane ATPase. *In* The Enzymes. P. D. Boyer, editor. Academic Press Inc., New York. 10:395.
4. Liu, S. S., H. L. Tsai, T. M. Tsou, and F. H. Gao. 1976. Comparative studies on the energy-transducing ATPase complex of biological membranes. I. Reconstitution of the submitochondrial membrane vesicles from the soluble mitochondrial F_1 and the depleted inner mitochondria membrane and the binding of ANS to the F_1 of rat liver mitochondria. *Acta Biochem. Biophys. Sinica.* 8:293–305.
5. Janoff, A. S., A. Huang, and E. J. McGroarty. 1979. Relationship of growth temperature and thermotropic lipid phase changes in ctyoplasmic and outer membranes from *Escherichia coli* K 12. *Biochim. Biophys. Acta.* 555:56.
6. Sebald, W., J. Hoppe, and E. Wachter. 1979. Amino acid sequence of the ATPase proteolipid from mitochondria, chloroplasts and bacteria (wild type and mutants). *In* Function and Molecular Aspects of Biomembrane Transport. E. Quagliariello et al., editors. Elsevier/North-Holland, Amsterdam, The Netherlands. 63.

ASSOCIATION OF THE BOVINE CARDIAC MITOCHONDRIAL ATPase WITH PHOSPHOLIPIDS

Reconstitution and Phospholipid Exchange Studies

Rhoderick E. Brown, and Carol C. Cunningham
Department of Biochemistry, Wake Forest University, School of Medicine, Winston Salem, North Carolina 27103 U.S.A.

The interaction of various diacyl phospholipids with a highly purified oligomycin-sensitive adenosine triphosphatase (ATPase) preparation (1) from bovine cardiac mitochondria has been examined. Because the phospholipid composition of submitochondrial particles is composed almost exclusively of acidic and neutral phospholipids (2, 3), the effects of these two types of phospholipids on the lipid-depleted mitochondrial ATPase were investigated (Table I). Acidic diacyl phospholipids increased the ATPase specific activity from two- to six-fold, whereas neutral diacyl phospholipids stimulated the ATPase specific activity from zero- to two-fold. Studies with both synthetic and naturally occurring diacyl phospholipids indicated that both the phospholipid fatty acyl side-chain region and the headgroup region affected ATPase activity (Table I). The following order of effectiveness was noted for diacyl phospholipid ATPase activators: asolectin > dioleoylphosphatidylglycerol (DOPG) > dimyristoylphosphatidylglycerol (DMPG) > phosphatidylserine (PS) > dioleoylphosphatidic acid (DOPA) > phosphatidylethanolamine (PE) > dioleoylphosphatidylcholine (DOPC) > dimyristoylphosphatidylcholine (DMPC). Two conclusions were drawn from these findings: (*a*) phospholipids containing fatty acyl side-chains with 18 carbon atoms activate the ATPase better than those containing 14 carbon atoms at 30°C; and (*b*) acidic diacyl phospholipids reactivate the highly purified ATPase better than neutral diacyl phospholipids.

The observations concerning the influence of acyl group composition on the activity of the highly purified ATPase are in agreement with earlier studies carried out with considerably less pure ATPase preparations (4). However, the precise structural characteristics of the fatty acyl chain (e.g. length vs. surface area/mol) which affect the ATPase remain to be clarified. On the other hand, the effect of the diacyl phospholipid headgroup region on the ATPase specific activity appears to be directly related to the ionic charge of the headgroup region. Cunningham and Sinthusek (5) have demonstrated that it is the net negative charge rather than any unique headgroup structure that is important for reactivation of the lipid-depleted ATPase. The relative levels of ATPase specific activity obtained in the present study (Table I) with DOPG, DOPA, and DOPC are very similar to those reported by Cunningham and coworkers (5, 6) with a less pure ATPase preparation and PG, PA, and PC of different fatty acyl chain compositions. However, the absolute levels of specific activity obtained in the present study (Table I) are ~ 10-fold

Dr. Brown's present address is the Department of Biochemistry, University of Virginia Medical School, Charlottesville, Virginia 22908 U.S.A.

TABLE I

THE ACTIVATING POTENTIAL OF VARIOUS DIACYL PHOSPHOLIPIDS WITH THE LIPID-DEPLETED ATPase*

Phospholipid	ATPase Specific Activity	Phospholipid Stimulated ATPase Specific Activity	Percent ATPase Specific Activity Oligomycin Sensitive
Asolectin	11.5	10.4	93-98
DOPG	12.0	9.5	10–20
DMPG	9.9	6.8	65–70
Beef brain			
PS	7.7	6.4	75–85
DOPA	7.0	5.0	60–70
Egg PE	4.1	2.5	80–85
DOPC	3.6	0.9	45–55
DMPC	2.1	—	45–55

*The mitochondrial ATPase (38–45p) was prepared and assayed according to the method of Serrano et al. (1). ATPase assay color development was achieved by replacing the ascorbic acid with 50 μl of a saturated solution of 1-amino-2-naphthol-sulfonic acid in 13.2% $Na_2S_2O_5$ and 0.5% Na_2SO_3 followed by incubation at 37°C for 10 min. The specific activities were determined from v vs. S plots at phospholipid concentrations that elicited zero order kinetics. Values are the average of three independent experiments.

TABLE II

THE EXCHANGE OF MITOCHONDRIAL PHOSPHOLIPID FOR DOPA OR DOPC IN THE 38–45 p ATPase*

	NMOL fatty acid per μG 38–45 p ATPase‡		% of original SMP phospholipid remaining bound to 38–45 p ATPase§	ATPase specific activity		% ATPase specific activity oligomycin sensitive	
	DOPA or DOPC	Mitochondrial		I‖	II	I	II
CONTROL							
ATPase	—	197.1	14.0	2.5	8.2	63	91
Asolectin-ATPase							
11.6 mM cholate	—	?	?	4.6	5.2	62	72
SAMPLES							
DOPA-ATPase							
5.8 mM cholate	1772	165.6	11.7	6.8	6.7	17	20
11.6 mM cholate	1244	106.0	7.5	5.4	5.5	15	26
DOPC-ATPase							
5.8 mM cholate	366	176.1	12.5	4.3	4.9	64	70
11.6 mM cholate	659	110.4	7.8	2.1	5.1	48	66

*The phospholipid exchange procedure was performed by incubating 38–45 p ATPase (2 mg) with synthetic phospholipid (4 mg) and sodium cholate (listed above) at 30°C for 90 min under nitrogen. The final volume (0.3 ml) contained 100 mM sucrose and was buffered as described by Serrano et al. (1). Unbound phospholipid and detergent were removed from the ATPase by centrifugation (300,000 g) for 90 min at 30°C into discontinuous sucrose gradients. The sucrose gradients contained 2 and 3 ml of 25% and 60% sucrose, respectively, plus ATPase buffer (1), but no sodium cholate.

‡After recovery from the interface region of the gradient, the particulate ATPase and associated phospholipid were analyzed for ATPase activity (1) and fatty acyl chain composition. Values are the average of three independent experiments.

§The amount of phospholipid in SMP is ~706 nmol/mg protein.

‖I—ATPase specific activities measured without asolectin in the assays; II-ATPase specific activities measured with 100 μg of asolectin in the assays.

higher than those reported for the partially-purified ATPase (5, 6). Therefore, the present study dramatically demonstrates the importance of negatively-charged phospholipid for ATPase function and implies that the ATPase may require a negatively-charged mitochondrial phospholipid [e.g. diphosphatidylglycerol (DPG)] for optimal in vivo activity.

Examination of the phospholipid remaining associated with the purified lipid-depleted ATPase showed that PC, PE, and DPG were present. No relative enrichment of any of these phospholipids compared to their distribution in submitochondrial particles (2, 3) was noted. Therefore, no preferential association between the ATPase and any one phospholipid could be found in the highly-purified mitochondrial ATPase.

The usefulness of a cholate-mediated phospholipid exchange procedure (7) for studying the phospholipid requirements of the ATPase has been evaluated. Application of the cholate-mediated phospholipid exchange procedure to the highly purified mitochondrial ATPase resulted in the displacement of almost 50% of the residual mitochondrial phospholipid remaining associated with the enzyme after purification (Table II). Approximately the same amount of total phospholipid substitution resulted using either DOPA or DOPC. Examination of the displaced mitochondrial phospholipid showed that PC, PE, and DPG were replaced with equal facility. Therefore, under the cholate-mediated phospholipid exchange conditions, the ATPase did not exhibit preferential affinity for any particular mitochondrial phospholipid. However, these results must be qualified by recognizing that the high concentrations of the negatively-charged sodium cholate may mask the affinity of the enzyme for a negatively-charged phospholipid such as DPG. This could explain why the ATPase exhibits no preference for DPG during isolation or under the cholate-mediated phospholipid exchange conditions even though the experiments in Table I suggest a requirement for negatively-charged diacyl phospholipid when the sodium cholate concentration is low.

This work is supported by grants 02887 and 00043 from the National Institute of Alcohol Abuse and Alcoholism and grant 1978-79-A-7 from the North Carolina Heart Association.

Received for publication 7 May 1981.

REFERENCES

1. Serrano, R., B. Kanner, and E. Racker. 1976. Purification and properties of the proton-translocating adenosine triphosphatase complex of bovine heart mitochondria. *J. Biol. Chem.* 251:2453–2461.
2. Kagawa, Y., and E. Racker. 1966. Partial resolution of the enzymes catalyzing oxidative phosphorylation. IX. Reconstruction of oligomycin-sensitive adenosine triphosphatase. *J. Biol. Chem.* 241:2467-2474.

3. Krebs, J. J. R., H. Hauser, and E. Carafoli. 1979. Asymmetric distribution of phospholipids in the inner membrane of beef heart mitochondria. *J. Biol. Chem.* 254:5308–5316.
4. Pitotti, A., F. Dabbeni-Sala, and A. Bruni. 1980. Phospholipid-dependent assembly of mitochondrial ATPase complex. *Biochim. Biophys. Acta.* 600:79–90.
5. Cunningham, C. C., and G. Sinthusek. 1979. Ionic charge on phospholipids and their interaction with the mitochondrial adenosine triphosphatase. *Biochim. Biophys. Acta.* 550:150–153.
6. Cunningham, C. C., and D. T. George. 1975. The relationship between the bovine heart mitochondrial adenosine triphosphatase, lipophilic compounds, and oligomycin. *J. Biol. Chem.* 250:2036–2044.
7. Warren, G. B., P. A. Toon, N. J. M. Birdsall, A. G. Lee, and J. C. Metcalfe. 1974. Reconstitution of a calcium pump using defined membrane components. *Proc. Natl. Acad. Sci. U. S. A.* 71:622–626.

RHODOPSIN-PHOSPHOLIPID RECONSTITUTION FROM OCTYL GLUCOSIDE-SOLUBILIZED SAMPLES

MARILYN L. JACKSON AND BURTON J. LITMAN
Department of Biochemistry, School of Medicine, University of Virginia, Charlottesville, Virginia 22908 U.S.A.

Rhodopsin and phospholipid, solubilized in the detergent octyl glucoside (OG), were reconstituted by removal of the detergent by dialysis. Sucrose density gradient centrifugation of samples reconstituted with disk lipids or egg phosphatidylcholine (PC) resulted in the separation of a phospholipid-rich (high phospholipid to protein ratio) and a protein-rich (low phospholipid to protein ratio) vesicle fraction, both of which deviated markedly from the phospholipid to protein ratio of the starting material. The disk lipid system, which has an initial phospholipid to protein ratio of ~70:1, had a final phospholipid to protein ratio of ~30:1 in the protein-rich fraction, while that of the PC system, in which the initial ratio ranged from 100:1 to 300:1, was in the range of 30:1 to 50:1. The ratio of the protein-rich fraction of the reconstituted PC system could be increased to as high as 100:1 by increasing the rhodopsin concentration of the starting material, but was not significantly affected by the addition of 3 mM $MgCl_2$, the rate of dialysis, or the salt concentration of the buffer.

To determine the origin of the heterogeneity of the reconstituted vesicles, aliquots of an OG-solubilized sample of rhodopsin and phospholipid were dialyzed to various fixed detergent concentrations encompassing the region of vesicle formation. The concentration of OG was determined; subsequently, the samples were centrifuged, and the supernatants were analyzed for protein and phospholipid content. Data for the rhodopsin-disk lipid system indicate that as the detergent is initially removed during dialysis, lipid-rich vesicles form and pellet; as the level of detergent is decreased further, protein-rich vesicles are formed (Fig. 1). By contrast, when the PC system was analyzed, rhodopsin and phospholipid showed identical pelleting behavior, implying a single vesicle population. However, the inhomogeneity of this system could be demonstrated by density gradients, which still yielded two vesicle populations. The difference in pelleting behavior of the two systems can be attributed to the difference in density of the highly unsaturated disk phospholipids and more saturated PC.

Solubilization of disks with OG results in 25–30 mol of phospholipid being associated with the initially solubilized rhodopsin; this is in good agreement with the number of phospholipid molecules calculated to form a boundary layer around rhodopsin (Stubbs and Litman, 1978). It appears that the most favorable packing of the mixed micelles in this system is represented by rhodopsin, surrounded by a boundary layer of phospholipid, and OG. Our centrifugation experiments indicate that disruption of phospholipid-rich micelles precedes disruption of protein-rich micelles. Thus, the phospholipid to protein ratio that exists in the protein-rich mixed micelles seems to determine the phospholipid to protein ratio observed in the reconstituted vesicle population. Helenius et al. (1977)

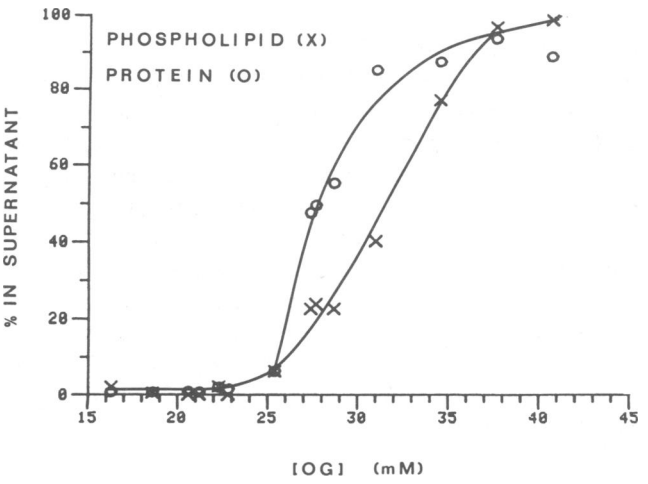

FIGURE 1 The percent phospholipid (×) and protein (O) in the supernatant as a function of the final, dialyzed OG concentration. Dialysis was carried out in 10 mM Tris, 10 mM sodium acetate, 50 mM potassium chloride (pH 7.0). The initial protein concentration was 1 mg/ml.