PRESERVATION OF ENERGY COUPLING IN SUBMITOCHONDRIAL PARTICLES DURING EXTRACTION AND REINSERTION OF CYTOCHROME C

William J. Arion and Barbara J. Wright

Department of Physiology, Biochemistry, and Pharmacology
New York State Veterinary College, Cornell University
Ithaca, New York 14850

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ABSTRACT

Cytochrome c was extracted from submitochondrial particles with the aid of cholate and KCl while completely retaining the $^{32}P_i$-ATP exchange activity. Reconstitution of oxidation coupled to phosphorylation was accomplished by exposing the depleted particles to an excess of cytochrome c in the presence of cholate and KCl, diluting with bovine plasma albumin, and finally washing the isolated particles with Mg^{++}-containing buffer to remove externally bound cytochrome c. With NADH, succinate, or phenazine methosulfate plus ascorbate, the P:O values obtained with reconstituted particles were 1.1, 0.64 and 0.63, respectively.

INTRODUCTION

Recent studies by Racker and coworkers (1) have established the asymmetric organization of the inner mitochondrial membrane. Succinate dehydrogenase, coupling factor 1(ATPase) and other protein coupling factors are attached to the M-side[1] of the membrane, cytochrome c resides on the C-side[1], while cytochrome oxidase and phospholipids are found on both surfaces of the membrane. In addition to increasing our understanding of molecular architecture of this organelle,these findings predict that successful reconstitution of a functional inner membrane

[1]The inner membrane can exist in two conformations (2): either in the "native" conformation as in intact mitochondria or in the "inverted" form as in submitochondrial particles. To avoid confusion, Racker and associates (1) have designated the side of the membrane which faces the matrix in intact mitochondria as the M-side, and the side which faces the outer membrane of the organelle as the C-side. These definitions are used here.

system from individual components must entail the reassembly of the components such that the topography of the native membrane is reproduced. The reconstitution of cytochrome c is a case in point. Through the use of a specific antibody prepared against cytochrome c, these workers have shown (1) that oxidation of ferrocytochrome c situated on the C-side is coupled to ATP synthesis, but that oxidation of ferrocytochrome c added to the M-side of submitochondrial particles is not. Therefore, success in reconstituting functional submitochondrial particles depends in some measure on developing a procedure whereby cytochrome c can be reinserted into its native location on the inside (i.e., on the C-side) without losing the capacity to catalyze oxidation phosphorylation. Reconstitution of oxidation was achieved in earlier studies (1) by removal and reinsertion of cytochrome c in the presence of cholate. However, because of the treatment conditions employed in these studies, oxidation was not coupled to ATP synthesis. The present report documents procedures with which the objective of preserving energy coupling during extraction and reconstitution of cytochrome c can be accomplished.

MATERIALS AND METHODS

Heavy layer beef heart mitochondria (3), F_1(4)[2], F_4(5), and SMP (6) were prepared as described in the references. Cholic acid ("enzyme grade") was obtained from Mann. Cytochrome c (type VI), NADH, ATP and crystalline yeast hexokinase were purchased from Sigma. Crystalline bovine plasma albumin and dithiothreitol were obtained from Calbiochem. All other chemicals were the purest grade commercially available. Procedures for defatting the bovine plasma albumin and assays for $^{32}P_i$-ATP exchange

[2]Abbreviations used are: F_1 and F_4, coupling factors 1 and 4 respectively; SMP, submitochondrial particles obtained after sonication of mitochondria in 10 mM $NaPP_i$ (pH 7.4); Tricine, N-tris (hydroxymethyl)-methyl glycine; PMS, N-methylphenasinium methyl sulfate; STE, buffer solution containing 0.25 M sucrose-10 mM Tricine-KOH-1 mM EDTA (pH 7.8); STE-DTT-Mg, STE containing 0.5 mM dithiothreitol and 10 mM $MgCl_2$.

activity, protein, and oxidative phosphorylation are described elsewhere (7).

Extraction of Cytochrome c from SMP - SMP (10 mg of protein) were added to a solution equilibrated at 4° containing 100 µmoles Tricine-KOH (pH 8.0), 10 µmoles unneutralized dithiothreitol, 5 mg sodium cholate (pH 7.6), and 400 µmoles KCl in a final volume of 2 ml. After 15 min. the solution was centrifuged for 30 min. at 165,000 x g. The pellet was suspended in 2 ml of cold STE-DTT-Mg. The particles were resedimented by centrifugation at 165,000 x g for 20 min. and finally suspended in 1 ml of STE.

Reinsertion of Cytochrome c into SMP - Heavy layer beef heart mitochondria were depleted of cytochrome c by repeated washings with KCl (8), and cytochrome c-depleted SMP were prepared in the usual way from these mitochondria. The cytochrome c-depleted SMP were exposed to the medium used to extract cytochrome c to which 0.45 mg cytochrome c had been added. After 10 min. at 4°, 40 mg of defatted bovine plasma albumin and enough water were added to bring the volume to 4 ml, and five min. later 40 µmoles (40 µl) of $MgCl_2$ were added. After five min. the samples were layered over 4 ml of cold STE-DTT-Mg previously placed in polycarbonate centrifuge tubes (IEC no. 2801), and the particles were recovered by centrifugation at 165,000 x g for 30 min. The cytochrome c-reconstituted SMP were resuspended in 2 ml of cold STE-DTT-Mg washed by centrifugation at 165,000 x g for 20 min. and finally suspended in 1 ml of STE.

RESULTS AND DISCUSSION

Succinate and NADH oxidase activities were decreased by more than 80% by treatment of SMP with cholate and KCl (Table I). Respiratory activity was fully restored by addition of cytochrome c, demonstrating that the losses in respiration were due only to removal of cytochrome c.

TABLE I

EFFECTS OF CHOLATE-KCl TREATMENT OF SMP ON RATES OF RESPIRATION AND $^{32}P_i$-ATP EXCHANGE ACTIVITY

Particle	Oxygen Uptake		$^{32}P_i$-ATP
	Succinate	NADH	Exchange Activity
	natoms per min per mg		nmoles AT ^{32}P formed per min. per mg
A. Untreated SMP	410	433	314
+ 30 µg cytochrome c	613	1200	
B. Cholate-KCl treated SMP	76	75	312
+ 30 µg cytochrome c	565	1230	

Procedures for polarographic assays of succinate and NADH oxidase are described elsewhere (7). Where indicated, 30 µg of cytochrome c were added directly to the polarograph chamber. Approximately 300 µg of SMP protein, 30 µg F_1 and 150 µg F_4 were used in the exchange assay.

The data in Table I also show the important feature that the $^{32}P_i$-ATP exchange activity was not significantly altered during extraction of cytochrome c.

Oxidative phosphorylation in SMP following extraction and reinsertion of cytochrome c is summarized in Table II. Rates of respiration by cytochrome c-reconstituted SMP were markedly greater than those of SMP prepared from cytochrome c-depleted mitochondria (Experiment I), whether NADH, succinate or ascorbate plus PMS were used as substrates. Rates of phosphorylation were correspondingly elevated as evidenced in the table by the essentially unchanged P:O values.

The procedure described clearly permits the reinsertion of cytochrome c under conditions yielding efficient energy transfer at all three coupling sites. In other experiments succinate and NADH oxidase activities of the cytochrome c-reconstituted SMP were found to be unaffected by additions

TABLE II

OXIDATIVE PHOSPHORYLATION IN SMP FOLLOWING EXTRACTION AND REINSERTION OF CYTOCHROME C

Particle	OXYGEN UPTAKE			P:O		
	NADH	Succ.	Asc. + PMS	NADH	Succ.	Asc. + PMS
	natoms per min per mg					
I. Cytochrome c-depleted SMP prepared from KCl-washed mitochondria						
A. "Control" ‡	158	110	325	0.99	0.80	0.54
B. Reconstituted	846	343	790	1.10	0.64	0.63
II. Cytochrome c-depleted SMP prepared by cholate-KCl extraction						
A. Reconstituted	352	205	315	0.82	0.79	0.60

‡ Cytochrome c-depleted SMP carried through the conditions used for reinsertion except KCl was omitted during the exposure to cytochrome c.

of cytochrome c antiserum, verifying that the cytochrome c is reinserted into its native location on the C-side of the membrane. It should be emphasized that neither the extraction nor the reinsertion of cytochrome c could be effected unless both KCl and cholate were present during treatment. When either of these reagents was omitted, the properties of the SMP were essentially unchanged from those of untreated preparations.

Experiment II of Table II establishes that the energy transfer system can tolerate repeated exposures to the cholate-salt media. In this exper-

iment cytochrome c-depleted SMP were prepared from SMP by treatment with cholate and KCl, frozen overnight at -70°, and the cytochrome c reinserted by a second exposure to cholate and salt. The efficiencies of energy transfer catalyzed by these reconstituted SMP were comparable to those of untreated SMP. This demonstration that the energy transfer system is preserved during repeated treatments with cholate and salt under the conditions described is of great importance to the success of future attempts to reconstitute oxidative phosphorylation from individual components.

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