Spontaneous Reactivation of Covalently Labeled Proton Adenosinetriphosphatase†

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ABSTRACT: Bovine heart mitochondrial adenosinetriphosphatase selectively labeled by [^{14}C]-*N,N'*-dicyclohexylcarbodiimide or [^{14}C]-7-chloro-4-nitro-2,1,3-benzoxadiazole was used together with other components to form reconstituted submitochondrial particles. When assayed for ATP hydrolysis under normal hydrolysis conditions, these labeled submitochondrial particles were found to increase slowly in specific activity with preincubation time, without losing the covalent label. But when assayed for oxidative phosphorylation, the ratio of the specific activity of the same labeled particles to that of the control particles was higher and was unaffected by preincubation. If the labeled particles had been treated by a simulated procedure for oxidative phosphorylation measurement before the ATPase assay, their specific activities for ATP hydrolysis were also found to be higher and unaffected by preincubation. These observations are difficult to reconcile with the alternating three-site model for proton adenosinetriphosphatase or any model which requires the sequential action of three identical sites for ATP hydrolysis and synthesis. A new model with one active and two latent interacting sites is proposed for interpreting the present data.

Recent studies on inner mitochondrial membranes reconstituted with NBD-Cl- (7-chloro-4-nitro-2,1,3-benzoxadiazole-) labeled MF_1-ATPase[1] show that these reconstituted vesicles can still catalyze oxidative phosphorylation although ATP hydrolysis is largely blocked (Steinmeier & Wang, 1979; Kohlbrenner & Boyer, 1982). These observations seem difficult to reconcile with the well-known alternating site models for oxidative phosphorylation and ATP hydrolysis (Adolfsen & Moundrianakis, 1976; Kayalar et al., 1977). For example, in Boyer's alternating three-site model (Gresser et al., 1982), the three catalytic sites are regarded as identical and participate in sequence with each site in turn going through three major reaction stages. Accordingly, inactivating one of the three sites in an ATPase complex by covalent labeling is expected to block the overall reaction catalyzed by that complex, and the degree of inhibition of the steady-state rate is expected to be the same for both ATP hydrolysis and oxidative phosphorylation. However, in view of the lability of the O-NBD label on this particularly reactive tyrosine residue in MF_1, there is the possibility that the label might fall off under the conditions of oxidative phosphorylation measurements.

In order to examine this possibility, the more stable N-NBD-labeled MF_1 and DCCD- (dicyclohexylcarbodiimide-) labeled MF_1 have been used to reconstitute the mitochondrial inner membrane vesicles in this work. These reconstituted vesicles are represented by MF_1-ASU, $(N\text{-NBD})_n MF_1$-ASU, and $(DCCD)_m MF_1$-ASU, where n and m denote the number of moles of N-NBD label and DCCD label, respectively, per mole of chemically modified MF_1.

Experimental Procedures

Materials

ATP, ADP, phosphoenolpyruvate, NADH, Hepes, Mes, EDTA, BSA, lactic acid, diadenosine pentaphosphate, hexokinase, pyruvate kinase, lactic dehydrogenase, adenosine 5'-(β,γ-methylenetriphosphate), oligomycin, and Sephadex G-50-80 were purchased from Sigma Chemical Co., and K-$H_2{}^{32}PO_4$ was from New England Nuclear. [^{14}C]NBD-Cl and [^{14}C]DCCD were purchased from Research Products International Corp. FCCP was purchased from Pierce Chemical Co.

Mitochondria were prepared from fresh bovine heart by the method of Low & Vallin (1963). MF_1-ATPase was prepared and stored as described by Knowles & Penefsky (1972). MF_1-depleted mitochondrial inner membrane vesicles (ASU particles) were prepared by the method of Racker & Horstman (1967). Oligomycin-sensitivity-conferring protein (OSCP) was prepared as described by Senior (1979).

Methods

Preparation of [^{14}C]*DCCD-MF*$_1$. A suspension of MF_1 in 2 M ammonium sulfate solution at 4 °C was centrifuged. The precipitate was redissolved in 50 mM Hepes–NaOH buffer (pH 7.5) containing 0.2 M sucrose, 2 mM EDTA, and 5 mM ATP at room temperature. The solution was centrifuge filtered through a Sephadex G-50-80 column (Penefsky, 1977; Cross & Nalin, 1982), which had been prewashed with the same buffer, for removing the ammonium sulfate. The filtrate was mixed with one-tenth of its volume of 0.1 M Mes solution (pH 4.0) to lower the pH to 6.6. A pentane–acetone solution of [^{14}C]DCCD (specific radioactivity 54 mCi/mmol) was evaporated to dryness in a stream of N_2. The residue was allowed to react with the above MF_1 solution at room temperature with slow stirring. The decrease of ATPase activity [initially between 50 and 70 μmol of ATP/(min·mg)] was monitored as a function of time. When the specific activity had decreased to a sufficiently low level, the reaction was

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[1] Abbreviations: AMPPCP, adenosine 5'-(β,γ-methylenetriphosphate); AP_5A, P^1,P^5-di(adenosine-5') pentaphosphate; ASU, submitochondrial particles prepared from bovine heart mitochondria by sonication in the presence of ammonia at pH 9 followed by steps involving Sephadex and urea treatments; BSA, bovine serum albumin; DCCD, *N,N'*-dicyclohexylcarbodiimide; DCCD-MF_1, MF_1 labeled by DCCD; DTT, DL-dithiothreitol; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; HK, hexokinase; LDH, L-lactic dehydrogenase; Mes, 2-(*N*-morpholino)ethanesulfonic acid; MF_1-ATPase, mitochondrial F_1 adenosinetriphosphatase; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole (*also named 4-chloro-7-nitrobenzofurazan*); N-NBD-MF_1, MF_1 *labeled* by NBD-Cl at an amino group; O-NBD-MF_1, MF_1 labeled by NBD-Cl at a Tyr residue; OSCP, oligomycin-sensitivity-conferring protein; PEP, phosphoenolpyruvate; PK, pyruvate kinase; S.A., specific activity; EDTA, ethylenediaminetetraacetic acid.

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stopped by centrifuge filtration. The filtrate was then assayed for protein concentration, ATPase activity, and radioactivity.

Preparation of [^{14}C]O-NBD-MF$_1$ and [^{14}C]N-NBD-MF$_1$. The previously reported procedure for preparing O-NBD-MF$_1$ (Steinmeier & Wang, 1979) was used. The unreacted [^{14}C]NBD-Cl (specific radioactivity 109 mCi/mmol) was removed by centrifuge filtration. The [^{14}C]N-NBD-MF$_1$-ASU was prepared by illuminating the [^{14}C]O-NBD-MF$_1$ solution with a 500-W projector lamp for 5–10 min at 0 °C at an intensity of 70 mW/cm^2. The nonprotein radioactive products were also removed by centrifuge filtration. The O → N transfer of NBD label during illumination was complete because the ATPase activity of the product was no longer sensitive to 2.5 mM DTT.

Reconstitution. The ASU particles were reconstituted with OSCP and MF$_1$ (either native or chemically modified) at 37 °C in 50 mM triethanolamine hydrochloride buffer (pH 7.5) containing 0.2 M sucrose, 2 mM EDTA, and 2 mM ATP. The three components at protein weight ratios of ASU:OSCP:MF$_1$ = 1:0.04:0.3 were used for reconstitution according to the reported procedure (Steinmeier & Wang, 1979; Kohlbrenner & Boyer, 1982). ASU particles were incubated with OSCP for 1 min at 37 °C. Then MF$_1$ was added and the mixture was incubated for another 3 min. The mixture was then centrifuged for 7 min in a Beckman airfuge at 30 psi (g_{av} = 135000). After the supernatant was removed, the pellets in the individual polyethylene airfuge tubes were frozen and stored at −70 °C. The ASU particles probably already contained OSCP, since the ATPase activity of unlabeled particles reconstituted in the absence of added OSCP was decreased to 6 or 7% of the uninhibited value by adding 0.2 μg of oligomycin/mL of the assay medium (22 μg/mg of protein).

Assay of Protein Concentration and Radioactivity. Protein concentration was determined by the Coomassie Blue binding method (Bradford, 1976). Radioactivity was assayed by liquid scintillation counting. The molar ratio of label to MF$_1$ was calculated by using a molecular weight of 360 000 for MF$_1$ (Lambeth et al., 1971) and the known specific radioactivity of the label.

Resuspension of the Reconstituted Inner Membrane Vesicles. In each experiment a frozen pellet of reconstituted membranes (with about 0.7 mg of protein) was homogenized with 175 or 350 μL of suspension medium by means of a glass plunger which had been specially ground to fit the polyethylene airfuge tubes. The suspension medium contained 50 mM Hepes–NaOH, pH 7.5, 0.2 M sucrose, and 10 mM AMPPCP. After the up and down motion of the plunger in the airfuge tube was repeated several times, the homogenate was transferred promptly to a screw-capped glass vial to minimize evaporation at room temperature throughout the experimental period. In the presence of 10 mM AMPPCP, it was found that the ATPase activity of the reconstituted MF$_1$-ASU stayed essentially constant at room temperature for 7 or 8 h.

Assay of ATPase Activity. ATPase activity was followed by taking 10- or 20-μL aliquots of the suspension at time invervals and assaying at 30 °C by an ATP-regenerating system coupled to the oxidation of NADH. The assay medium contained 50 mM Hepes–NaOH, pH 8.0, 3 mM $MgCl_2$, 50 mM KCl, 2 mM ATP, 2 mM PEP, 0.4 mM NADH, 21 units/mL PK, 11 units/mL LDH, and 1.5 mM KCN (added immediately before the measurement). The rate of ATP hydrolysis was computed from the linear decrease of A_{340} due to the oxidation of NADH. Because of the 100- or 200-fold dilution factor, the concentration of AMPPCP in the assay mixture was only 0.1 or 0.2 mM and was found to have negligible effect on the observed specific activity.

For assaying the ATPase activity under simulated oxidative phosphorylation conditions, a 10- or 20-μL aliquot of the suspension of reconstituted vesicles was added to 50 or 100 μL of a simulated oxidative phosphorylation medium in an airfuge tube and homogenized continually for 3 min by the up and down motion of the specially made glass plunger at 25 °C in exactly the same way as for oxidative phosphorylation measurements described below. At the end of 3 min, a 50-μL aliquot was taken out and added to 2 mL of ATPase assay medium for spectrophotometric measurement of ATP hydrolysis. The simulated oxidative phosphorylation medium contained 50 mM Hepes–NaOH, pH 7.5, 0.2 M sucrose, 10 mM ADP, 10 mM NADH, 10 mM $MgCl_2$, 23.3 mM KH_2PO_4, and 20 mM glucose.

Assay of Oxidative Phosphorylation. Rates of oxidative phosphorylation by the reconstituted vesicles were measured at room temperature (ca. 25 °C) in a medium containing 50 mM Hepes–NaOH, pH 7.5, 0.2 M sucrose, 20 mM glucose, 23.3 mM KH_2PO_4 (0.5 mCi of ^{32}P/mmol of P_i), 1 mg/mL defatted bovine serum albumin (BSA), 50 units of HK/mL, 100 μM AP_5A, 10 mM ADP, 10 mM NADH, and 10 mM $MgCl_2$. In each assay, a 10-μL aliquot of the suspended vesicles was added to 50 μL of this oxidative phosphorylation medium in an airfuge tube and mixed continually for 3 min by the up and down motion of the glass plunger. At 3.00 min of reaction time, 10 μL of 3 M $HClO_4$ was injected into the tube, and the suspension was again mixed with the plunger. The tube was then stoppered and kept on ice for chromatographic analysis at a later time along with all the other oxidative phosphorylation samples. Net ATP synthesis was determined from the formation of glucose 6-[^{32}P]phosphate.

The analysis was performed by spotting a 10-μL aliquot of each acidified reaction mixture on Whatman No. 541 chromatography paper with 4-cm-wide channels and developing overnight with the solvent system of Wood (1968). Composition of the developing solvent was 1-butanol–1-propanol–acetone (80% w/v)–formic acid (30% w/v)–trichloroacetic acid at a ratio of 40:20:25:25:1.5, plus 0.05 g of tetrasodium salt of EDTA/100 mL of the solvent. The developed paper was dried, and its UV-absorbing spots were marked with pencil, photocopied, cut into 1 × 4 cm strips, and counted with 5 mL of Aquasol in a Beckman LS-233 liquid scintillation counter. With this developing solvent, glucose 6-phosphate migrates faster than ADP but slower than AMP and P_i, resulting in a clean separation.

Results

The specific ATPase activities of DCCD-labeled reconstituted inner membrane vesicles (submitochondrial particles) are listed in Table I. The specific activity (S.A.) of labeled MF$_1$ samples used for the reconstitution were 5.2 units/mg for (DCCD)$_{1.09}$MF$_1$ and 0.52 unit/mg for (DCCD)$_{1.80}$MF$_1$. These values correspond to 10.1% and 1.0%, respectively, of that for the control MF$_1$. The S.A. of ASU used for the reconstitution was 0.24 unit/mg and was unaffected by further sonication. This value was subtracted from all observed S.A. values listed in Tables I and V. Table I shows that the S.A. values for (DCCD)$_{1.09}$MF$_1$-ASU and (DCCD)$_{1.80}$MF$_1$-ASU are much higher, ranging from 1.42 to 2.24 for the former and 0.45 to 1.41 for the latter. These higher S.A. values could not be due to the preferential binding of unlabeled MF$_1$ by ASU during reconstitution, even though the soluble ATPase was present in 2.5-fold excess. This is because there was not enough of unlabeled MF$_1$ present in the reconstitution mixture to engender such high S.A. values. For example, the

Table I: ATPase Activity of DCCD-Labeled Reconstituted Submitochondrial Particles

preincubation time at 22 °C (h)	μmol of ATP hydrolyzed/[min·(mg of protein)] control MF_1-ASU	$(DCCD)_{1.09}$-MF_1-ASU	$(DCCD)_{1.80}$-MF_1-ASU
(A) Assayed under ATP Hydrolysis Conditions[a]			
0	6.20	1.42 (0.23)[b]	0.45 (0.073)[b]
1	6.39	1.56 (0.24)	0.73 (0.11)
3	6.32	2.04 (0.32)	1.00 (0.16)
5	6.39	2.05 (0.32)	1.00 (0.16)
7.5	6.39	2.24 (0.35)	1.41 (0.22)
(B) Assayed after Simulated Oxidative Phosphorylation Conditions[c]			
0	5.41	1.76 (0.33)	1.18 (0.22)
1	5.46	1.87 (0.34)	1.30 (0.24)
2	5.46	2.04 (0.37)	1.33 (0.24)
7.5	5.25	1.99 (0.38)	1.26 (0.24)

[a] Reconstituted particles were resuspended in 50 mM Hepes buffer, pH 7.5, containing 0.2 M sucrose, 2 mM EDTA, and 10 mM AMPPCP at room temperature. Aliquots were taken at time intervals and assayed for ATPase activity as described under Methods. [b] The numbers in parentheses denote the ratio of the specific activity of the DCCD-labeled particles to that of control MF_1-ASU. [c] Reconstituted particles were resuspended in 50 mM Hepes buffer, pH 7.5, containing 0.2 M sucrose and 10 mM AMPPCP. Aliquots were taken out at indicated time intervals and added to a simulated oxidative phosphorylation buffer containing 50 mM Hepes, pH 7.5, 0.2 M sucrose, 10 mM $MgCl_2$, 20 mM glucose, 23.3 mM KH_2PO_4, 10 mM ADP, and 10 mM NADH. The suspension was continually mixed with a glass plunger for 3 min and then assayed for ATPase activity as described under Methods.

$(DCCD)_{1.80}MF_1$ preparation could not contain more than 1% of unlabeled MF_1, because its S.A. was only 1% of that of the control MF_1. Consequently, even if all unlabeled MF_1 in the reconstitution mixture was preferentially bound to ASU, it could only raise the S.A. of the reconstituted submitochondrial particles to 2.5 × 0.007 × 6.4 = 0.11 unit/mg, which is an order of magnitude lower than most of the S.A. values listed in Table I. The ATPase activity of our reconstituted MF_1-ASU was 87% inhibited by 2 μg of oligomycin/mg of total protein. These reconstituted particles seem quite leaky to protons, because only an 8% increase in ATPase activity was observed after the addition of 0.2 μM FCCP. But oxidative phosphorylation was completely abolished by 17 μM FCCP.

Another puzzling aspect of the data in Table I is that the specific activities determined under ATP hydrolysis conditions slowly increase with time and seem to approach gradually a limiting value, whereas those determined under simulated oxidative phosphorylation conditions stay more or less unchanged near this limiting value. Can the observed partial reactivation of these labeled submitochondrial particles be due to the slow dissociation of the covalent label?

In order to test this possibility, the soluble enzyme $(DCCD)_{1.13}MF_1$ was incubated under normal experimental conditions for 43 and 48 h, followed by centrifuge filtration (Penefsky, 1977) through a Sephadex G-50-80 column to remove any radioactive small molecules which might have been hydrolyzed off. Table II shows that after 43 h, neither its specific activity nor its [^{14}C]DCCD/protein ratio had changed. The reconstituted submitochondrial particles $(DCCD)_{1.13}MF_1$-ASU were also incubated under normal conditions for 12 h, followed by centrifugation in a Beckman airfuge and resuspension of the pellet. Table II shows that the [^{14}C]DCCD/protein ratio remained unchanged after 12 h.

Table II: Stability of DCCD Label in Soluble MF_1 and in Reconstituted Submitochondrial Particles Measured by Gel Filtration and Centrifugation

preincubation time at 22 °C (h)	S.A. [μmol of ATP/(min·mg)]	protein concn (mg/mL)	DCCD label (μM)	DCCD/protein (nmol/mg)
(A) Soluble Enzyme $(DCCD)_{1.13}MF_1$[a]				
0	4.41	2.97	9.3	3.1
43	4.36	1.70	5.57	3.3
48		2.54	7.08	2.8
(B) Reconstituted Particle $(DCCD)_{1.13}MF_1$-ASU[b]				
0		3.64	0.47	0.129
12		3.15	0.41	0.130

[a] [^{14}C]DCCD-labeled MF_1 (with molar ratio DCCD/MF_1 = 1.13/1) was incubated in 50 mM Hepes buffer, pH 7.5, containing 0.2 M sucrose, 2 mM EDTA, and 6 mM AMPPCP at 25 °C. At the indicated time intervals, the suspension was centrifuge filtered through a Sephadex G-50-80 column which had been preequilibrated with 50 mM Hepes buffer containing 0.2 M sucrose, 2 mM EDTA, and 5 mM ATP at pH 7.5. [b] ASU particles reconstituted with OSCP and $(DCCD)_{1.13}MF_1$ were first resuspended in the above buffer. At the indicated time intervals, an aliquot of the suspension was centrifuged in a Beckman airfuge for 7 min at 30 psi. The pellet was resuspended in 50 mM Hepes buffer, pH 7.5, containing 0.2 M sucrose and 10 mM ATP instead of 10 mM AMPPCP. The resulting suspension was then assayed for protein concentration and radioactivity.

There is still the possibility that the radioactive labels might have been hydrolyzed off, but the resulting radioactive products were still adsorbed to the submitochondrial particles by noncovalent binding. This possibility was tested by incubating three types of labeled reconstituted submitochondrial particles, $(O\text{-}NBD)_{0.78}MF_1$-ASU, $(DCCD)_{1.0}MF_1$-ASU, and $(N\text{-}NBD)_{2.1}MF_1$-ASU, for 2 h at room temperature, followed by treatment with 8 M urea, centrifugation, gel filtration of the supernatant, and subsequent determination of protein concentration and radioactivity in the filtrate. The results summarized in Table III show that while the labile O-NBD label of the reconstituted vesicles can be removed after treatment with DTT as expected, both the DCCD label and the N-NBD label remained quantitatively attached to the protein by a covalent bond after 2 h of preincubation and subsequent urea treatment. Consequently we have to conclude that the observed reactivation of labeled proton adenosinetriphosphatase is due to structural rearrangement within this enzyme complex without losing the normally inhibitory covalent labels.

A very interesting feature of the data in Table I is that although it took 7.5 h under ATP hydrolysis conditions for the S.A. ratios of $(DCCD)_{1.09}MF_1$-ASU and $(DCCD)_{1.80}MF_1$-ASU to increase to 0.35 and 0.22, respectively, these values were reached within 3 min under simulated oxidative phosphorylation conditions. It seems that the said structural rearrangement within the proton ATPase is greatly accelerated by the assay procedure of oxidative phosphorylation and that an equilibrium or steady-state distribution of the conformational states of this protein complex can be reached within 3 min under these conditions. Comparison of these zero time S.A. ratios with those for the soluble enzymes $(DCCD)_{1.09}MF_1$ and $(DCCD)_{1.80}MF_1$ (0.10 and 0.01, respectively) suggests that such structural rearrangement had probably already taken place to a limited extent during the reconstitution and subsequent resuspension of the reconstituted particles.

Such an interpretation is also consistent with the oxidative phosphorylation data in Table IV. In spite of the large ex-

Table III: Stability of Covalent Labels in Reconstituted Submitochondrial Particles Tested by Treatment with Urea[a]

preincubation time at 22 °C (min)	nmol of label/mg of urea-released soluble protein		
	$(O\text{-}NBD)_{0.78}$-MF_1-ASU	$(DCCD)_{1.0}$-MF_1-ASU	$(N\text{-}NBD)_{2.1}$-MF_1-ASU
(A) Under ATP Hydrolysis Conditions[b]			
0		1.16	1.89
120		1.16	1.90
(B) Under Oxidative Phosphorylation Conditions[c]			
0	0.52 (−DTT), 0.15 (+DTT)[d]	1.21	1.95
90	0.10 (+DTT)[d]		
120		1.23	1.92

[a] Preincubated particles in 25 μL of appropriate buffer were mixed with 150 μL of 8 M urea ar toom temperature. After homogenization the mixture was centrifuged in a Beckman airfuge for 7 min at 30 psi. The supernatant was then centrifuge filtered through a Sephadex G-50-80 column which had been equilibrated with a buffer, containing 50 mM Hepes, 0.2 M sucrose, 2 mM EDTA, and 8 M urea, to remove radioactive small molecules (if present). The eluate was assayed for total protein concentration and radioactivity. [b] The particles were resuspended in a buffer containing 50 mM Hepes, pH 7.5, 0.2 M sucrose, 2 mM EDTA, and 10 mM AMPPCP. [c] The particles were resuspended in a simulated oxidative phosphorylation buffer containing 50 mM Hepes, pH 7.5, 0.2 M sucrose, 10 mM $MgCl_2$, 20 mM glucose, 23.3 mM KH_2PO_4, 5 mM ADP, and 10 mM NADH. [d] The particles were resuspended in the simulated oxidative phosphorylation buffer that included 57 mM DTT. After mixing with 8 M urea solution, 10 μL more of 1.0 M DTT was added.

Table IV: Oxidative Phosphorylation by DCCD-Labeled Reconstituted Submitochondrial Particles[a]

preincubation at 22 °C (h)	nmol of ATP synthesized/[min·(mg of protein)]		
	control MF_1-ASU	$(DCCD)_{1.09}$-MF_1-ASU	$(DCCD)_{1.80}$-MF_1-ASU
0	165	72 (0.43)[b]	54 (0.34)[b]
0	3 (+ oligomycin)[c]		
1	158	54 (0.34)	45 (0.28)
2	156	56 (0.36)	45 (0.29)
4	155	62 (0.40)	46 (0.30)

[a] Reconstituted submitochondrial particles were resuspended at time zero in 50 mM Hepes buffer, pH 7.5, containing 0.2 M sucrose and 10 mM AMPPCP at room temperature. At the indicated time intervals, 10 μL of the suspension was mixed with 50 μL of oxidative phosphorylation medium (composition given under Methods) in an airfuge tube and continually mixed with a plunger for 3 min to ensure the adequately rapid supply of air for the oxidation of NADH. At 3.00 min, 10 μL of 3 M $HClO_4$ was injected, and the suspension was again mixed with the same (unwashed) plunger. The tube was stoppered and kept on ice for later spotting on chromatographic paper. [b] Ratio of the oxidation phosphorylation rate of the DCCD-labeled particles to that of the control MF_1-ASU. [c] About 70 nmol of oligomycin/mg of total protein was added.

perimental errors inherent in the sampling of small volumes (10 μL) of suspended particles, the general trends of observed values obtained over a large number of ATP hydrolysis and oxidative phosphorylation experiments have always been the same. Table IV shows that well within 3 min under oxidative phosphorylation conditions, the reconstituted vesicles can reach the steady-state distribution of conformational states and hence the observed oxidative phosphorylation rates are insensitive to preincubation time. The ratios of observed oxidative phosphorylation rates of the DCCD-labeled particles to that of the control MF_1-ASU are also much higher than the corresponding zero times ratios for ATP hydrolysis assayed under hydrolysis conditions, but not very different from the ratios for ATP hydrolysis assayed under simulated oxidative phosphorylation conditions. Because of its interference with the method of ATPase assay based on coupled oxidation of NADH, hexokinase was omitted in the simulated oxidative phosphorylation medium.

Table V: Effect of Ligand Exchange and Respiration on the Acceleration of Spontaneous Reactivation of DCCD-Labeled Submitochondrial Particles[a]

preincubation[b] time (h)	preincubation[b] temp (°C)	preassay treatment at 25 °C	S.A. [μmol of ATP hydrolyzed/[min·(mg of protein)]] control MF_1-ASU	S.A. [μmol of ATP hydrolyzed/[min·(mg of protein)]] DCCD-MF_1-ASU[c]
0.2	1	none	7.58	0.92 (0.12)[d]
0.6	1	medium A[e] + 10 mM ADP + 10 mM NADH	3.48	0.76 (0.22)
1.3	1	medium A + 10 mM ADP	3.31	0.57 (0.17)
2.0	1	medium A + 10 mM NADH	4.06	0.58 (0.14)
2.0	1	medium A + 10 mM ATP	3.36	0.42 (0.13)
2.8	1	none	6.92	1.03 (0.15)
2.8	23	none	7.49	1.60 (0.21)

[a] These particles were reconstituted without adding isolated OSCP. [b] The composition of preincubation medium is given in footnote *a* of Table I. [c] MF_1 of S.A. 49.1 units/mg was labeled with nonradioactive DCCD until only 4.2% of the initial S.A. was left when the reaction was terminated by gel filtration. The DCCD/MF_1 ratio of this batch of labeled ATPase was estimated from our previously established ratio vs. % S.A. curve to be about 1.5. [d] Same as footnote *b* of Table I. [e] Medium A contained 50 mM Hepes–NaOH, 0.2 M sucrose, 10 mM $MgCl_2$, 23.3 mM K_2HPO_4, and 20 mM glucose, pH 7.5. In the preassay treatment, 10 μL of the preincubated particles was mixed with 50 μL of preassay treatment medium (medium A plus the additional components as listed) in an airfuge tube, and the mixture was homogenized for 3 min by the up and down motion of a special glass plunger. Subsequently, a 25-μL aliquot of the mixture was injected into 2 mL of assay medium for measuring the ATPase activity. The concentrations of NADH in the assay media were so preadjusted that a few seconds after sample injection all assay mixtures contained 0.4 mM NADH initially for monitoring the ATPase reaction.

Factors which may cause the acceleration of spontaneous reactivation of the labeled submitochondrial particles have been explored by varying the composition of the simulated oxidative phosphorylation medium. The data from one batch of submitochondrial particles reconstituted in the absence of additional OSCP are summarized in Table V. Since the specific activities of the unlabeled control particles are also affected by the exchange of tightly bound adenine nucleotides (Harris et al., 1978; Tamura & Wang, 1983), only the S.A. ratios (values in parentheses) can be compared directly. The S.A. ratios in Table V suggest that the spontaneous reactivation of labeled particles is probably accelerated by nucleotide exchange which may be facilitated by respiration. A pair of experiments with ATP replacing ADP in the preassay treatment medium is also included in Table V, although the resulting S.A. ratio cannot be interpreted in a simple way, because in the presence of Mg^{2+} the free ATP was continually hydrolyzed to ADP and P_i by the particles during the 3-min treatment.

Similar measurements of the rates of ATP hydrolysis and oxidative phosphorylation as functions of preincubation time were also made with N-NBD-labeled reconstituted submito-

Table VI: ATP Hydrolysis and Synthesis by N-NBD-Labeled Reconstituted Submitochondrial Particles

preincubation time at 22 °C (h)	μmol of ATP hydrolyzed/[min·(mg of protein)] control MF_1-ASU	(N-NBD)$_{1.18}$-MF_1-ASU
(A) ATP Hydrolysis Assayed under Hydrolysis Conditions[a]		
0	9.12	1.82 (0.20)[b]
0	9.86 (+0.2 μM FCCP)	
1.25	8.43	2.32 (0.28)
4.5	8.49	2.58 (0.30)
8	8.07	2.75 (0.34)
(B) ATP Hydrolysis Assayed after Simulated Oxidative Phosphorylation Conditions[c]		
0	6.99	2.74 (0.39)[b]
1	7.88	2.80 (0.36)
2	6.87	2.93 (0.43)
4	7.90	2.51 (0.32)
6	7.13	2.63 (0.37)
(C) ATP Synthesis via Oxidative Phosphorylation[d]		
0.5	0.140	0.046 (0.33)[e]
1.5	0.149	0.048 (0.32)

[a] Same as footnote *a* of Table I. [b] Same as footnote *b* of Table I. [c] Same as footnote *c* of Table I. [d] Same as footnote *a* of Table IV. [e] Ratio of the oxidative phosphorylation rate of N-NBD-labeled particles to that of the control MF_1-ASU.

chondrial particles. Table VI shows that for (N-NBD)$_{1.18}$$MF_1$-ASU the effects of preincubation time on observed S.A. for ATP hydrolysis, assayed either under hydrolysis conditions or under simulated oxidative phosphorylation conditions, and for ATP synthesis, respectively, are qualitatively the same as on the values in Tables I and IV. Therefore, the data in Table VI also support the above interpretation.

Discussion

The present results show that the ATPase activities of DCCD-labeled and N-NBD-labeled submitochondrial particles assayed in the usual procedure increase slowly with preincubation time without losing the covalent label. Unless one adopts the highly improbable assumption that the DCCD-labeled carboxyl group, the N-NBD-labeled amino group, and the O-NBD-labeled phenolic group are all nonessential for the catalytic mechanism, it seems very difficult to reconcile this observation with the alternating three-site model (Gresser et al., 1982), or any mechanistic model which requires the sequential action of three identical sites for ATP hydrolysis and synthesis, for reasons already mentioned in the introduction.

In view of the available information on the structure of MF_1-ATPase (Amzel et al., 1982), it seems justifiable to intrepret the present results in terms of a hypothetical model with one active and two latent catalytic sites in the following way. Let us assume that both ATP hydrolysis and ATP synthesis take place at the active catalytic site of one specially positioned β-subunit which interacts with the smaller subunits as well as with the two latent catalytic sites in a unique way and that the catalytic properties of this active catalytic site can be affected by the state of ligation of the latent catalytic sites as well as the regulatory sites (Minkov et al., 1980; Cross et al., 1982; Tamura & Wang, 1983).

For MF_1-ATPase in solution, the rate of subunit rearrangement is presumably so slow that catalytic hydrolysis of ATP takes place essentially at the same active site of the specially positioned β-subunit. Consequently, the whole enzyme is inactivated and stays inactivated when one of the essential functional groups of this specially positioned β-subunit is selectively labeled.

In submitochondrial particles reconstituted with either native MF_1 or selectively labeled MF_1, an intramolecular subunit rearrangement could take place at an appreciable rate so that the active state of a labeled β-subunit may become a latent state and the latent state of an unlabeled β-subunit may become an active state. In this way the selectively labeled enzyme could regain some of the lost catalyst activity without losing its covalent label. The extent of this type of spontaneous reactivation of covalently labeled proton ATPase would be limited by the fraction of its β-subunits that is not labeled. But since the standard free energy change for a labeled β-subunit to switch from an active to latent state may not be the same as that for an unlabeled β-subunit, the equilibrium ratio of the specific activity of the labeled submitochondrial particles to that of the control particles could differ from the simple statistical value.

Under normal conditions of ATP hydrolysis, the rate of subunit rearrangement may be quite slow, so could be the rate of spontaneous reactivation. But during the 3-min homogenization in actual or simulated oxidative phosphorylation medium, rearrangement could be so accelerated that the specific activities of the selectively labeled submitochondrial particles for oxidative phosphorylation and ATP hydrolysis, respectively, could both be increased to their equilibrium or steady-state values within the 3-min respiration period irrespective of the duration of preincubation. This acceleration of reactivation under oxidative phosphorylation conditions may be responsible for the intriguing difference between the observed S.A. ratio of the labeled particles to that of the unlabeled particles for oxidative phosphorylation and the corresponding S.A. ratio observed normally for ATP hydrolysis at zero preincubation time (Steinmeier & Wang, 1979; Kohlbrenner & Boyer, 1982).

Within experimental uncertainties, ATP hydrolysis by the labeled particles after treatment with simulated oxidative phosphorylation medium and oxidative phosphorylation by the labeled particles without pretreatment are both enhanced by about the same factor relative to the corresponding S.A. ratio observed normally for ATP hydrolysis at zero preincubation time. This finding substantiates the widely accepted assumption that both the hydrolysis and synthesis of ATP take place at the same catalytic site.

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Registry No. ATPase, 9000-83-3; DCCD, 538-75-0; NBD-Cl, 10199-89-0.

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Purification and Characterization of a Long-Chain Acyl Coenzyme A Thioesterase from *Rhodopseudomonas sphaeroides*†

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ABSTRACT: A long-chain acyl coenzyme A thioesterase has been purified over 10000-fold (38% yield) from photoheterotrophically grown cells of the facultative phototrophic organism *Rhodopseudomonas sphaeroides*. This enzyme, designated thioesterase I, has a native molecular mass (M_r) of 22400 as estimated by gel filtration and apparently consists of two subunits of M_r 12500 each as revealed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The enzyme will only hydrolyze acyl thio esters of coenzyme A (CoA) and displays a strict specificity for acyl coenzyme A substrates where the acyl moiety is $\geq C_{12}$. Palmitoyl coenzyme A and stearoyl coenzyme A are the preferred saturated acyl-CoA substrates while vaccenoyl coenzyme A is the preferred unsaturated substrate. The purified enzyme has a pH optimum of 8.0, is stabilized by 25% (w/v) glycerol, and is inhibited (90%) by treatment with 10 μM diisopropyl fluorophosphate. The physical and biochemical properties of the enzyme resemble those reported for the *Escherichia coli* low molecular weight thioesterase, and it is proposed that the *R. sphaeroides* thioesterase participates in the cellular mechanism for the direct utilization of exogenously supplied fatty acids for membrane phospholipid biosynthesis.

The cytosolic fraction of cells of *Escherichia coli* contains two distinct acyl-CoA[1] thioesterases, designated thioesterases I and II,[2] which are easily distinguished by size, sensitivity to inhibition by DFP, and substrate specificity (Kass et al., 1967; Barnes et al., 1970; Barnes & Wakil, 1968; Bonner & Bloch, 1972). The functions of these enzymes in the lipid metabolism of *E. coli* are unknown. In bacteria such as *E. coli*, which only possess a type II fatty acid synthetase (Bloch & Vance, 1977), the intermediates and products of fatty acid synthesis occur covalently attached to ACP (Vagelos, 1971; Prescott & Vagelos, 1972; Bloch & Vance, 1977), and the freely dissociable acyl-ACP products of de novo fatty acid synthesis are known to efficiently serve as immediate substrates for the bacterial *sn*-glycerol-3-phosphate acyltransferase, the enzyme that catalyzes the first committed step in bacterial phospholipid synthesis (Ailhaud & Vagelos, 1966; van den Bosch & Vagelos, 1970; Goldfine et al., 1967; Goldfine & Ailhaud, 1971; Lueking & Goldfine, 1975a; Cronan, 1978; Rock & Cronan, 1982). Thus, under conditions where cellular fatty acids are being produced de novo, a need for an acyl-CoA thioesterase, as well as for the acyl-CoA substrates themselves, is not readily apparent and not surprisingly, an intracellular pool of these intermediates has not been detected in *E. coli* (Klein et al., 1971; Frerman & Bennett, 1973; Rock & Jackowski, 1982).

The ability of bacteria to directly utilize exogenously supplied fatty acids for phospholipid synthesis suggests a specific function for acyl-CoA thioesterases in the lipid metabolism of procaryotes. Studies conducted with *E. coli* have shown that fatty acid transport is accompanied by an activation of the fatty acid to its acyl-CoA form (Overath et al., 1969; Klein et al., 1971; Frerman & Bennett, 1973). Thus, if one assumes that the direct utilization of exogenous fatty acids for phospholipid synthesis requires the entry of the fatty acids into an intracellular acyl-ACP pool, a conversion of acyl-CoA to acyl-ACP must occur and could result from the combined actions of an acyl-CoA thioesterase and the more recently identified acyl-ACP synthetase (Ray & Cronan, 1976; Spencer et al., 1978; Rock & Cronan, 1979). Whether such a process actually occurs in *E. coli*, however, is unclear, since in vitro studies have shown that this organism's *sn*-glycerol-3-phosphate acyltransferase can independently utilize both acyl-CoA and acyl-ACP substrates (van den Bosch & Vagelos, 1970;

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[1] Abbreviations: ACP, acyl carrier protein (reduced form); acyl-ACP, acylated acyl carrier protein; BSA, bovine serum albumin; CoA, coenzyme A; acyl-CoA, acyl coenzyme A; cmc, critical micellar concentration; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DFP, diisopropyl fluorophosphate; $NaDodSO_4$, sodium dodecyl sulfate; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; Tris, tris(hydroxymethyl)aminomethane.

[2] For the reasons previously discussed by Spencer et al. (1978), we have chosen to utilize the nomenclature of Barnes et al. (1970) for the designations of the *Escherichia coli* acyl-CoA thioesterases.

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