Catalytic Hydrolysis and Synthesis of Adenosine 5′-Triphosphate by Stereoisomers of Covalently Labeled F_1-Adenosinetriphosphatase and Reconstituted Submitochondrial Particles†

Jui H. Wang,* Jennifer Cesana,‡ and Joe C. Wu

Bioenergetics Laboratory, State University of New York, Buffalo, New York 14214-3094

Received February 20, 1987; Revised Manuscript Received April 23, 1987

ABSTRACT: Bovine heart F_1-adenosinetriphosphatase (F_1) was labeled specifically and precisely with 7-chloro-4-nitro-2,1,3-[^{14}C]benzoxadiazole ([^{14}C]NBD-Cl). The stereospecifically labeled F_1 (*O*-β'-[^{14}C]-NBD-F_1) was partially reactivated by LiCl treatment, which could cause rearrangement of the β subunits to form *O*-β',β''-[^{14}C]NBD-F_1. Both labeled enzymes were used to combine with F_1-deficient submitochondrial particles (ASU) to form the reconstituted particles *O*-β'-NBD-F_1–ASU and *O*-β',β''-NBD-F_1–ASU, respectively. A comparison of the observed steady-state rates of catalytic ATP hydrolysis and oxidative phosphorylation by these specifically labeled submitochondrial particles (SMP) with those of the unlabeled control samples suggests that oxidative phosphorylation involves more active sites of F_1 than catalytic ATP hydrolysis. A comparison of the observed ATPase activity of uncoupled labeled SMP and the activity for ATP-driven reverse electron transport in coupled labeled SMP with the corresponding values of the unlabeled control samples shows that the observed fractional inhibition ATP hydrolysis is the same for both the coupled SMP and uncoupled SMP and is determined only by the state of stereospecific labeling of F_1. The effect of preincubation under simulated oxidative phosphorylation conditions on the ATPase activity of the unperturbed, specifically NBD-labeled submitochondrial particles was also examined. The data show that respiration-generated proton flux does not cause the β subunits in bovine heart proton-ATPase to continue switching places with each other during oxidative phosphorylation. Samples of NBD-F_1 with specific labels on its nonhydrolytic β'' subunits but none on its hydrolytic β' subunit were prepared by a three-cycle process. Both this specifically labeled enzyme, *O*-β''-NBD-F_1, and the unlabeled control, F_1, were used to reconstitute the submitochondrial particles. It was found that for the first time stereospecifically labeled submitochondrial particles have been prepared that exhibit a lower catalytic efficiency relative to the unlabeled control particles for ATP synthesis than for ATP hydrolysis. These results led to the following conclusions: (1) Due to interaction with the smaller subunits in MF_1, the three β subunits become functionally distinguishable. Efficient hydrolysis of ATP takes place at the directly active site of β' subunit when and only when proper ligands are bound at the indirectly active sites of β'' subunits. (2) Because of conformation changes driven by the proton flux, all three active sites of F_0F_1 catalyze ATP synthesis, though probably not with the same efficiency. (3) Internal rotation of $\alpha_3\beta_3$ relative to $\gamma\delta\epsilon$ did not occur during the steady-state oxidative phosphorylation in submitochondrial particles.

In the interpretation of experimental data for oxidative phosphorylation and ATP hydrolysis by submitochondrial particles (SMP),[1] it has often been assumed that the same active sites of F_1 catalyze both the synthesis and hydrolysis of ATP so that the sequence of F_1-catalyzed steps in one process is the exact reversal of that in the other. Accordingly, any covalent label on F_1 in the F_0F_1 complex that inhibits ATP hydrolysis is also expected to inhibit ATP synthesis. Although oxidative phosphorylation is measured under coupled conditions, whereas ATP hydrolysis is often measured under uncoupled conditions, the observed ratio *r* of the catalytic efficiency of the partially labeled F_0F_1 complex to that of the unlabeled enzyme complex should be the same for both phosphorylation and hydrolysis if the two processes involve the same number of catalytic sites in F_1. For example, if the presence of a proton gradient or uncoupling agent could affect the catalytic efficiency of F_1, it would change the efficiency of the unlabeled F_1 molecules in the control sample as well as in a partially labeled sample by exactly the same factor. Since all specifically labeled F_1 molecules are inactivated, the observed value of *r* should be equal to the fraction of F_1 molecules that remain unlabeled in the reconstituted SMP sample, which should be the same in both phosphorylation and hydrolysis measurements.

† This work was supported in part by General Medical Sciences Research Grant GM 31463.

* Author to whom correspondence should be addressed.

‡ Undergraduate Research Participant, Department of Chemistry.

[1] Abbreviations: AC, *N*-acetyl-L-cysteine; AP_5A, P^1,P^5-bis(5′-adenosyl) pentaphosphate; ASU, submitochondrial particles prepared from bovine heart mitochondria by sonication at pH 9 followed by steps involving urea treatment; BSA, bovine serum albumin; DCCD, dicyclohexylcarbodiimide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ETPH, phosphorylating electron-transport particles prepared in the presence of 5 mM $MgCl_2$ and 10 mM $MnCl_2$; F_1 or F_1-ATPase, F_1-adenosinetriphosphatase; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; G6P, glucose 6-phosphate; HBHM, heavy bovine heart mitochondria; HK, hexokinase; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; LDH, L-lactic dehydrogenase; *n*, molar ratio of label to F_1; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; *O*-NBD-F_1, F_1 labeled with NBD-Cl at its essential Tyr-β311 residue; OSCP, oligomycin-sensitivity-conferring protein; PEP, phosphoenolpyruvate; P_i, inorganic phosphate; PK, pyruvate kinase; PMS, phenazine methosulfate; *r*, ratio of the specific activity of the labeled enzyme to that of the unlabeled control; SMP, submitochondrial particles; Re-F_1, rearranged F_1; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/87/0426-5527$01.50/0

However, some published observations are difficult to reconcile with such a picture. Steinmeier and Wang (1979) showed that when submitochondrial particles are reconstituted by combining ASU particles with NBD-labeled F_1, their activities for ATP $\rightleftarrows$ P_i exchange, ATP-driven reverse electron transport, and membrane-bound ATPase are almost completely inhibited, but the remaining activity for net ATP synthesis is 35–65% of the control value. Subsequent studies by other investigators also showed that specific covalent inhibitors generally inhibit catalytic ATP hydrolysis much more than oxidative phosphorylation (Kohlbrenner & Boyer, 1982; Matsuno-Yagi & Hatefi, 1984; Soong & Wang, 1984).

In order to find the cause of the above discrepancy, submitochondrial particles have been reconstituted in this work by combining ASU particles with different stereoisomeric forms of covalently labeled F_1, and the steady-state rates of catalytic ATP hydrolysis, electron transfer, oxidative phosphorylation, and ATP-driven reduction of NAD^+ by succinate by the reconstituted SMP have been measured and compared with the corresponding rates of the control SMP.

Experimental Procedures

Materials

ATP, ADP, BSA, EDTA, NBD-Cl, NAD^+, NADH, $NADP^+$, *N*-acetyl-L-cysteine, rotenone, oligomycin, valinomycin, PMS, Hepes, Sephadex G-50-80, cytochrome *c* (equine), PEP, pyruvate kinase, lactic dehydrogenase, hexokinase, and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. FCCP was from Pierce Chemical Co.

[^{14}C]NBD-Cl was purchased from Research Chemicals International. An aliquot of this [^{14}C]NBD-Cl was converted to 7-(dimethylamino)-4-nitro-2,1,3-benzoxadiazole by reaction with an excess of dimethylamine and purified chromatography on a silica column (Wang et al., 1986). Spectrometric and radiochemical assays of the purified product gave a specific radioactivity of 93 ± 1 mCi/mmol for this shipment of [^{14}C]NBD-Cl.

Mitochondria were prepared from fresh bovine heart by the method of Löw and Vallin (1963). ETPH was prepared by the procedure of Beyer (1967). F_1-ATPase was prepared from frozen mitochondria and stored as described by Knowles and Penefsky (1972). ASU particles were prepared by a modified version of the procedure of Racker and Horstman (1967) as described below.

Heavy bovine heart mitochondria (HBHM) were prepared and stored in 0.25 M sucrose and 10 mM Tris-HCl (pH 7.4) medium at −70 °C (Beyer, 1967). The stored HBHM containing 600 mg of protein were homogenized at 2 °C with 40 mL of 2 mM Tris-HCl, 0.05 M sucrose, and 0.6 mM EDTA (pH 7.8 at 22 °C). The mixture was adjusted to pH 9.2 at 2 °C with 0.5 M NH_4OH. About half of the mixture was transferred to a 35-mL beaker, covered with parafilm, flushed with N_2, and chilled by immersion in a dry ice–acetone mixture until a thin layer of ice was formed along the inside wall of the beaker. The chilled mixture was then sonicated for 90 s at a 115-W power output with intermittent chilling periods to keep the temperature below 5 °C. The debris was then removed by centrifugation at 18000*g* for 10 min in a JA-20 rotor (12 400 rpm). The supernatant suspension was pipetted out and again centrifuged at 100000*g* in a Ti-75 rotor (40 000 rpm) for 45 min at 4 °C. The new pellets were suspended in 20 mL of 0.25 M sucrose and 10 mM Tris–SO_4 at pH 7.5 (22 °C) and 4 °C. The suspension was again centrifuged at 100000*g* for 45 min at 4 °C. The resulting pellets were resuspended in 15 mL of the same buffer to give a protein concentration of 10 mg/mL. This suspension was chilled to 2 °C and mixed with an equal volume of cold 0.1 M Tris–SO_4 (pH 8.0), 4 mM EDTA, and 4 M urea (freshly prepared) solution. After exactly 40 min of incubation at 2–4 °C, the mixture was centrifuged at 163000*g* in a Ti-75 rotor (50 000 rpm) for 10 min at 4 °C. The resulting pellets were rinsed with cold 0.25 M sucrose, then homogenized with 4 mL of 0.25 M sucrose and 4 mM potassium succinate (pH 7.5) at a protein concentration of 20 mg/mL, and stored under liquid N_2. The ASU particles prepared by this procedure contain endogenous OSCP so that it is not necessary to add OSCP for their subsequent reconstitution with various forms of F_1.

Methods

Assay of Soluble Forms of F_1-ATPase. The ATPase activity was assayed by coupled oxidation of NADH in a medium containing 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, and 11 units/mL LDH. The steady-state rate of ATP hydrolysis was computed from the observed linear decrease of A_{340} due to the coupled oxidation of NADH at 30 °C, with 6220 as the molar absorbance of NADH. Protein concentrations were determined by the Coomassie Blue binding method (Bradford, 1976).

Labeling of F_1 by [^{14}C]NBD-Cl. In one experiment, 50 μL of a stock solution of [^{14}C]NBD-Cl in acetone was placed in a glass vial containing a Teflon-coated stirring bar and evaporated to dryness in a stream of N_2. A solution containing 10.8 mg of F_1 in 400 μL of buffer A (50 mM Hepes–NaOH, 2 mM EDTA, 25% glycerol, pH 7.0) was centrifugally filtered through Sephadex G-50-80 that had been preequilibrated with buffer A containing 5 mM ATP. The initial ATPase activity of F_1 in the filtrate was 71.4 μmol mg^{-1} min^{-1}. Half of the gel filtered F_1 solution was added to the invisible film of [^{14}C]NBD-Cl with the stirring bar rotating. The reaction was allowed to take place in the dark at 22 °C. The other half of the F_1 solution was kept under the same conditions as those for control. At intervals, 1-μL aliquots of the reaction mixture (containing 14.9 μg of F_1) were taken for ATPase assay. The reaction was terminated in about 1 h when the ATPase activity of the reaction mixture had dropped to about 5% of that of the control by centrifugal gel filtration through Sephadex G-50-80 that had been preequilibrated with buffer A containing 5 mM ATP. The control F_1 was treated similarly, and its ATPase activity at the end of the experiment was 72.3 μmol mg^{-1} min^{-1}. The radioactivity of the stereospecifically labeled enzyme *O*-β′-[^{14}C]NBD-F_1 was assayed by liquid scintillation counting, with counting efficiency determined by means of a [^{14}C]toluene internal standard supplied by New England Nuclear Corp. The molar ratio *n* of NBD label to F_1 was calculated by using a molecular weight of 3.5×10^5 for F_1 and the specific radioactivity of the [^{14}C]NBD-Cl (93 ± 1 mCi/mmol).

Preparation of F_1 with Rearranged Labeled β Subunits (Re-F_1). It was shown that the intrinsically identical β subunits of bovine heart F_1-ATPase are functionally distinct, probably due to different subunit interactions (Wang, 1985; Wang et al., 1986). When the highly inhibited enzyme with specific covalent label on its catalytic β subunit was forced to rearrange by LiCl treatment, its ATPase activity could be increased 20-fold without losing its covalent label. For this work, the rearranged enzyme *O*-β′,β″-[^{14}C]NBD-F_1 was prepared by adding buffer A solution at 0 °C containing 6–15 mg of the enzyme/mL and 5 mM ATP to one-third its volume of vigorously shaken 12 M LiCl solution in buffer A at 0 °C. After

3.5 min at 0 °C, the mixture was centrifugally filtered at 25 °C through Sephadex G-50-80 that had been preequilibrated with buffer A containing 5 mM ATP. The rearranged enzymes were either used immediately or stored under liquid N_2.

Reconstitution of Submitochondrial Particles (SMP). In a typical experiment, 2.4 mg (protein) of ASU particles were mixed with 0.9 mg of F_1 in 255 μL of 50 mM Hepes–NaOH buffer (pH 7.5) containing 0.25 M sucrose, 2 mM EDTA, and 6 mM ATP. After the mixture was incubated for 7 min at 30 °C, 0.60 μg of oligomycin in 6 μL of ethanol solution (0.25 μg of oligomycin/mg of ASU) was added, followed by 7.8 μL of 0.1 M $MgCl_2$ solution (final $[Mg^{2+}]$ = 3 mM). After 7 min at 25 °C, the mixture was centrifuged for 7 min at 130000*g* in a Beckman Airfuge. The pellets were freed from the supernatant and homogenized with 140 μL of buffer B (10 mM KP_i, 20 mM potassium succinate, 0.25 M sucrose, 20 mM glucose, and 0.2 mM EDTA, pH 7.5). Like ETPH, this suspension of reconstituted SMP can be stored under liquid N_2 for months without noticeable change in its catalytic activities.

Assay of ATP Hydrolysis Catalyzed by SMP. The steady-state rate of ATP hydrolysis catalyzed by SMP was also measured by coupled oxidation of NADH as described above for the soluble enzyme, except that the assay medium for this case also contained 1.5 mM KCN to suppress respiration and 12.5 μM FCCP to dissipate the proton gradient. Figure 1A shows that the absorbance at 340 nm became a linear function of time about 40 s after the injection of SMP and vigorous shaking with a Vortex mixer.

Assay of ATP-Driven Reverse Electron Transport. ATP-driven reverse electron transport was measured by monitoring the increase in A_{340} due to the reduction of NAD^+ by succinate (Ernster & Lee, 1967). The assay medium contained 50 mM Hepes–NaOH (pH 7.5), 0.25 M sucrose, 3 mM $MgCl_2$, 10 mM KCl, 6.7 mM potassium succinate, 3 mM ATP, 10 mM PEP, 21 units/mL PK, 1 mM NAD^+, 11 units/mL LDH, and 2 mg/mL defatted BSA. In measurements of reverse electron transport and oxidative phosphorylation with coupled SMP, the addition of BSA improved the rates slightly but was not essential. In measurements of ATPase and electron transport with FCCP-uncoupled SMP, BSA had no effect on the rates and hence was omitted. Each assay was started by injecting 10 μL of 1 M KCN into 3 mL of the medium containing 0.25–0.60 mg (total protein) of the reconstituted SMP. The rate of reduction of NAD^+ by succinate was computed from the linear portion of the recorder trace. After about 10 min, 80 μL of 0.3 mM rotenone in ethanol solution was injected in the mixture to stop the reaction. In every assay, A_{340} became constant within 10 s after the injection of rotenone and stayed constant for at least 10 min before the measurement was terminated.

Assay of Oxidative Phosphorylation. The steady-state rates of oxidative phosphorylation were monitored continuously in an ADP-regenerating system coupled to the reduction of NAD^+. The assay medium contained 10 mM KP_i, pH 7.5, 20 mM potassium succinate, 0.25 M sucrose, 20 mM glucose, 3 mM $MgCl_2$, 0.2 mM EDTA, 1 mg of defatted BSA/mL (may be omitted), 3 μM rotenone, and 0.4 mM $NADP^+$. Shortly before the start of each assay, ADP (0.1 or 0.2 mM), AP_5A (0.05 or 0.10 mM), HK (40 units/mL, sulfate-free), and G6P-dehydrogenase (8 units/mL, sulfate-free) were injected into 2 mL of the above assay medium at 30 °C. The reaction was started by injecting 5 μL of SMP (50–100 μg of total protein) followed by vigorous shaking for 3 s with a Vortex mixer and was promptly monitored continually for the

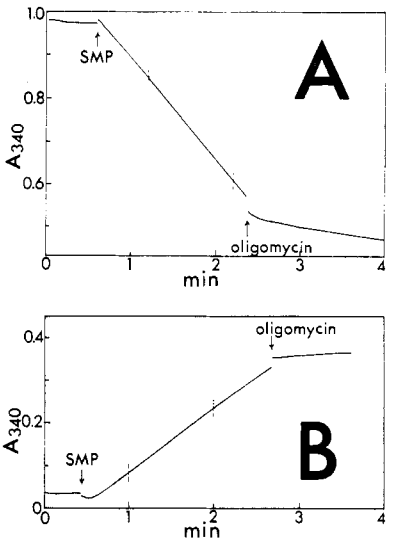

FIGURE 1: Continuous monitoring of catalytic ATP hydrolysis and oxidative phosphorylation by reconstituted submitochondrial particles. (A) Catalytic ATP hydrolysis by reconstituted SMP. Assay medium: 50 mM Hepes–NaOH, pH 8.0, 3 mM $MgCl_2$, 50 mM KCl, 2 mM ATP, 2 mM PEP, 0.4 mM NADH, 21 units/mL PK and 11 units/mL LDH, total volume 2.00 mL, 30 °C. Shortly before the assay 5 μL of 5 mM FCCP in ethanol and 10 μL of 0.3 M KCN were added and mixed. At the indicated time 2 μL of reconstituted SMP (15.3 mg/mL) was injected into the medium and vigorously shaken. The linear segment of the recorder trace (between the two vertical broken lines) was used to compute the specific ATPase activity of the FCCP-uncoupled SMP. (B) Oxidative phosphorylation by reconstituted SMP. Assay medium: 10 mM potassium phosphate, 20 mM potassium succinate, 0.25 M sucrose, 20 mM glucose, 3 mM $MgCl_2$, 0.2 mM EDTA, 1 mg of defatted BSA/mL, 3 μM rotenone, 0.4 mM $NADP^+$, pH 7.5. Shortly before the assay, ADP (0.2 mM), AP_5A (0.10 mM), HK (40 units/mL, sulfate-free), and G6P-dehydrogenase (8 units/mL, sulfate-free) were injected into 2 mL of the above assay medium at 30 °C. The reaction was started by injecting 10 μL of reconstituted SMP (15.3 mg/mL) at the indicated time, followed by vigorous shaking for 3 s with a Vortex mixer. The steady-state phosphorylation rate was computed from the linear section of the recorder trace between the two vertical broken lines.

increase of A_{340} with time due to the coupled reduction of $NADP^+$ by G6P. The steady-state rate was computed from the linear portion of the recorder trace, with 6220 as the molar absorbance of NADP. A typical example of such a measurement is shown in Figure 1B.

Since SMP usually has adenylate kinase activity, it was necessary to add AP_5A to the medium as an inhibitor (Lienhard & Secemski, 1973). Assays with medium ADP concentration above 0.2 mM were avoided because of the incomplete suppression of the adenylate kinase reaction by a low concentration of AP_5A (0.1 mM).

Reconstitution of Submitochondrial Particles with F_1 Containing NBD-Labeled β″ Subunits and Unlabeled β′ Subunit. A 600-μL sample of Tyr-β′311-[^{14}C]NBD-F_1 was prepared in the dark as described under Methods. It had the following characteristics: c = 16.9 mg of protein/mL; ATPase activity (μmol of ATP min^{-1} mg^{-1}) = 1.24 (–DTT), 52.6 (+2.5 mM DTT); r = 0.02; n = 1.03. A 210-μL aliquot of this sample was scrambled by 70 μL of 12 M LiCl at 0 °C for 3.5 min. The LiCl was then removed immediately by centrifugal gel filtration at room temperature through Sephadex G-50-80 that had been preequilibrated with buffer A containing 10 mM ATP. Characteristics of the once-scrambled sample: c = 8.66 mg/mL; ATPase activity = 33.4 (–DTT), 51.3 (+2.5 mM DTT); r = 0.65; n = 1.00. This sample was gel filtered to remove ATP, again labeled with [^{14}C]NBD-Cl in the dark, then gel filtered to remove excess [^{14}C]NBD-Cl, and again

Table I: Oxidative Phosphorylation and Catalytic ATP Hydrolysis by ETPH and Reconstituted F_1–ASU at 30 °C[a]

sample	additional reagent	steady-state rates: ATP synthesis (nmol mg^{-1} min^{-1})	steady-state rates: ATP hydrolysis (μmol mg^{-1} min^{-1})
ETPH	none	254	1.20
ETPH	1.5 mM KCN	1.3	
ETPH	5 μM FCCP	0.0	
ETPH	5 μM valinomycin	6.7	
ETPH	5 μg of oligomycin/mL	0.0	0.041
F_1–ASU	none	160	2.49
F_1–ASU	none	148	
F_1–ASU	2.5 μg of oligomycin/mL	7.9	0.33
F_1–ASU	5 μg of oligomycin/mL		0.00

[a] Compositions of the assay media are given under Experimental Procedures. The concentrations of ADP in the oxidative phosphorylation media are 0.1 mM for ETPH and 0.2 mM for F_1–ASU.

made 10 mM in ATP. Characteristics of the twice-labeled sample: c = 7.31 mg/mL; ATPase activity = 0.86 (–DTT), 57.6 (+DTT); r = 0.01; n = 1.63. This 390-μL sample was again scrambled by 130 μL of 12 M LiCl at 0 °C for 3.5 min and gel filtered as before. Characteristics of the rescrambled sample: c = 3.24 mg/mL; ATP activity = 16.4 (–DTT), 37.6 (+2.5 mM DTT); r = 0.44; n = 1.52. This rescrambled sample was again gel filtered to remove ATP, labeled for a third time by [^{14}C]NBD-Cl, and again made 10 mM in ATP. Characteristics of the three times labeled sample: c = 3.20 mg/mL; ATPase activity = 0.40 (–DTT), 46.2 (+2.5 mM DTT); r = 0.01; n = 2.00. The resulting 510-μL sample was again scrambled with 170 μL of 12 M LiCl as before. Characteristics of the three times scrambled sample: c = 1.65 mg/mL; ATPase activity = 9.35 (–DTT); 19.5 (+2.5 mM DTT); r = 0.48; n = 1.85.

The three times scrambled sample was gel filtered to remove ATP. The filtrate (750 μL) was mixed with 1.8 μL of 8 mM *N*-acetyl-L-cysteine (AC), and the mixture was kept in the dark at room temperature (final [AC] = 20 μM). After 68 min of reaction time, the solution was gel filtered to remove AC, made 10 mM in ATP, and assayed. Characteristics: c = 1.17 mg/mL; ATPase activity = 20.6 (–DTT), 35.7 (+2.5 mM DTT); r = 0.58; n = 1.72. The sample was again gel filtered to remove ATP and again reacted with 20 μM AC in the dark at room temperature. After 93 min, the solution was again gel filtered, made 10 mM in ATP, and assayed. Characteristics: c = 0.92 mg/mL; ATPase activity = 28.3 (–DTT), 28.0 (+2.5 mM DTT); r = 1.0; n = 1.30. The final product of this labeled F_1-ATPase may be represented by the formula (Tyr-β''311-[^{14}C]NBD)$_{1.3}F_1$. The value of r and n determined at each step in the entire preparative procedure is summarized schematically in Figure 2. Another independent batch of this type of specifically labeled F_1 was prepared with the formula (Tyr-β''311-[^{14}C]NBD)$_{2.1}F_1$.

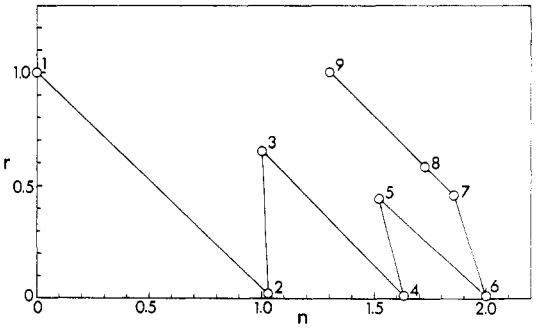

FIGURE 2: Changes in r and n during the preparation of (Tyr-β''311-[^{14}C]NBD)$_{1.3}F_1$. The numbers indicate the sequence of steps in the preparative procedure from the starting F_1-ATPase represented by 1 to the final product represented by 9 as described under Experimental Procedures.

RESULTS

Characterization of Reconstituted Submitochondrial Particles. For comparison, the observed steady-state rates of oxidative phosphorylation using succinate as the substrate and of catalytic ATP hydrolysis by ETPH and by the reconstituted submitochondrial particles F_1–ASU are listed in Table I. The observed inhibition of oxidative phosphorylation in ETPH by low concentrations of KCN, FCCP, valinomycin, and oligomycin justifies the use of the present assay method, which is much faster than the conventional [^{32}P]P_i method. It also gives the instantaneous rate as a continuous function of reaction time up to 15–20 min before the concentration of dissolved O_2 falls below the K_d value. The rate of oxidative phosphorylation could be further increased by raising the ADP concentration, but that would also require higher concentration of AP_5A in the medium, which would be unnecessarily costly for the purpose of this work.

As expected, the reconstituted F_1–ASU have a lower rate of oxidative phosphorylation but a higher rate of catalytic ATP hydrolysis than ETPH, but both rates are still very sensitive to oligomycin.

Catalytic Hydrolysis and Oxidative Phosphorylation by Reconstituted Submitochondrial Particles. Since the labeling of F_1-ATPase by NBD-Cl in the dark is highly specific (Ferguson et al., 1975; Wang et al., 1986) and for $n < 1$ is almost exclusively on Tyr-β311 (Andrews et al., 1984; Sutton & Ferguson, 1985), a careful study of *O*-β'-NBD-F_1, *O*-β',-β''-NBD-F_1, and the reconstituted SMP may resolve the discrepancy discussed above. The catalytic activities of (*O*-β'-NBD)$_{0.97}F_1$, (*O*-β',β''-NBD)$_{0.95}F_1$, the control F_1 samples,

Table II: Catalytic Activities of [^{14}C]NBD-Labeled F_1 and Reconstituted Submitochondrial Particles at 30 °C[a]

soluble enzyme	Soluble Enzymes: F_1	*O*-β'-NBD-F_1	Re-F_1	*O*-β',β''-NBD-F_1
ATP hydrolysis (μmol of ATP mg^{-1} min^{-1})	72.3	1.52	64.3	23.8
n	0	0.97 ± 0.01	0	0.95 ± 0.01
r	(1)	0.021	(1)	0.37
SMP	**Reconstituted SMP: F_1–ASU**	***O*-β'-NBD-F_1–ASU**	**Re-F_1–ASU**	***O*-β',β''-F_1–ASU**
ATP hydrolysis (μmol of ATP mg^{-1} min^{-1})	3.51 ± 0.09	0.432 ± 0.08	3.97 ± 0.04	1.38 ± 0.08
r	(1)	0.12	(1)	0.35
oxidative phosphorylation (nmol of ATP mg^{-1} min^{-1})	111 ± 5	48 ± 2	111 ± 12	82 ± 5
r	(1)	0.43	(1)	0.71

[a] The compositions of media and assay protocols are given under Experimental Procedures. Additional abbreviations: n, molar ratio of NBD label to F_1; Re-F_1, rearranged F_1; *O*-β',β''-NBD-F_1, rearranged NBD-F_1.

Table III: Effect of Oligomycin and Preincubation on the ATPase and Reverse Electron Transport Activities of Reconstituted SMP at 30 °C[a]

	Soluble Enzymes F_1[b]	*O*-β'-NBD-F_1[c]	*r*
ATPase [μmol of ATP min^{-1} (mg of protein)$^{-1}$]	48.6 ± 2.0	1.45 ± 0.01	0.030
	SMP Reconstituted without Oligomycin		
		F_1–ASU	*O*-β'-NBD-F_1–ASU	*r*
ATPase (–oligomycin) [μmol of ATP min^{-1} (mg of total protein)$^{-1}$]		5.15 ± 0.10	0.461 ± 0.004	0.090
reverse electron transport (–oligomycin) [nmol of NADH min^{-1} (mg of total protein)$^{-1}$]		7	<1	
reverse electron transport (+0.25 μg of oligomycin)[d]		29.4	2.78	0.095
	SMP Reconstituted with Oligomycin			
		F_1–ASU	*O*-β'-NBD-F_1–ASU	*r*
ATPase		4.02 ± 0.12	0.622 ± 0.009	0.155
reverse electron transport		31.8 ± 0.2	2.99 ± 0.08	0.094
ATPase (assayed after preincubation under oxidative phosphorylation conditions)[e]		4.08 ± 0.00	0.917 ± 0.023	0.225

[a] The compositions of media and assay protocols are given under Experimental Procedures. [b] The control F_1 was prepared by reacting the stereospecifically labeled F_1 (*O*-β'-NBD-F_1) with 10 mM DTT in the dark for 10 min at 26 °C. All preparations and subsequent assays were conducted in a darkened room to minimize O → N transfer of the NBD label. [c] The stereospecifically [^{14}C]NBD-labeled F_1-ATPase with $n = 1.03$ and $r = 0.02$ had been prepared from bovine heart mitochondrial F_1-ATPase and stored under liquid nitrogen. [d] Exactly 0.25 μg of oligomycin was added to each reconstituted SMP sample (>10 mg/mL) per milligram of total protein and incubated for 5 min at 26 °C before the aliquots were taken for assay. [e] Each 100-μL sample was shaken in simulated oxidative phosphorylation medium exposed to air for 12 min at 28 °C before the assay. Composition of the preincubation medium: Hepes–NaOH, 50 mM; sucrose, 175 mM; glucose, 35 mM; EDTA, 0.35 mM; potassium succinate, 20 mM; $MgCl_2$, 3 mM; ADP, 2 mM; rotenone, 3 mM; pH 7.5.

and the reconstituted SMP have been determined and summarized in Table II.

Table II also shows that the ratio, *r*, of the specific activity of the labeled SMP to that of the unlabeled control SMP is much higher for oxidative phosphorlyation than for catalytic ATP hydrolysis and that for phosphorylation the *r* values are less affected by LiCl treatment than those for hyrolysis. The *r* values are 0.43 and 0.71 for *O*-β'-NBD-F_1–ASU and *O*-β',β''-NBD-F_1–ASU, respectively. For the unperturbed samples *O*-β'-NBD-F_1–ASU and F_1–ASU, the value of *r* for oxidative phosphorylation (0.43) is clearly much higher than that for ATP hydrolysis (0.12). These data seem to suggest that oxidative phosphorylation may involve more active sites of F_1 than catalytic ATP hydrolysis, but the more decisive evidence presented in the next three sections is still required before a definite conclusion can be reached.

It may also be noticed that, for ATP hydrolysis, the value $r = 0.12$ for *O*-β'-NBD-F_1–ASU (Table II) is 6 times as large as $r = 0.021$ for *O*-β'-NBD-F_1 (Table I). This is partly due to the presence of a small amount of unlabeled F_1 in ASU particles. In the absence of oligomycin, the ATPase of ASU was about 0.24 μmol mg^{-1} min^{-1}. While this represents less than 7% of the ATPase activity of F_1–ASU, it could represent more than 50% of the ATPase activity of *O*-β'-NBD-F_1–ASU. A similar enhancement in the value of *r* was observed when DCCD-F_1 was reconstituted with ASU (Soong & Wang, 1984). It could also be due partly to a small fraction of enzyme molecules that underwent spontaneous rearrangement of its subunits during reconstitution.

Previous studies show (Wang, 1985; Wang et al., 1986) that only one of the three active sites on the β subunits of F_1-ATPase (β' site) is directly responsible for the hydrolysis of ATP, although its hydrolytic efficiency is dependent on ligand binding by the other two sites (β'' sites) due to interaction between the subunits (Grubmeyer et al., 1982; Cross et al., 1982; Gresser et al., 1982; Fellous et al., 1984). When F_1-ATPase is labeled with low concentrations of NBD-Cl in the dark and in the presence of ATP at pH 7, the first NBD label goes to Tyr-β'311 at the hydrolytic site because of its much higher reactivity and completely inhibits the ATPase activity of the labeled molecule (Ferguson et al., 1975). But when the β subunits are forced to rearranged by LiCl treatment, some of the labeled β' subunits in the hydrolytic state may switch places or roles with unlabeled β'' subunits in the nonhydrolytic state due to a change in their mode of interaction with the smaller subunits (Williams et al., 1984). Consequently, the enzyme may recover some of its initial ATPase activity without losing the label (Wang, 1985). The recent isolation of the geometric isomers of labeled F_1-ATPase with contrasting properties further substantiates this interpretation (Wang et al., 1986).

On the other hand, the above results would be expected if all three active sites on β subunits catalyze oxidative phosphorylation, even if they do not have the same catalytic efficiency. Such an assumed model would indeed predict the SMP reconstituted with (*O*-β'-NBD)$_{0.97}F_1$ to have a much higher normalized *r* value for oxidative phosphorylation than for ATP hydrolysis and the *r* value for phosphorylation to be less sensitive to LiCl treatment than that for catalytic ATP hydrolysis as observed (Table II).

ATP-Driven Reverse Electron Transport Catalyzed by Reconstituted Submitochondrial Particles. In order to explore the possible dependence of the catalytic efficiency of the F_0F_1 complex on the state of energization of SMP, the rate of catalytic ATP hydrolysis by reconstituted SMP, both directly in uncoupled SMP and indirectly through the coupled reduction of NAD^+ with succinate by coupled SMP, has been measured. The results, which are summarized in Table III, show that the ratio, *r*, of the specific activity of the labeled enzyme complex to that of the unlabeled control is essentially independent of the state of energization of the reconstituted SMP.

Effect of Preincubation under Oxidative Phosphorylation Conditions on the ATPase Activity of O-β'-NBD-F_1–ASU. There is the possibility that each F_1-ATPase molecule has only one catalytic site for both hydrolysis and phosphorylation, but the respiration-generated proton flux causes the β subunits to swtich roles continually so that all three β subunits catalyze steady-state oxidative phosphorylation. In order to test this possibility, unperturbed (*O*-β'-NBD)$_{0.97}F_1$–ASU was stirred gently in simulated oxidative phosphorylation medium for 12 min in the dark at 28 °C and then assayed for ATPase activity in the usual way. If the respiration-generated proton flux had indeed caused the β subunits in membrane-bound F_1-ATPase to switch roles, the preincubated sample should exhibit a value of *r* for its ATPase activity similar to that for its oxidative

Table IV: Catalytic Activities of Submitochondrial Particles Reconstituted from (Tyr-β″311-[^{14}C]NBD)$_n$F$_1$ and ASU[a]

SMP	n	control "unlabeled" F$_1$–ASU	(*O*-β″-[^{14}C]-NBD)$_n$F$_1$–ASU
ATP hydrolysis (μmol of ATP mg^{-1} min^{-1})	1.3	1.88	1.84 r = 0.98
	2.1	2.51	2.49 r = 0.99
oxidative phosphorylation (nmol of ATP mg^{-1} min^{-1})	1.3	269	166 r = 0.61
	2.1	156	91 r = 0.58

[a] The compositions of media and assay procedures are the same as those for Table II. All measurements were conducted at 30 °C.

phosphorylation activity. The results listed in the last line of Table III show that this is not the case. The small increase in r observed after preincubation is similar in magnitude to that due to slow spontaneous reactivation reported previously (Soong & Wang, 1984).

ATP Hydrolysis and Oxidative Phosphorylation by Reconstituted SMP Containing NBD-Labeled β″ Subunits and Unlabeled β′ Subunit. In order to conduct a more direct test of the possibility that more active sites of F$_1$-ATPase may be involved in oxidative phosphorylation than in catalytic ATP hydrolysis, F$_1$-ATPase with [^{14}C]NBD-labeled Tyr-311 in β″ subunits and unlabeled β′ subunit was used to reconstitute the submitochondrial particles. Because of their low reactivity, the β″ subunits in F$_1$ cannot be labeled directly with [^{14}C]NBD-Cl with satisfactory specificity. The (Tyr-β″311-[^{14}C]-NBD)$_n$F$_1$ used for reconstitution with ASU was prepared by three cycles of a previously reported label–scramble–removal process (Wang et al., 1986) described under Methods.

A 100-μL sample of this (Tyr-β″311-[^{14}C]NBD)$_n$F$_1$ was mixed with 2.2 μL of 0.5 M DTT and incubated for 5 min. This DTT-treated sample was found to contain only 0.14 mol of NBD label per F$_1$ and was used as the "unlabeled" control F$_1$. Both *O*-β″-[^{14}C]NBD-F$_1$ and the unlabeled control F$_1$ were used to reconstitute with ASU particles and assayed as described under Methods. The results, with n = 1.3 and 2.1, respectively, show for the first time that the catalytic efficiency of covalently labeled F$_1$-ATPase relative to that of the unlabeled control F$_1$ can be lower for oxidative phosphorylation than for ATP hydrolysis (Table IV).

Several experiments were also performed to treat (Tyr-β″311-[^{14}C]NBD)$_{1.3}$F$_1$ again with LiCl to see whether the labeled subunit can be moved back from the β″ to the β′ position. Although the experimental errors became larger because of the further decrease in ATPase activity of the samples, the results indicate that r stays near 1 and hence the labeled subunit did not move back to the β′ position. These results suggest that LiCl-facilitated rearrangement of the β subunits is probably not merely a random-walk process but that, after the β′ subunit has been labeled by NBD-Cl, it may have a chemical preference for the β″ position.

Discussion

The data in Table III show that the observed values of r for catalytic ATP hydrolysis are not significantly affected by the state of energization of the reconstituted SMP. Essentially the same value of r was observed for ATP hydrolysis either directly in FCCP-uncoupled SMP or indirectly through reverse electron transport in oligomycin-recoupled SMP. The rate of catalytic ATP hydrolysis is lowered by the state of specific labeling by the same amount in both energized and de-energized SMP. Consequently, a comparison of the r values for ATP hydrolysis and oxidative phosphorylation by the same unlabeled and labeled SMP could provide some information on whether these two oppositely directed catalytic processes have exactly the same reaction path.

Values in Table II may be used for such a comparison. For the pair of samples F$_1$–ASU and *O*-β′-NBD-F$_1$–ASU, r = 0.12 for ATP hydrolysis; but for oxidative phosphorylation, r = 0.43. A direct comparison of the basal respiration rates at 30 °C has been made previously with succinate as the substrate (Steinmeier & Wang, 1979). The observed rates are 457 ± 16 and 464 ± 12 ng (atoms of O) mg^{-1} min^{-1} for F$_1$–ASU and *O*-β′-NBD-F$_1$–ASU, respectively. Respiration of F$_1$–ASU in the presence of ADP (5 mM), 2,4-dinitrophenol (0.3 mM), and FCCP (0.5 μM) was stimulated by 1.02-, 1.10-, and 1.09-fold respectively. Similar measurements on *O*-β′-NBD-F$_1$–ASU gave stimulation ratios of 0.97, 1.06, and 1.12, respectively. These results indicate that, in the oxidative phosphorylation experiments of this work, respiration rate is always essentially maximum and invariant in both labeled and unlabeled SMP.

The observed higher value of r for oxidative phosphorylation than for ATP hydrolysis suggests that the two catalytic processes might not have precisely the same reaction path as it is assumed in the alternating sites model (Gresser et al., 1982). An attempt was made to defend the alternating-sites model with a single catalytic route for both the synthesis and hydrolysis of ATP by postulating that because of different conformational changes the NBD label inhibits the rate-limiting step in hydrolysis without hindering the rate-limiting step in phosphorylation. However, since all β subunits are assumed to be equivalent according to this model, we would not expect the value of r to change after the labeled F$_1$ has been rearranged by LiCl treatment. Table II shows that, with the pair of rearranged samples Re-F$_1$–ASU and *O*-β′,β″-F$_1$–ASU, the value of r for ATP hydrolysis increased from 0.12 to 0.35. On the other hand, according to the other model (Wang, 1985), treatment with LiCl could cause the labeled subunit in the catalytic position (β′) to change place with an unlabeled subunit in the auxiliary or regulatory position (β″) and hence could lead to partial reactivation of the ATPase without losing the covalent label. That the r value of untreated samples is higher for oxidative phosphorylation and is enhanced less by LiCl treatment suggests that the β″ subunits may also catalyze phosphorylation driven by the proton flux, although they could catalyze with lower efficiency.

An alternative interpretation would be to assume that only β′ catalyzes both the hydrolysis and synthesis of ATP, but that during oxidative phosphorylation the β subunits were scrambled by proton flux so that both the labeled and unlabeled β subunits had their turn at the catalytic position (β′) and the auxiliary positions (β″). Consequently, the r value of the untreated samples for oxidative phosphorylation should be higher than that for ATP hydrolysis and should be enhanced less by further treatment with LiCl. In order to check this possibility, the reconstituted SMP were preincubated under simulated oxidative phosphorylation conditions and subsequently assayed for ATPase activity. Table II shows that the observed value r = 0.23 for catalytic ATP hydrolysis by submitochondrial particles that had been preincubated for 12 min under simulated oxidative phosphorylation conditions is higher than the value r = 0.16 for the unpreincubated samples only by a factor of 1.4. This increase in r by preincubation is about the same as that observed in previous experiments with DCCD-labeled SMP (Soong & Wang, 1984). On the other hand, the value r = 0.43 for oxidative phosphorylation is higher

than that for ATP hydrolysis by the same unpreincubated samples by a factor of 3.6. Had the β subunits indeed switched roles in each catalytic cycle, we would expect the r for ATP hydrolysis by the preincubated samples also to increase by a factor of 3.6 because the F_0F_1 complex turned over 40 000 times during the 12-min preincubation period. These results enable us to conclude that the β' and β'' subunits did not switch roles during oxidative phosphorylation.

Decisive evidence for the conclusion that the β'' subunits in F_0F_1 also catalyze ATP synthesis driven by the proton flux has been provided by experiments with SMP reconstituted with F_1 containing NBD-labeled β'' subunits and unlabeled β' subunit (Table IV). Since after scrambling with LiCl, the [^{14}C]NBD label in F_1 is still covalently attached to Tyr-β311 (Andrew et al., 1984; Wang et al., 1986), we may conclude from the r values in Table IV that the NBD label attached to Tyr-β''311 inhibits oxidative phosphorylation but does not inhibit catalytic ATP hydrolysis in the reconstituted submitochondrial particles. This implies that although only the β' subunit catalyzes ATP hydrolysis directly, both β' and β'' subunits catalyze oxidative phosphorylation.

If all three β subunits catalyze ATP synthesis with equal efficiency, the expected value of r for (Tyr-β''311-NBD)$_{1.3}F_1$-ASU should be $r = (3 - 1.3)/3 = 0.57$. The observed value of $r = 0.61$ could be because β' subunit catalyzes ATP synthesis with a slightly higher efficiency.

It is not clear how all three β subunits of F_1 in the F_0F_1 complex can catalyze ATP synthesis, whereas only the β' subunit catalyzes efficient ATP hydrolysis directly. One possibility is that interactions between the subunits could provide the catalytic site on β' with a unique mechanism for the rapid formation or decomposition of certain necessary intermediates along the reaction path or for the rapid release of products during the steady-state hydrolysis of ATP (Gresser et al., 1982), whereas during oxidative phosphorylation the same formation or decomposition or release could be effected rapidly by conformation changes driven by the proton flux so that all three sites can function effectively.

It has been suggested that the functional differentiation of the intrinsically identical β subunits for steady-state ATP hydrolysis may be due to the closer interaction of a particular $\alpha\beta$ pair ($\alpha'\beta'$) that interacts more closely with the smaller subunits $\gamma\delta\epsilon$ (Williams et al., 1984; Soong & Wang, 1984; Stan-Lotter & Bragg, 1986; Boekema et al., 1986; Wu et al., 1987). Since preincubation under simulated oxidative phosphorylation conditions did not cause the β' and β'' subunits to switch roles (Table III), we may conclude that the $\alpha_3\beta_3$ moiety of F_1 did not rotate relative to the $\gamma\delta\epsilon$ moiety during oxidative phosphorylation. Although such switching of roles could occur under other conditions, (e.g., during LiCl treatment) or in other energy-transducing membranes, the present results show that this type of internal rotation is not necessary for the coupling of proton flux to the synthesis of ATP.

ACKNOWLEDGMENTS

The mitochondria and F_1-ATPase used in this work were prepared in our laboratory by Betty Stone. The specific radioactivity of the [^{14}C]NBD-Cl was redetermined in our laboratory by Dr. Vijay Joshi.

REFERENCES

Andrews, W. M., Hill, F. C., & Allison, W. S. (1984) *J. Biol. Chem. 259*, 14378–14382.
Beyer, R. E. (1967) *Methods Enzymol. 10*, 189–190.
Boekema, E. J., Berden, J. A., & van Heel, M. G. (1986) *Biochim. Biophys. Acta 851*, 353–360.
Bradford, N. M. (1976) *Anal. Biochem. 72*, 248–257.
Cross, R. L., Grubmeyer, C., & Penefsky, H. S. (1982) *J. Biol. Chem. 257*, 12101–12105.
Ernster, L., & Lee, C. P. (1967) *Methods Enzymol. 10*, 729–738.
Fellous, G., Godinot, C., Baubichon, H., DiPietro, A., & Gautheron, D. C. (1984) *Biochemistry 23*, 5294–5299.
Ferguson, S. J., Lloyd, W. J., & Radda, G. K. (1975) *Eur. J. Biochem. 54*, 117–126.
Gresser, M. J., Myers, J. A., & Boyer, P. D. (1982) *J. Biol. Chem. 257*, 12030–12038.
Grubmeyer, C., Cross, R. L., & Penefsky, H. S. (1982) *J. Biol. Chem. 257*, 12092–12100.
Knowles, A. F., & Penefsky, H. S. (1972) *J. Biol. Chem. 247*, 6617–6623.
Kohlbrenner, W. E., & Boyer, P. D. (1982) *J. Biol. Chem. 257*, 3441–3446.
Lienhard, G. E., & Secemski, I. I. (1973) *J. Biol. Chem. 248*, 1121–1123.
Löw, H., & Vallin, I. (1963) *Biochim. Biophys. Acta 69*, 361–374.
Matsuno-Yagi, A., & Hatefi, Y. (1984) *Biochemistry 23*, 3508–3514.
Racker, E., & Horstman, L. L. (1967) *J. Biol. Chem. 242*, 2547–2551.
Soong, K. S., & Wang, J. H. (1984) *Biochemistry 23*, 136–141.
Stan-Lotter, H., & Bragg, P. D. (1986) *Arch. Biochem. Biophys. 248*, 116–120.
Steinmeier, R. D., & Wang, J. H. (1979) *Biochemistry 18*, 11–18.
Sutton, R., & Ferguson, S. J. (1985) *Eur. J. Biochem. 148*, 551–554.
Wang, J. H. (1985) *J. Biol. Chem. 260*, 1374–1377.
Wang, J. H., Joshi, V., & Wu, J. C. (1986) *Biochemistry 25*, 7996–8001.
Williams, N., Hullihen, J. M., & Pedersen, P. L. (1984) *Biochemistry 23*, 780–785.
Wu, J. C., Chuan, H., & Wang, J. H. (1987) *J. Biol. Chem. 262*, 5145–5150.