Determination of the Roles of Active Sites in F_1-ATPase by Controlled Affinity Labeling†

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ABSTRACT: The affinity reagents 3′-*O*-(5-fluoro-2,4-dinitrophenyl)[α-^{32}P]ATP (FDNP-[α-^{32}P]ATP) and 3′-*O*-(5-fluoro-2,4-dinitrophenyl)[8-^{14}C]ATP (FDNP-[^{14}C]ATP) were synthesized and used to characterize the structure and function of the three active sites in F_1-ATPase. FDNP-[α-^{32}P]ATP was found to bind covalently to F_1 up to two DNP-[α-^{32}P]ATP labels per F_1 in the absence of Mg^{2+} without decreasing the ATPase activity. However, when $MgCl_2$ was subsequently added to the reaction mixture, the enzyme could be further labeled with concomitant decrease in ATPase activity that is consistent with the complete inactivation of one enzyme molecule by an affinity label at the third ATP-binding site. Partial hydrolysis of the FDNP-[^{14}C]ATP-labeled enzyme and sequencing of the isolated peptide indicated that the affinity label was attached to Lys-β301 at all three active sites. Samples of F_1 with covalent affinity label on Lys-β301 were also used to reconstitute F_1-deficient submitochondrial particles. The reconstituted particles were assayed for ATPase and oxidative phosphorylation activities. These results show that the catalytic hydrolysis of ATP either by F_1 in solution or by F_0F_1 complex attached to inner mitochondrial membrane takes place essentially at only one active site, but promoted the binding of ATP at the other two active sites, and that ATP synthesis during oxidative phosphorylation takes place at all three active sites.

Kinetic data on the catalytic hydrolysis of ATP by F_1-ATPase in solution have frequently been interpreted by the well-known alternating sites model based on three or two strongly interacting but functionally equivalent β subunits in the enzyme molecule (Gresser et al., 1982; O'Neal & Boyer, 1984). This model is consistent with the fact that each F_1 molecule has three β subunits of identical amino acid sequence (Sarate et al., 1981; Kanazawa et al., 1982; Runswick & Walker, 1983) and that there is an exchangeable nucleotide binding site on each β subunit (Esch & Allison, 1979; Cross & Nalin, 1982; Satre et al., 1982). It is also consistent with the observed threefold external symmetry of the molecular complex formed between F_1 and anti-α or anti-β monoclonal antibodies (Ehrig et al., 1986).

On the other hand, the alternating sites model is not consistent with X-ray diffraction data (Amzel et al., 1982) and electron microscopy data (Boekema et al., 1986), nor with the results of chemical modification studies (DiPietro et al., 1983; Lotscher & Capaldi, 1984; Fellous et al., 1984; Matsuno-Yagi & Hatefi, 1984a,b; Wang, 1985, 1986; Bullough & Allison, 1986) and cold denaturation experiments (Williams et al., 1984) as well as circular dichroism (Roux et al., 1984) and kinetic (Hammes, 1983; Leckland & Hammes, 1987) data. Consequently, some investigators do not believe that three or two β subunits catalyze sequentially and alternately in each reaction cycle during the steady-state hydrolysis of ATP by F_1. However, all investigators seem to agree that there is strong interaction between the subunits of F_1-ATPase so that the binding of ATP or ADP at one site may affect the activity of another site through ligand-induced conformation change.

In an attempt to resolve this controversy, the affinity labeling reagents 3′-*O*-(5-fluoro-2,4-dinitrophenyl)[α-^{32}P]ATP (FDNP-[α-^{32}P]ATP)[1] and 3′-*O*-(5-fluoro-2,4-dinitrophenyl)[8-^{14}C]ATP (FDNP-[^{14}C]ATP) have been synthesized and used in the present work to determine the roles of the three active sites in F_1-ATPase during the catalytic hydrolysis and synthesis of ATP.

Experimental Procedures

Materials

ATP, ADP, EDTA, NAD^+, NADH, NADP, rotenone, oligomycin, Hepes, Sephadex G-50–80, PEP, pyruvate kinase, lactic dehydrogenase, hexokinase, glucose-6-phosphate dehydrogenase, and 1,5-difluoro-2,4-dinitrobenzene were purchased from Sigma Chemical Co. [γ-^{32}P]ATP, [α-^{32}P]ATP, and [8-^{14}C]ATP were purchased from New England Nuclear, Du Pont. FCCP was from Pierce Chemical Co. ETPH, ASU particles, F_1-ATPase, and reconstituted SMP were prepared as described previously (Wang et al., 1986, 1987).

Methods

Assay of Soluble Forms of F_1-ATPase. The catalytic activities of modified and control F_1-ATPase were determined 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. Normally an aliquot of F_1 sample was injected into a 2-mL solution of the above assay medium without $MgCl_2$ and incubated for 5 min before $MgCl_2$ was added to initiate the coupled reactions. The steady-state rate

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[1] Abbreviations: ASU, submitochondrial particles prepared from bovine heart mitochondria by sonication at pH 9 followed by steps involving urea treatment; DFDNB, 1,5-difluoro-2,4-dinitrobenzene; DNP-ATP-F_1, F_1 labeled by FDNP-ATP; EDTA, ethylenediaminetetraacetic acid; FCCP, carbonyl cyanide *p*-(trifluoromethyl)phenylhydrazone; FDNP-ATP, 3′-*O*-(5-fluoro-2,4-dinitrophenyl)-ATP; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; LDH, lactic dehydrogenase; *n*, molar ratio of covalent label to F_1; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole (also named 4-chloro-7-nitrobenzofurazan); OPA, *o*-phthalaldehyde; PEP, phosphoenolpyruvate; PK, pyruvate kinase; *r*, ratio of the specific activity of the labeled enzyme to that of the unlabeled control; SMP, submitochondrial particles; TFA, trifluoroacetic acid; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate [2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene)adenosine 5′-triphosphate].

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of ATP hydrolysis was computed from the observed linear decrease of A_{340} due to the coupled oxidation of NADH at 30 °C by using 6220 as the molar absorbance of NADH. Protein concentrations were determined by the Coomassie Blue binding method (Bradford, 1976).

Assay of ATP Hydrolysis and Oxidative Phosphorylation 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 measurement also contained 1.5 mM KCN to suppress respiration and 12.5 μM FCCP to dissipate the proton gradient. The steady-state rates of oxidative phosphorylation were monitored continuously in an ADP-regenerating system coupled to the reduction of NADP (Wang et al., 1987).

Syntheses of FDNP-[α-^{32}P]ATP and FDNP-[^{14}C]ATP. A typical preparation is described as follows. [α-^{32}P]ATP (1.6 μmol of disodium salt, specific radioactivity of 160 mCi/mmol) was dissolved in 200 μL of water containing 21 mg of Na_2CO_3 and 17 mg of $NaHCO_3$, pH 9.4. To this continually stirred solution was added dropwise 1.56 μmol of DFDNB in 50 μL of acetone. The reaction was allowed to proceed for 18 h at 36 °C. The progress of reaction was monitored by taking small aliquots at intervals and analyzing the content of each by thin-layer chromatography on precoated TLC plates (Si250F, analytical, J. T. Baker Chemical Co.). The developing solvent was ethanol plus 1 M ammonium acetate (aqueous) at pH 7.5 (5:2 v/v). A pale yellow reaction product (R_f = 0.28, as compared to R_f = 0.11 for [α-^{32}P]ATP) was gradually formed in about 5 h after the reaction was initiated. The condensation reached the optimum at 18 h. The condensation product was separated from the reaction mixture by applying the mixture to the same TLC plate and developing it with the same solvent. The product was harvested by scraping off the band at R_f = 0.28 on the developed TLC plate and subsequently extracted from the silica gel with 3 × 2 mL water. The extracts were pooled and concentrated by lyophilization and kept at −20 °C. On the basis of the radioactivity, the yield of this product was estimated to be about 35%. The purity of product was estimated from the ratio of radioactivity to the absorbance at 259 nm. A molar extinction coefficient 19 300 (15 400 for adenosine and 3900 for the FDNP group which was obtained with the model compound 2,4-dinitro-5-fluoro-1-methoxybenzene). These measurements gave an approximate molar ratio of 0.98. The procedure for the synthesis of FDNP-[^{14}C]ATP was the same except that [8-^{14}C]ATP was used in place of FDNP-[α-^{32}P]ATP.

Preparation of DNP-[α-^{32}P]ATP-F_1 and DNP-[^{14}C]ATP-F_1 (Figure 1). The labeling of F_1 by FDNP-[α-^{32}P]ATP was conducted in buffer A (50 mM Hepes–NaOH, pH 7.0, 2 mM EDTA, 25% glycerol) with or without 3 mM $MgCl_2$. In a typical preparation, a suspension of MF_1 in a sucrose buffer [50 mM Hepes–NaOH, 0.20 M sucrose, 2 mM EDTA, 5 mM ATP, 2 M $(NH_4)_2SO_4$, pH 7.0] containing about 10 mg of the protein was centrifuged. The pellet was redissolved in 150 μL of buffer A and desalted two times by centrifugal gel filtration through Sephadex G-50–80 that had been preequilibrated with buffer A. With this process about three endogenous nucleotides were retained in each F_1 molecule (27). The F_1 sample was subsequently diluted with buffer A to a final concentration of 57.4 μM in F_1. A 40-μL solution of FDNP-[α-^{32}P]ATP in water (286 μM) was mixed into 200 μL of the above solution containing an equimolar amount of F_1, and the labeling was allowed to progress for 2 h. At the end of the 2-h incubation, the unreacted and noncovalently bound labeling reagent was removed by incubating the reaction mixture with 15 mM ATP for 15 min followed by centrifugal gel filtration through Sephadex equilibrated with buffer A. The control F_1 was treated exactly in the same way, except that FDNP-[α-^{32}P]ATP in the mixture was replaced by TNP-ATP. The monitoring of ATPase activity of control F_1 indicated that the above process successfully removed all of the noncovalently bound TNP-ATP. MF_1 can be labeled with FDNP-[α-^{32}P]ATP with a molar ratio of covalent label to F_1 up to 2, without significant decrease in its ATPase activity, by repeating the above procedure several times. After the ratio reached 2, further incubation of the enzyme with labeling reagent did not increase the extent of covalent labeling. However, if 3 mM $MgCl_2$ was introduced to the reaction mixture at this stage of labeling, the molar ratio of covalently bound label to F_1 could be further increased with concomitant decrease of ATPase activity. DNP-[^{14}C]ATP-F_1 was prepared similarly by labeling F_1 with FDNP-[8-^{14}C]ATP.

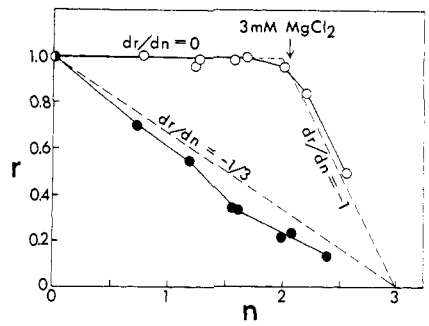

FIGURE 1: Preparation of DNP-[α-^{32}P]ATP-F_1. Upper curve (○): (DNP-[α-^{32}P]ATP_n-F_1 was prepared by incubating F_1 with FDNP-[α-^{32}P]ATP in buffer A in the absence ($n < 2$) or presence ($n > 2$) of additional 3 mM $MgCl_2$ as described under Methods. At the end of labeling, the unreacted and noncovalently bound labeling reagent was were removed by incubating the reaction mixture with 15 mM ATP for 15 min followed by centrifugal gel filtration. A control F_1 was treated in exactly the same way, except that FDNP-[α-^{32}P]ATP in the mixture was replaced by TNP-ATP. After gel filtration, the eluate were assayed for protein concentration, radioactivity, and ATPase specific activity. r represents the ratio of specific activity of labeled F_1 to that of control F_1, and n represents the molar ratio of label to F_1. The upper broken lines are reference lines with slopes equal to 0 and −1, respectively. Lower curve (●): Control F_1 and FDNP-ATP-labeled F_1 were scrambled by incubation of the enzyme in buffer A + 0.5 M $Mg(NO_3)_2$ + 10 mM ATP for 8 min at 0 °C followed by the centrifugal gel filtration through Sephadex G-50–80 that had been preequilibrated with buffer A + 5 mM ATP. Then the n and r values of the labeled enzymes were determined.

Assay of the Hydrolysis of Substoichiometric Amount of ATP by F_1 or DNP-[α-^{32}P]ATP-F_1. The hydrolysis of ATP catalyzed by F_1 or modified F_1 at subequivalent concentrations of ATP was assayed by monitoring the ^{32}P remaining in the enzyme complex at intervals (Cross et al., 1982). For these experiments, F_1 or $(DNP\text{-}[\alpha\text{-}^{32}P]ATP)_{1.8}$-$F_1$, where 1.8 denotes the molar ratio of covalently bound DNP-[α-^{32}P]ATP label to F_1, was usually dissolved in a Mg^{2+} buffer (0.25 M sucrose, 40 mM MES, 40 mM Tris, 1 mM K_2HPO_4, and 0.5 mM $MgSO_4$, pH 7.5) with a final concentration of 0.85 μM in the enzyme. To this F_1 or labeled F_1 solution, [γ-^{32}P]ATP with a specific radioactivity of about 1600 mCi/mmol was added to form a final concentration of 0.5 μM in [γ-^{32}P]ATP. The molar ratio of [γ-^{32}P]ATP to enzyme in this solution was thus equal to 0.59. The mixture was quickly stirred with a vortex mixer for 5 s and then incubated at room temperature (28 °C). After 40 s of incubation to permit the formation of a complex between the enzyme and its substrate, the unbound ligand was removed on a Sephadex G-50–80 column. The eluate was

further incubated at the same temperature. At each incubation time indicated in Figure 3, a 50-μL aliquot of the reaction mixture was taken out, and the reaction in each aliquot was quenched by the addition of 1 mM (final concentration) quercetin (Cantley & Hammes, 1976). The hydrolytic products released from F_1 in each aliquot were removed by centrifugal gel filtration. The eluate was collected for determination of the value of bound [^{32}P]/[enzyme] by measuring the protein concentration and radioactivity. To avoid the possible complication due to double isotopic labeling (γ-^{32}P in bound substrate and α-^{32}P in labeled F_1), the (DNP-[α-^{32}P]ATP)$_{1,8}$-F_1 used in these experiments had usually been aged until almost all radioisotope in labeled enzyme was decayed (radiospecific activity of 3.3 mCi/mmol). The TLC analysis of the radioisotope remained in Sephadex gel after the centrifugal gel filtration indicated that >99% of radioactive material was inorganic phosphate. Only less than 1% of it remained as [γ-^{32}P]ATP.

Identification of the Specifically Labeled Amino Acid Residue. The location of the DNP-[^{14}C]ATP label in F_1 was determined by separation of the subunits and by pepsin digestion of the labeled F_1 followed by isolation and sequencing of the principal radioactive peptide as described previously (Joshi & Wang, 1987).

Preparation of F_1-ATPase with Rearranged β Subunits. The rearrangement or scrambling of β subunits in F_1-ATPase was usually conducted by adding a solution containing 3–5 mg of the enzyme/mL and 10 mM ATP in buffer A (50 mM Hepes–NaOH, pH 7.0, 2 mM EDTA, 25% glycerol) at 0 °C to one-seventh of its volume of vigorously shaken 4.0 M $Mg(NO_3)_2$ solution in buffer A at 0 °C. After 8 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 scrambled enzyme was either used immediately or stored in liquid nitrogen.

Results

Steady-State Hydrolysis by (DNP-ATP)$_n$-F_1. The F_1 sample with three endogenous nucleotides per enzyme could be labeled with FDNP-[α-^{32}P]ATP up to two DNP-[α-^{32}P]-ATP/F_1 in a 25% glycerol solution containing 2 mM EDTA. It seemed surprising that this labeling was not accompanied by loss of ATPase activity (Figure 1). However, if 3 mM $MgCl_2$ was added to the mixture at this stage, further labeling by additional FDNP-[α-^{32}P]ATP did occur with $dr/dn = -1$, which implies that during this second phase of the experiment each additional covalent label completely inactivated a fully active F_1 molecule for catalyzing ATP hydrolysis. Sequencing data on similarly prepared DNP-[^{14}C]ATP-F_1 show that only Lys-β301 was labeled both before and after the addition of $MgCl_2$.

For the removal of unreacted and noncovalently bound FDNP-[α-^{32}P]ATP, the labeled enzyme was preincubated with 15 mM ATP in the 25% glycerol buffer for 15 min prior to centrifugal gel filtration. Since preliminary experiments show that this process completely removed TNP-ATP bound to the enzyme (data not shown), the possibility that noncovalent binding could be involved in the above results can be ruled out. The preincubation of labeled F_1 with 15 mM ATP was essential for the observation of full ATPase activity. The measured rates of hydrolysis by (DNP-ATP)$_{1,8}$-F_1 fit precisely the equation $1/k = 0.0133(1 + 41/[ATP])$, whereas the rates of hydrolysis of ATP by the control F_1 give $1/k = 0.0133(1 + 17/[ATP])$.

The steady-state hydrolysis of ITP by the F_1 labeled with FDNP-[α-^{32}P]ATP in the absence of Mg^{2+} was also measured.

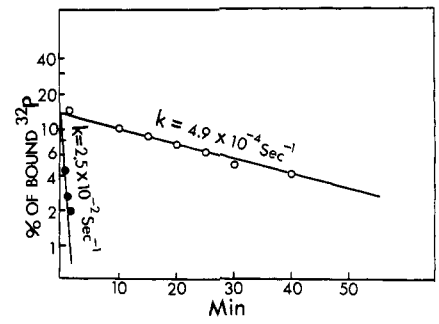

FIGURE 2: Rate of dissociation of hydrolytic product from F_1 and (β-DNP-[α-^{32}P]ATP)$_{1,8}$-F_1. F_1 (○) or (β-DNP-[α-^{32}P]ATP)$_{1,8}$-F_1 (●) at 0.85 μM was incubated with 0.5 μM [γ-^{32}P]ATP in a buffer containing 0.25 M sucrose, 40 mM MES, 40 mM Tris, 1 mM K_2HPO_4, and 0.5 mM $MgSO_4$, pH 7.5. After 40 s of incubation to permit the formation of a complex between the enzyme and its substrate, unbound ligand was removed by centrifugal gel filtration at $t = 0$. At the time indicated, aliquots of the mixture were taken, out and the reaction in each aliquot was quenched by the addition of 1 mM quercetin. The ^{32}P remaining bound to enzyme was detected after centrifugal gel filtration. The results are expressed as percent of the originally added ^{32}P (0.59 μmol/μmol of F_1) that remained bound to the enzyme. The ordinate is in logarithmic scale.

The specific ITPase activities observed were 40.1, 42.6, and 41.4 μmol min^{-1} mg^{-1} for (DNP-[α-^{32}P]ATP)$_{0.92}$-F_1, (DNP-[α-^{32}P]ATP)$_{1,83}$-F_1 and unlabeled F_1, respectively.

Unisite Hydrolysis by F_1 and (DNP-[α-^{32}P]ATP)$_{1,8}$-F_1. To examine the effect of DNP-ATP label on the efficiency of unisite catalysis for ATP hydrolysis, (DNP-[α-^{32}P]ATP)$_{1,8}$-F_1 had been prepared by labeling F_1 with FDNP-[α-^{32}P]ATP in the absence of Mg^{2+}, and the labeled enzyme was subsequently used for reaction with a substoichiometric amount of [γ-^{32}P]ATP as described under Methods. With subequivalent concentrations of [γ-^{32}P]ATP, the catalytic hydrolysis may be represented by

$$F_1 + [\gamma\text{-}^{32}P]ATP \rightleftarrows F_1\cdot[\gamma\text{-}^{32}P]ATP \rightleftarrows F_1\cdot ADP\cdot[^{32}P]P_i \overset{k}{\rightleftharpoons} F_1 + ADP + [^{32}P]P_i$$

Since the release of products from the enzyme is rate-limiting (32), and since in a very dilute solution the rate of recombination of the released product with the enzyme is negligible, the rate of dissociation of [^{32}P]P_i from the F_1·$^{32}P_i$ complex can be measured by monitoring the ^{32}P that remained bound at various time intervals. The experimental values of the first-order rate constants for the dissociation of [^{32}P]P_i from F_1·^{32}P and from DNP-ATP-F_1·^{32}P, respectively, are summarized in Figure 2, which shows that the labeled F_1 releases bound [^{32}P]P_i 50 times as fast as the unlabeled F_1.

Location of the Covalently Bound Radioactive Group in FDNP-[^{14}C]ATP-Labeled F_1. Dissociation of the FDNP-[^{14}C]ATP-labeled enzyme with 1.7 labels/F_1 by 6 M guanidine hydrochloride and separation of the subunits of HPLC showed that 65% of the radioactive label was on β subunit, 12% on α subunit, 3% on the smaller subunits, and 20% fell off the protein. A typical chromatogram is shown in Figure 3. Partial hydrolysis of the FDNP-[^{14}C]ATP-labeled enzyme with either 1.5, 1.7, or 2.5 labels/F_1, respectively, by pepsin and separation of the peptides showed that in all three cases the hydrolysis contained principally only one and the same radioactive peptide as illustrated in Figure 4. For the labeled F_1 sample with $n = 1.7$ and 2.5, respectively, 64% of all the bound radioactive label was associated with this peptide. For the other F_1 sample with $n = 1.5$, 72% of the radioactivity was associated with this peptide. The labeled enzyme with $n = 1.5$ and 1.7, respectively, was prepared in the absence of $MgCl_2$; that with $n = 2.5$ was prepared with the addition of

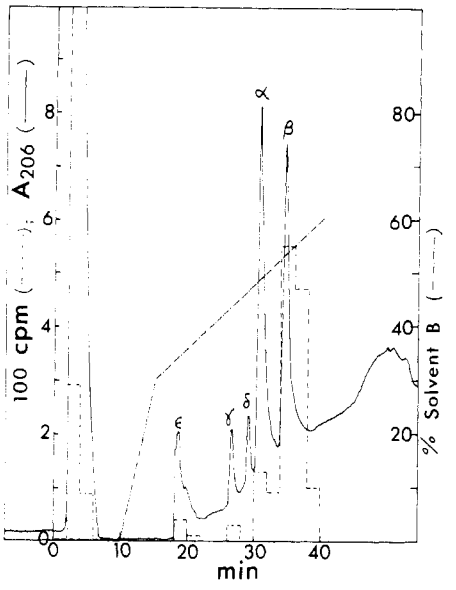

FIGURE 3: Separation of the subunits of DNP-[^{14}C]ATP-F_1 by reverse-phase HPLC. A 25-μg sample of the (DNP-[^{14}C]ATP)$_{1.72}$-F_1 was dissociated in 6 M guanidine hydrochloride solution at pH 7.9 and applied onto a Vydac C-4 column that had been preequilibrated with 30% CH_3CN + 70% aqueous 0.1% TFA. The column was subjected to linear gradient elution as illustrated at a flow rate of 1 mL/min. The fractions were collected for the assay of radioactivity by liquid scintillation counting. Solvent A contained 70% aqueous 0.1% TFA + 30% CH_3CN; solvent B contained 20% aqueous 0.1% TFA + 80% CH_3CN.

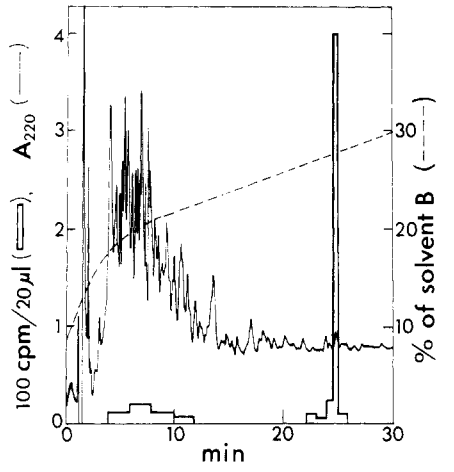

FIGURE 4: Separation of the major radioactive peptide from the pepsin digest of (DNP-[^{14}C]ATP)$_{1.5}$-F_1 by HPLC. After overnight dialysis of the labeled F_1 against 1% formic acid solution, the sample was incubated with 1% its weight (1.5 μg) of pepsin at 30 °C for 5 h. The resulting sample was lyophilized and redissolved in 200 μL of 0.1% TFA, passed through a 0.45-μm filter, and separated by a C_{18} NOVA-PAK column that was fitted with a guard column. The major radioactive peptide peak shown, which contained 72% of all the radioactivity, was collected manually. The mobile phase was a mixture of solvents A and B with programmed ratio indicated by the broken curve. The flow rate was 1 mL/min. Solvent A contained 0.125% trifluoroacetic acid and 0.1% morpholine in water; solvent B contained 0.1% trifluoroacetic acid and 0.1% morpholine in acetonitrile. The chromatograms of the pepsin digest of (DNP-[^{14}C]ATP)$_{1.7}$-F_1 and (DNP-[^{14}C]ATP)$_{2.5}$-F_1 were essentially the same.

$MgCl_2$ after n had almost reached 2. The percent of labeled Lys-β301 is likely to be higher than 64–72%, since there might be overlapping peptides also containing Lys-β301 but with different chain lengths.

The sequencing of this ^{14}C-labeled peptide by Edman degradation is summarized below. The phenylthiazolinone from the first Edman cycle gave, after hydrolysis and reaction with

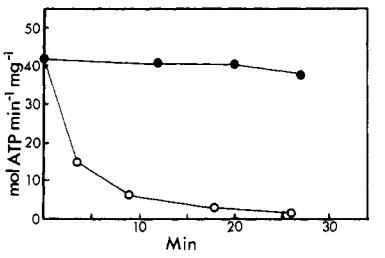

FIGURE 5: Inactivation and reactivation of F_1-ATPase by treatment with Mg^{2+} and centrifugal gel filtration. (○) Specific activity of F_1 dissolved in buffer A (50 mM Hepes, 2 mM EDTA, 25% glycerol, pH 7.0) containing 0.5 M $Mg(NO_3)_2$ and 10 mM ATP at 0 °C ([F_1] = 4.29 mg/mL); (●) specific activity of the sample taken from the above solution at time indicated and centrifugally gel filtered through Sephadex G-50–80 that had been preequilibrated with buffer A containing 5 mM ATP.

o-phthalaldehyde, a strong Thr peak. There were also a number of minor peaks due to contaminants soluble in benzene–ethyl acetate (2:1) which were found absent in the products of subsequent cycles. The second, third, and fourth cycles gave Thr, Thr, and Lys. The fifth cycle gave only background fluorescence, and the sixth cycle gave Gly. No fluorescence was detected after the sixth cycle. The failure of the fifth cycle to yield a fluorescent derivative suggests that the fifth amino acid residue in this peptide was labeled by the highly hydrophilic FDNP-[^{14}ATP] so that its derivative could not be extracted into the benzene–ethyl acetate layer. Although this batch of labeled F_1 has n = 1.5, the radioactive hexapeptide isolated from pepsin digest was not significantly contaminated by the corresponding nonradioactive hexapeptide because of the difference in thier elution times. This is also consistent with the observation that throughout the six Edman cycles the ^{14}C radioactivity stayed in the aqueous phase; all the PTH derivatives extracted into the organic phase were nonradioactive. Therefore, the sequence of the labeled peptide is Thr-Thr-Thr-Lys-Lys*-Gly, with the ^{14}C label attached to Lys*. A comparison of this sequence with the known sequence of the β subunit of bovine F_1-ATPase (Runswick & Walker, 1983) indicated that the labeled residue was Lys-β301. Since this peptide was found to be the only major radioactive peptide in the pepsin digest of all three labeled F_1 samples with n = 1.5, 1.7, and 2.5, respectively, we have to conclude that up to n = 2.5 essentially only Lys-β301 residues in each F_1 molecule were labeled in the above experiments.

When the FDNP-[α-^{32}P]ATP-labeled F_1 was treated by the same separation procedure, it was found that all the ^{32}P radioactivity fell off the labeled protein. However, the 3′-*O*-(2,4-dinitrophenyl)adenosine moiety of the label must be still covalently attached to F_1, because similar experiments with FDNP-[8-^{14}C]ATP showed that the ^{14}C radioactivity was still attached to Lys-β301 as described above. This unexpected observation deserves further investigation, because it might be relevant to the catalytic mechanism, although it is difficult to think of a reasonable mechanism for cleaving the triphosphate from the covalently attached label.

It is interesting to note that Hollemans et al. (1983) had previously concluded from their labeling experiments with 8-azido-[α-^{3}H]ATP and bovine heart mitochondrial F_1 that Lys-β301, Ile-β304, and Tyr-β311 were at the active site of F_1. The present results show that by successive additions of equivalent amounts of FDNP-[^{14}C]ATP to F_1 solution this affinity reagent labels specifically only Lys-β301 at each active site both in the absence and in the presence of Mg^{2+}.

Partial Dissociation and Reassociation of Subunits in F_1. The best way to rearrange the labeled and unlabeled subunits

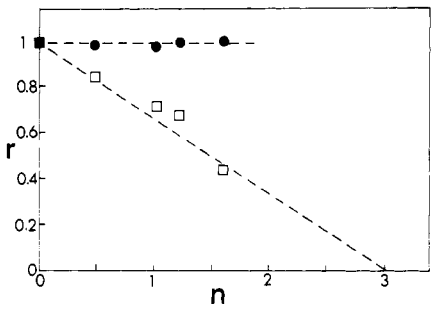

FIGURE 6: Effect of labeling Lys-β301 at the regulatory sites of F_1 on the ATPase and oxidative phosphorylation activities of the reconstituted SMP. The specifically labeled SMP was prepared by labeling F_1-ATPase with the affinity reagent FDNP-[α^{32}P]ATP in the absence of Mg^{2+} and subsequent reconstitution of (DNP-[α-^{32}P]ATP)$_n$-F_1 with ASU. (●) ATP hydrolysis; (□) oxidative phosphorylation. The upper broken line represents $r = 1$ for ATP hydrolysis. The lower broken line represents $r = 1 - n/3$ for oxidative phosphorylation.

of F_1-ATPase molecule seems to be forcing the subunits to dissociate partially and subsequently making them reassociate. In our previous procedure developed specifically for this purpose, F_1-ATPase was treated with 3.0 M LiCl at 0 °C for 3–4 min, and subsequently the LiCl was removed by centrifugal gel filtration at 25 °C (Wang, 1985). These treatments showed resonable success in scrambling the β subunits in F_1. However, it was found that some irreversible damage of F_1 was always unavoidable due to the treatment with LiCl. After extensive experimentation, a better procedure was found for scrambling the subunits in F_1-ATPase. It was mainly based on the finding by Pedersen and his colleagues (Pederson et al., 1987) that in a solution containing Mg^{2+} at high concentration the ATPase activity of F_1 was completely inhibited due mainly to the dissociation of some of the subunits in the enzyme. Following this hint, we found that incubation of F_1 in buffer A containing 0.5 M $Mg(NO_3)_2$ at 0 °C for 2 min resulted in almost total inactivation of the enzyme (Figure 6). This is most likely due to the dissociation of the subunits in F_1, because if the inactivated F_1 was subjected to centrifugal gel filtration, 99% of the initial activity of the enzyme was restored (Figure 6, upper curve). For example, after unlabeled F_1 with ATPase activity of 42.3 units/mg was treated with 0.5 M $Mg(NO_3)_2$ for 18 min at 0 °C, its activity decreased to 2.5 units/mg. But after subsequent centrifugal gel filtration in the presence of ATP at room temperature, its ATPase activity was restored to 41.9 units/mg. This procedure seems to be a better method than the 3 M LiCl treatment, which gave only 65–70% recovery of initial activity, and provided us with a better control in the scrambling experiments.

$Mg(NO_3)_2$-Induced Rearrangement of Specifically Labeled and Unlabeled β Subunits in F_1-ATPase. F_1-ATPase was labeled specifically at Lys-β301 by the affinity reagent FDNP-[α-^{32}P]ATP as described above. After each labeled sample was treated with 0.5 M $Mg(NO_3)_2$ to induce subunit rearrangment, an r vs n curve with slope dr/dn equal approximately to $-1/3$ was obtained (lower curve in Figure 1). The broken line with $dr/dn = -1/3$ represents the hypothetical case of completely random distribution of the affinity label among all three active sites but with steady-state ATP hydrolysis taking place at only one of the three sites.

Catalytic Hydrolysis and Oxidative Phosphorylation by DNP-[α-^{32}P]ATP-Labeled Reconstituted Submitochondrial Particles. Samples of (DNP-[α-^{32}P]ATP)$_n$-F_1 with n varying from 0 to 1.61 were prepared by labeling F_1 with FDNP-[α-^{32}P]ATP in the absence of Mg^{2+} as before and used to reconstitute with ASU to form specifically labeled submitochondrial particles (SMP). These SMP were assayed for catalytic ATP hydrolysis and oxidative phosphorylation. The observed specific activities are summarized in Table I. The data in Table I show that these specifically labeled SMP have the same ATPase activity as that of the unlabeled control SMP, but their phosphorylation activities are lower than those of the control SMP by a factor of approximately $(1 - n/3)$.

Table I: Catalytic Activities of Submitochondrial Particles Reconstituted from FDNP-[α-^{32}P]ATP-Labeled F_1 and ASU[a]

	ATPase activity (μmol mg^{-1} min^{-1})			oxidative phosphorylation activity (nmol mg^{-1} min^{-1})		
n	control unlabeled F_1-ASU	(DNP-[α-^{32}P]-ATP)$_n$-F_1-ASU	r	control unlabeled F_1-ASU	(DNP-[α-^{32}P]-ATP)$_n$-F_1-ASU	r
0.5	3.46	3.42	0.99	353	285	0.84
1.03	3.64	3.73	0.98	300	214	0.72
1.23	3.91	3.91	1.00	393	267	0.68
1.61	4.00	4.09	1.02	287	124	0.44

[a] The compositions of media and assay protocols are given under Methods. n represents the molar ratio of the covalent label to F_1, and r represents the ratio of specific activity for ATP hydrolysis or synthesis of labeled F_1-ASU to that of control SMP.

To minimize the adenylate kinase reaction, we had to keep the concentration of ADP in the assay medium at or below 0.2 mM and hence obtained rates below V_{max}. However, since our purpose was to measure the ratio of the activity of the labeled SMP to that of the unlabeled control SMP, it is not very important whether we use V_{max} or V as long as both the labeled and unlabeled SMP were measured under identical conditions.

Discussion

The F_1-ATPase used in the present work was desalted by centrifugal gel filtration twice through Sephadex G-50–80 that had been preequilibrated with buffer A. The so desalted F_1 was found by incubation with 8 M urea at 0 °C and subsequent anion-exchange chromatography to contain 2.8 ± 0.1 endogenous adenine nucleotides per F_1 (Tamura & Wang, 1983). To prevent possible exchange of the added ATP analogue in solution with the endogenous tightly bound adenine nucleotides, the labeling of F_1 with FDNP-[α-^{32}P]ATP or FDNP-[^{14}C]ATP was performed in steps with the molar ratio of free reagent to F_1 equal to or less than 1 at any time during each reaction period (see Methods). In this way it was found that in the absence of Mg^{2+} only Lys-β301 in F_1 was labeled by FDNP-[α-^{32}P]ATP continually until $n = 2$ with no significant decrease in ATPase activity.

After the addition of Mg^{2+} at this stage, the third Lys-β301 was labeled with concomitant decrease in ATPase activity. The slope of -1 for this segment of the r vs n curve shows that every label covalently attached to F_1 during this stage completely inactivates a fully active ATPase molecule. Therefore, we may conclude that the other two Lys-β301 residues that were covalently labeled by FDNP-ATP before the addition of $MgCl_2$ must be at the auxiliary or regulatory sites which could be labeled by an ATP analogue without inactivating the ATPase.

The observation that the two regulatory sites were labeled without affecting the steady-state ATPase activity may seem puzzling and deserves further discussion. The data in Figure 2 show that the covalent labels at the regulatory sites can indeed enhance the rate of product release at the hydrolytic site. However, the unisite rate at the hydrolytic site is enhanced only 50-fold by the affinity label at the regulatory site,

whereas an enhancement of several orders of magnitude by ATP itself was reported (Cross et al., 1982). The much lower enhancement by this affinity label as an effector is not really surprising if we recall that the affinity label does not have exactly the same structure as ATP and that it is also attached to the ϵ amino group of Lys-β301 by a covalent bond, which could prevent its spontaneous orientation for optimal effector action. On the other hand, since the ATP analogue at each regulatory site was attached to the flexible side chain of Lys-β301, it could be displaced by ATP (15 mM) in the 15-min preincubation period before each assay so that full steady-state rate for ATP hydrolysis could still be observed. Kinetic measurements with $(DNP\text{-}ATP)_{1.8}\text{-}F_1$ and the linear equations given under Results suggest that ATP can indeed compete with the covalently attached label for the regulatory sites.

It is generally agreed that each F_1 has not more than six binding sites for adenine nucleotides with $K_d < 1$ mM (Wise et al., 1983). Absorbance measurements showed that the F_1 sample prepared as described under Methods already contained 2.8 ± 0.1 endogenous adenine nucleotides per F_1 (Tamura & Wang, 1983). Therefore, the labeled enzyme $(DNP\text{-}[\alpha\text{-}^{32}P]ATP)_{1.8}\text{-}F_1$ that was prepared from this F_1 could have at most only 1.4 vacant sites per F_1. It is difficult to see how this labeled enzyme could hydrolyze ATP at the full steady-state rate by the alternating sites mechanism. In an attempt to defend the alternating equivalent sites model, we had considered the possibility that one of the three endogenous nucleotides exchanged immediately with medium ATP as soon as the modified F_1 was injected into the assay mixture containing 2 mM ATP and 3 mM Mg^{2+} (Kironde & Cross, 1987), and in this way each labeled F_1 could still have two free active sites that could alternate in catalyzing the hydrolysis of ATP. However, when these labeled enzymes were used to catalyze the hydrolysis of ITP as described under Results, we found that both $(DNP\text{-}[\alpha\text{-}^{32}P]ATP)_{0.92}\text{-}F_1$ and $(DNP\text{-}[\alpha\text{-}^{32}P]ATP)_{1.83}\text{-}F_1$ have essentially the same ITPase activity as the unlabeled F_1. Since ITP does not exchange with endogenous ATP bound at the high affinity sites (Perlin et al., 1984), this labeled F_1 really had only 1.2 sites left for binding ITP, but it still exhibited full ITPase activity. Consequently, this possibility is also ruled out.

Since there are only three Lys-β301 residues at the active sites of each F_1 molecule, the observation that F_1 with two of its three Lys-β301 residues covalently labeled by FDNP-ATP in the absence of Mg^{2+} can still hydrolyze ATP at the full steady-state rate shows that the catalysis does not take place via the alternating sites mechanism. The complete inactivation of each F_1 molecule by covalently labeling its third Lys-β301 residue in the presence of Mg^{2+} indicates that only this third Lys-β301 is at the site where steady-state hydrolysis of ATP takes place. The observed 50-fold enhancement of the rate of product release at the hydrolytic site by DNP-ATP labels covalently attached to the first and second Lys-β301 residues suggests that these residues may indeed by at the regulatory sites and that when the ATP analogues in these sites are displaced by the ideal effector ATP, the rate of product release at the hydrolytic site could be raised by several orders of magnitude as observed (Cross et al., 1982).

Previous studies on NBD-labeled F_1 (Steinmeyer & Wang, 1979; Kohlbrenner & Boyer, 1982; Soong & Wang, 1984; Matsuno-Yagi & Hatefi, 1984a,b) show that with one NBD label on Tyr-β311 at the hydrolytic site, the ATPase activity of F_1 is almost completely inhibited, but if this labeled enzyme is recombined with ASU particles, the reconstituted SMP still exhibits about 35–60% of the oxidative phosphorylation activity of the control SMP. On the other hand, if the NBD label is put on Tyr-β311 at one or both of the nonhydrolytic sites, and subsequently reconstituted with ASU, the labeled submitochondrial particles show full ATPase activity but only 62% of the oxidative phosphorylation activity of the control SMP (Wang et al., 1987). On the basis of these observations, it was concluded that, unlike hydrolysis, synthesis of ATP by ATP synthase takes place at all three active sites.

In the present work, F_1 was labeled with the affinity reagent $FDNP\text{-}[\alpha\text{-}^{32}P]ATP$ and subsequently treated with 0.5 M $Mg(NO_3)_2$ for 8 min at 0 °C to induce the rearrangement of the labeled and unlabeled β subunits. As shown by the lower curve in Figure 1, the resulting enzyme samples with n covalent labels/F_1 molecule were found to have specific ATPase activities lower than those of the corresponding control unlabeled enzyme by an approximate factor of $(1 - n/3)$. Since it has already been shown above that the three active sites in F_1 do not alternate in catalyzing steady-state ATP hydrolysis, this observation implies that the treatment with 0.5 M $Mg(NO_3)_2$ has caused an almost random distribution of the covalent label among the three Lys-β301 residues of F_1 with a slight preference of the labeled Lys-β301 to be at the hydrolytic position.

The earlier observation that NBD-labeled SMP exhibited lower r values for ATP hydrolysis than for oxidative phosphorylation cannot be due to the difference in free energy states of the SMP, because FCCP-uncoupled SMP and ATP-energized SMP were found to have the same r values for ATP hydrolysis. These previous results were explained in terms of the model F_1 with one specific active site for catalyzing efficient ATP hydrolysis when the other two active sites are also occupied by ATP, but with all three active sites catalyzing oxidative phosphorylation (Wang et al., 1987). It is possible that during oxidative phosphorylation the repeated conformation changes driven by the proton flux make the promotional effect on the ATP-occupied regulatory site(s) unnecessary and that consequently all three active sites can catalyze ATP synthesis.

In contrast to the data of direct labeling by NBD-Cl, Table I shows that when ASU was reconstituted with F_1 that were labeled with FDNP-ATP specifically at the regulatory sites, the resulting particles exhibited lower r values for oxidative phosphorylation than for ATP hydrolysis. Since FDNP-ATP covalently labels only Lys-β301 in F_1 under our experimental conditions, the r values in Table I are again most simply explained in terms of the same model. For ATP hydrolysis, the catalytic activity of the labeled SMP should be approximately equal to that of the unlabeled control SMP, i.e., $r \approx 1$, because all the labeled Lys-β301 residues are at the regulatory sites. However, for oxidative phosphorylation the catalytic activities of the labeled SMP should be significantly lower than those for the unlabeled control SMP, because some of the phosphorylation sites are strongly inhibited by the affinity label(s). If the three active sites are equally efficient in catalyzing oxidative phosphorylation, the r value of the modified SMP with n labels per F_1 should be equal to $(1 - n/3)$. Figure 6 shows that deviations of the observed r values for ATP hydrolysis and oxidative phosphorylation from the straight lines $r = 1$ and $r = 1 - n/3$, respectively, are probably within the experimental uncertainties. The present results show that the catalytic paths for the hydrolysis and synthesis of ATP are closely related but not identical.

It is interesting to note that although both Lys-β162 and Lys-β301 are at the active site of F_1-ATPase, FDNP-ADP labels Lys-β162 (Chuan & Wang, 1988), whereas FDNP-ATP

labels Lys-β301. This is presumably because the binding of ADP and ATP analogues engenders different protein conformations at the active sites. The present example could serve as a reminder for us to be cautious in drawing conclusions from labeling data. The observation that different amino acid residues are labeled or that the labeled protein species have different net electric charge could be because the labeled residues are at different sites of the protein, but it could also be because the labeled residues are at the same site of the protein in diffferent conformational states. For these two cases, the conformational difference between the two labeled F_1 molecules could favor the C-5″ of bound FDNP-ADP for nucleophilic attack by the amino group of Lys-β162, but favor the C-5″ of bound FDNP-ATP for nucleophilic attack by the amino group of Lys-β301.

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