Geometric Isomers of Covalently Labeled Mitochondrial F_1-Adenosinetriphosphatase with Different Properties†

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ABSTRACT: Two geometric isomers of covalently labeled F_1-adenosinetriphosphatase (F_1-ATPase) have been prepared by reaction with 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl): a directly labeled product denoted by O-β'-NBD-F_1 and an indirectly prepared product denoted by O-β''-NBD-F_1. The normal isomer O-β'-NBD-F_1 is highly inhibited, and its label can be removed by 20 μM N-acetyl-L-cysteine (AC) at the expected rate with $dr/dn \approx -1$, where n is the molar ratio of the label to F_1 and r is the ratio of the ATPase activity of the labeled enzyme to that of the unlabeled control enzyme. But O-β''-NBD-F_1 is almost fully active, and its label can be removed by 20 μM AC at much slower rates with $dr/dn \approx 0$. Cleavage of either isomer with pepsin and subsequent amino acid analysis of the isolated radioactive polypeptides show that the label is attached to Tyr-β311 in both isomers. At pH 9 the label in O-β'-NBD-F_1 spontaneously transfers from Tyr-β311 to the presumably nearby Lys-β162 in the dark with a half-time of $^1/_2$ h, but the label in O-β''-NBD-F_1 does not transfer under the same conditions. The existence of geometric isomers of O-NBD-F_1 with contrastingly different properties invalidates models for F_1 with three equivalent β subunits but is consistent with the model based on one principal catalytic β' subunit and two auxiliary β'' subunits. A possible mechanism for promoting the catalytic efficiency of β' through protein conformation change induced by ATP and/or ADP is suggested.

Kinetic data on the catalytic hydrolysis of ATP by F_1-ATPase[1] have often been interpreted by the well-known alternating sites model based on the presence of three or two strongly interacting but functionally equivalent β subunits in the enzyme molecule (Gresser et al., 1982; O'Neal & Boyer, 1984). The model is consistent with the fact that the three β subunits have identical amino acid sequences (Sarate et al., 1981; Kanazawa et al., 1982; Runswick & Walker, 1983) and that there is a nucleotide binding site on each of them (Esch & Allison, 1979; Cross & Nalin, 1982; Satre et al., 1982). There is also convincing evidence for strong interaction between the β subunits (Cross et al., 1982; Grubmeyer et al., 1982).

Recently Melese and Boyer (1985) found that the ^{32}P label and ^{14}C label were attached to separate β subunits if CF_1-ATPase was first photolabeled with [β,γ-^{32}P]-2-azido-ATP and subsequently labeled with [^{14}C]DCCD but that some β subunits contained both labels if CF_1 was first labeled with [^{14}C]DCCD and subsequently photolabeled with [β,γ-^{32}P]-2-azido-ATP. They cited this observation as supporting evidence for the model with three equivalent alternating sites. But if the photolabeling of CF_1 by 2-azido-ATP caused a structural change that prevented subsequent labeling of the same β subunit by DCCD whereas the labeling by DCCD did not prevent the subsequent photolabeling by 2-azido-ATP, their data will also be expected if CF_1 has nonequivalent β subunits with occasional spontaneous switching of their catalytic and regulatory roles. Direct evidence is still needed to show that the three subunits indeed catalyze sequentially in each reaction cycle during steady-state catalysis.

On the other hand, the arrangment of subunits in F_1 could make the β subunits nonequivalent. Indeed, X-ray diffraction data indicate that the three β subunits in F_1 are not structurally equivalent (Amzel et al., 1982). This conclusion is also consistent with the results of chemical modification studies (Di Pietro et al., 1982; Lötscher & Capaldi, 1984; Fellous et al., 1984; Matsuno-Yagi & Hatefi, 1984; Snyder & Hammes, 1984; Soong & Wang, 1984; Wang, 1985). Cold denaturation studies suggest that the γ subunit may "tag" one out of three $\alpha\beta$ pairs in the F_1 molecule (Williams et al., 1984). Circular dichroism (Roux et al., 1984) and fluorescence studies (Ferguson et al., 1975; Wang, 1984, 1986) suggest that the binding of ADP to the regulatory site(s) of β subunits can change the protein conformation at the catalytic site. But compelling evidence is still needed to show that the β subunits in each F_1 molecule are indeed functionally not equivalent. The results reported in this paper may enable us to decide between the alternative models.

Experimental Procedures

Materials

N-Acetyl-L-cysteine, ATP, ADP, DTT, EDTA, Hepes, lactic acid dehydrogenase, Mes, NBD-Cl, pepsin, *o*-phthaldialdehyde, and Sephadex G-50-80 were purchased from Sigma Chemical Co. [^{14}C]NBD-Cl was from Research Products International Corp. and was found (see below) to have a specific radioactivity of 77 mCi/mmol (Wang, 1985). Other special reagents included acetonitrile (HPLC grade, Baker), morpholine (Gold Label, Aldrich), and TFA

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[1] Abbreviations: AC, *N*-acetyl-L-cysteine; β, β subunit of F_1-adenosinetriphosphatase; DCCD, dicyclohexylcarbodiimide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; F_1 or F_1-ATPase, F_1-adenosinetriphosphatase; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; LDH, L-lactic acid dehydrogenase; Mes, 2-(*N*-morpholino)ethanesulfonic acid; n, molar ratio of label to F_1; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; *N*-NBD-F_1, F_1 labeled with NBD-Cl at its essential Lys residue; *O*-NBD-F_1, F_1 labeled with NBD-Cl at its essential Tyr residue; PEP, phosphoenolpyruvate; PK, pyruvate kinase; r, ratio of the specific activity of the labeled enzyme to that of the unlabeled control; TFA, trifluoroacetic acid; TNT-ATP, 2',3'-*O*-(2,4,6-trinitrocyclohexadienylidene)adenosine 5'-triphosphate.

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(HPLC/spectrograde, Pierce Chemical Co.).

Mitochondrial F_1-ATPase was prepared from fresh bovine heart and stored as described by Knowles and Penefsky (1972). *O*-[^{14}C]-NBD-F_1 and *N*-[^{14}C]NBD-F_1 were prepared as previously described (Wang, 1985). The labeled proteins were normally prepared in buffer A solution containing 50 mM Hepes–NaOH, pH 7.0, 2 mM EDTA, and 25% (v/v) glycerol and stored under liquid nitrogen.

Methods

Biochemical Assays. Protein concentrations were determined by the Coomassie Blue binding method (Bradford, 1976). The ^{14}C radioactivity was assayed by liquid scintillation counting, with counting efficiency determined by means of a [^{14}C]toluene internal standard. The molar ratio *n* of label to F_1 was calculated by using a molecular weight of 3.5×10^5 for F_1 and the known specific radioactivity of the label (77 mCi/mmol).

The ATPase activity was assayed by an ATP-regenerating system coupled to the oxidation of NADH in an assay 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 rate of ATP hydrolysis was computed from the observed linear decrease of A_{340} due to the oxidation of NADH at 30 °C by using 6220 as the molar absorbance of NADH.

Isolation of O-[^{14}C]NBD-Labeled Peptide. All operations were conducted in a darkened room. In a typical experiment, 1.6 mg of *O*-[^{14}C]NBD-F_1 in buffer A was dialyzed against 500-fold its volume of aqueous formic acid (1% v/v) at 4 °C for 5 h according to the procedure of Sutton and Ferguson (1985b). The dialyzed solution was mixed with 20 μg of pepsin and incubated at 30 °C for 4 h. The resulting solution was subsequently lyophilized. The residue was redissolved in 500 μL of 0.1% TFA and centrifuged through a 0.45-μm filter (Rainin). An aliquot of the filtrate was applied to a Du Pont Zorbax Bio-Series PEP-RP1 HPLC column and eluted with mixtures of solvent I (H_2O, 0.125% TFA, 0.1% morpholine) and solvent II (CH_3CN, 0.1% TFA, 0.1% morpholine). All solvents were prefiltered through a 0.45-μm Millipore membrane. Absorbance at 220 nm and radioactivity of the eluate were monitored concurrently. (See Figure 3.)

Amino Acid Analysis. The isolated radioactive polypeptide fraction was lyophilized in a vacuum hydrolysis tube (Pierce Chemical Co.). After the residue was mixed with constant boiling HCl (6 N), the tube was sealed under vacuum and incubated at 150 °C for 6 h. The excess HCl was subsequently removed by lyophilization, the residue was dissolved in 100 μL of borate buffer, pH 9.2, and the amino acids were derivatized with *o*-phthaldialdehyde (Hill et al., 1979). A 100-μL sample of the derivatized amino acids was applied to a Waters Radial-PAK NOVA-PAK C_{18} cartridge (8 mm × 10 cm) and assayed by comparing the fluorescence intensity of the elution peaks with those of the freshly prepared standards.

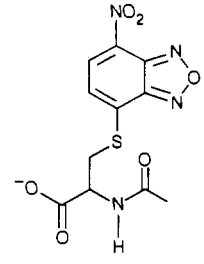
Fluorescence Emission Spectra. A Hitachi–Perkin-Elmer fluorescence spectrophotometer, Model MPF-2A, was used to measure the fluorescence emission of *N*-NBD-F_1 and *N*-NBD-β with 440-nm excitation light at a slit width of 6 nm.

Determination of the Specific Radioactivity of [^{14}C]NBD-Cl. Quantitative treatment of the chemical modification data requires an exact knowledge of the specific radioactivity of the NBD-Cl used. Pure NBD-Cl can be converted quantitatively to 7-(*N*,*N*-dimethylamino)-4-nitro-2,1,3-benzoxadiazole [NBD-N(CH_3)$_2$] by its reaction with a large excess of dimethylamine in methanol solution at room temperature. A linear plot of the maximal absorbance at 475 nm due to NBD-N(CH_3)$_2$ vs. the initial molar concentration of NBD-Cl yielded a molar absorbance of A_{475} = 22 414 for NBD-N(CH_3)$_2$. The [^{14}C]NBD-Cl received from the supplier contained a substantial amount of radioactive impurity that did not react with dimethylamine and was removed by adsorption on a silica column (silica powder, 60–200 mesh, Baker analyzed, 0.5 cm × 10 cm). Pure [^{14}C]NBD-Cl was eluted from the column with chloroform. The specific radioactivity of [^{14}C]NBD-Cl in the commercial sample was computed from the observed dpm of the eluted [^{14}C]NBD-N(CH_3)$_2$ sample, determined with internal [^{14}C]toluene standard, and its absorbance observed at 475 nm. It was found to be 76 ± 1 mCi/mmol. The specific activity was also determined by titrating F_1 with the same commercial NBD-Cl and assuming that below 85% inhibition of the activity each NBD label completely inactivates an F_1 molecule (Ferguson et al., 1975). A linear plot of the fraction of initial ATPase activity remaining vs. the number of labels covalently attached to each F_1 yielded a specific radioactivity of 77 mCi/mmol, which is within experimental errors in agreement with the above value.

Results

Preparation of O-β′-NBD-F_1 and O-β′,β″-NBD-F_1. Previous studies show that when the highly inhibited F_1, specifically labeled with NBD-Cl at its β′ subunit, was treated for a few minutes with 3 M LiCl at 0 °C and subsequently renatured by centrifugal gel filtration in the presence of 5 mM ATP at 25 °C, the specific ATPase activity of the enzyme relative to that of control F_1 can be increased by a large factor without losing the covalent label (Wang, 1985). Presumably the brief perturbation by LiCl causes rearrangement or scrambling of the β subunits so that some of the unlabeled β subunits are now in the catalytic β′ position whereas some of the labeled β subunits are now in the auxiliary or regulatory β″ position. In the present work, the scrambled enzyme *O*-β′,β″-NBD-F_1 was prepared by mixing a solution of *O*-β′-NBD-F_1 in buffer A containing 5 mM ATP with an equal volume of 6 M LiCl in buffer A in the dark at 0 °C for 3.5 min and immediately filtering the mixture through a Sephadex G-50-80 gel column that had been preequilibrated with 5 mM ATP in buffer A in the dark at 25 °C. The product *O*-β′,β″-NBD-F_1 was used at the starting material for preparing *O*-β″-NBD-F_1 described below.

Selective Removal of NBD Label from the Catalytic β′ Subunit. When *O*-β′-[^{14}C]NBD-F_1 was incubated with 0.5 mM *N*-acetyl-L-cysteine (AC) for a few minutes in the dark at pH 7 and subsequently gel filtered through Sephadex G-50-80, it was found that all the radioactive labels were removed and the enzyme regained its full ATPase activity as expected. However, when the same concentration of AC was injected into an assay mixture containing *O*-β′-NBD-F_1 without subsequent gel filtration, it not only made little change in the observed ATPase activity (see Figure 1) but also prevented DTT from removing the inhibition. These observations suggest that the reaction product of AC and the labeled enzyme



is probably still bound at the catalytic site of the enzyme and

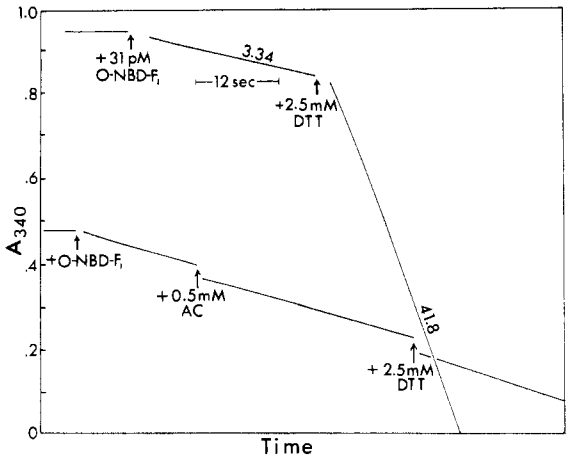


FIGURE 1: Effect of *N*-acetyl-L-cysteine (AC) on specific ATPase activity of *O*-NBD-F_1. The conditions of ATPase assay are given under Methods. (Upper trace) The addition of 2.5 mM DTT increases the observed specific activity (μmol of ATP hydrolyzed min^{-1} mg^{-1}) of *O*-NBD-F_1 from 3.34 to 41.8 due to rapid removal of the NBD label as expected. (Lower trace) The addition of 0.5 mM *N*-acetyl-L-cysteine (AC) has little effect on the observed specific acitivty of *O*-NBD-F_1, but it eliminates the expected enhancement of specific activity by subsequent addition of 2.5 mM DTT.

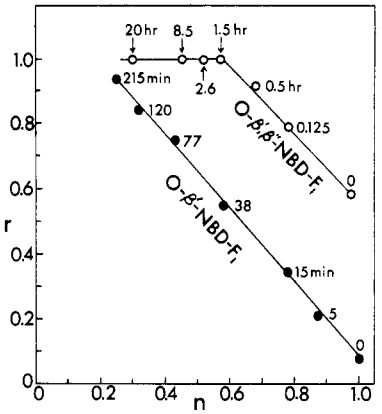


FIGURE 2: Selective removal of *O*-NBD label from *O*-NBD-F_1 by AC in the dark at 25 °C. *O*-β'-[^{14}C]NBD-F_1 (4.97 μM in buffer A) was prepared by direct labeling of F_1 with [^{14}C]NBD-Cl in the dark at pH 7.0. *O*-β',β''-[^{14}C]NBD-F_1 (3.68 μM) was prepared by incubation of *O*-β'-[^{14}C]NBD-F_1 with 3 M LiCl and 5 mM ATP in buffer A in the dark at 0 °C for 3.5 min and subsequent centrifugal filtration in the dark at 25 °C through Sephadex G-50-80 that had been preequilibrated with buffer A containing 5 mM ATP. For each set of measurements, 20 μM AC was added at time 0 and aliquots of the reaction mixture were taken at the indicated time intervals. Each aliquot was immediately centrifugally filtered through G-50-80 that had been preequilibrated with buffer A containing 5 mM ATP and assayed for protein concentraiton, radioactivity, and ATPase specific activity. n represents the molar ratio of NBD label to F_1. r represents the ratio of the specific ATPase activity of *O*-NBD-F_1 before and after treatment with 2.5 mM DTT, respectively.

inhibits the activity of F_1 just like the covalently attached *O*-NBD label, but this type of inhibition would not be relieved by DTT. The reaction of *O*-NBD label with 20 μM AC can be greatly reduced by inorganic phosphate or ATP. The inhibition of F_1-ATPase by the above negatively charged product shown in Figure 1 may be taken as further evidence for the presence of the labeled Tyr at the catalytic site.

Following this hint, we found that the *O*-β'-NBD label in the scrambled product *O*-β',β''-NBD-F_1 can be removed by very low concentrations of AC in a highly selective and reproducible way. The results of a typical set of measurements are summarized in Figure 2, in which the observed ratio of the ATPase activity of the labeled enzyme to that of the same but with all the *O*-NBD label removed by DTT is plotted against the number n of labels remaining per F_1. As expected, the removal of NBD label from *O*-β'-NBD-F_1 proceeded at moderate rate with $dr/dn \approx -1$. On the other hand, the removal of NBD label from *O*-β',β''-NBD-F_1 by 20 μM AC was clearly biphasic: First, the removal of label (presumably from the catalytic β' subunit) proceeded at similar moderate rate with $dr/dn \approx -1$; then the removal of label (presumably from the auxiliary β'' subunit) proceeded at a much slower rate with $dr/dn \approx 0$. These experimental results, which had been repeated more than 10 times and were always reproducible, are in agreement with the model of F_1-ATPase with a single catalytic site and two auxiliary sites.

Preparation of O-β''-NBD-F$_1$. If the reaction of AC with *O*-β',β''-([^{14}C]NBD)$_n$$F_1$ is terminated by gel filtration when most of the labels on β' have been removed, presumably *O*-β''-NBD-F_1, a geometric isomer of *O*-β'-NBD-F_1 with very different biochemical properties, could be obtained.

For this purpose, a sample of F_1 with specific activity 71.4 u/mg (1 u = 1 μmol of ATP hydrolyzed/min) was labeled with [^{14}C]NBD-Cl until only less than 10% of its initial ATPase activity was left. The reaction was stopped by gel filtration, and the isolated product *O*-β'-[^{14}C]NBD-F_1 exhibited a speficic activity of 4.16 u/mg with n = 1.07 and r = 0.060. All operations were in a darkened room.

The *O*-β'-[^{14}C]NBD-F_1 was treated with 3 M LiCl, and the scrambled product *O*-β',β''-[^{14}C]NBD-F_1 exhibited a specific acitivity of 13.5 u/mg with n = 1.03 and r = 0.40. In order to minimize the rate of O → N transfer of the label, the treatment with LiCl was conducted in a buffer containing 50 mM Mes–NaOH, pH 6.0, 2 mM EDTA, and 25% (v/v) glycerol.

The *O*-β',β''-[^{14}C]NBD-F_1 was then allowed to react with 20 μM AC in the above pH 6.0 Mes buffer for 4 h at 25 °C in the absence of ATP. The reaction mixture was subsequently filtered through Sephadex G-50-80 that had been preequilibrated with the same buffer, and the filtrate was stored under liquid nitrogen. After 2 days, the frozen sample was thawed and again filtered through a Sephadex G-50-80 column that had been preequilibrated with the Mes bufer containing 5 mM ATP.

The final product, which may be tentatively denoted by *O*-β''-[^{14}C]NBD-F_1, has the following characteristics: n = 0.52; ATPase activity = 50.7 units/mg in the absence of DTT and 50.7 units/mg in the presence of 2.5 mM DTT; hence r = 1.0. A sample of the unlabeled F_1 that had been treated with 3 M LiCl and subsequently gel filtered under similar conditions showed an ATPase activity of 56.6 units/mg. Obviously most of the 0.52 label per F_1 in the final product is no longer at the catalytic site, because within experimental uncertainties the final labeled enzyme has full ATPase activity.

Is the label still attached to the same Tyr residue but now in an auxiliary β'' subunit, or has it moved to a nonessential residue in the catalytic β' subunit? In order to answer this question, it was necessary to identify the labeled amino acid residue in *O*-β''-[^{14}C]NBD-F_1.

Location of the Radioactive Label in O-β''-[^{14}C]*NBD-F*$_1$. Previous work showed that after O-β'-[^{14}C]NBD-F_1 was reduced with sodium hydrosulfite, the reduced radioactive label was attached to Tyr-β311 (Andrews et al., 1984). But after the labeled enzyme was reduced with zinc and methylviologen, the reduced radioactive label was attached to Tyr-β197 (Ho & Wang, 1983). Recently, Sutton and Ferguson (1985b) avoided the reduction step and cleaved *O*-β'-[^{14}C]NBD-F_1 directly with pepsin in 1% formic acid solution. They isolated a radioactive peptide with the [^{14}C]NBD label attached to

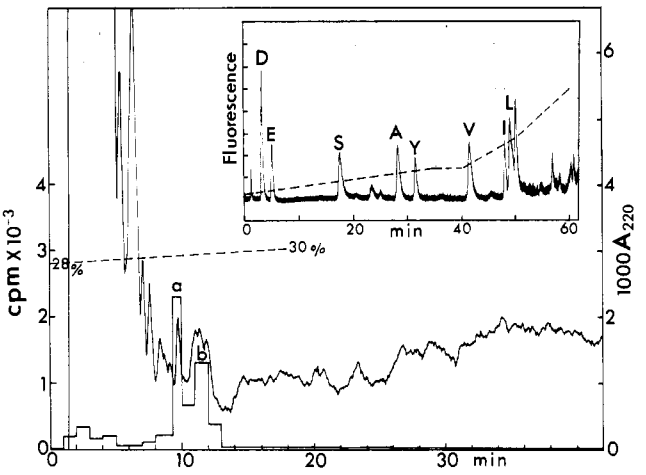


FIGURE 3: Isolation of the prinicipal radioactive polypeptide cleaved from *O*-β''-[^{14}C]NBD-F_1 with pepsin and determination of its amino acid composition by HPLC. (Main figure) A 20-μL sample of dialyzed pepsin digest of *O*-β''-[^{14}C]NBD-F_1 containing about 20 μg of the protein in aqueous 0.1% TFA solution was applied to a Zorbax Bio-Series PEP-RP1 column that had been preequilibrated with a mixture of 72% solvent I (H_2O, 0.125% TFA, 0.1% morpholine) and 28% solvent II (CH_3CN, 0.1% TFA, 0.1% morpholine). The column was then eluted at a rate of 1.0 mL/min with a linear gradient of 72% I + 28% II to 65% I + 35% II over a 60-min period. The experimental trace represents A_{220}, the block diagram represents cpm, and the broken straight line represents compositions of the mobile phase. Total radioactivity recovered, 95%; fraction a, 28%; fraction b, 23%. (Inset) Amino acid analysis of fraction a by the procedure described under Methods. A 100-μL sample of the derivatized amino acids was applied to a Radial-PAK NOVA-PAK C_{18} (8 mm × 10 cm) column and eluted at 1.0 mL/min with the following mixtures of solvent III (0.0125 M Na_2HPO_4, pH 7.2) and solvent IV (acetonitrile): 0–35 min, 12–24% IV; 35–40 min, 24% IV, 40–50 min, 24–38% IV; 50–60 min, 38–60% IV. The experimental trace represents fluorescence intensity; the broken straight line represents compositions of the mobile phase.

Table I

amino acid	D	E	S	A	Y	V	I	L
pmol	144	83	86	149	54	126	70	88
ratio to I	2.1	1.2	1.2	1.7	0.8	1.8	(1)	1.3
expected ratio	2	1	1	2	1	2	(1)	1

Tyr-β311. Their observation shows that initially Tyr-β311 is labeled by NBD-Cl and suggests that during the subsequent reaction with methylviologen free radical the reduced radioactive label may be transferred to Tyr-β197.

In order to identify the labeled amino acid residue, *O*-β''-[^{14}C]NBD-F_1 was dialyzed in 1% formic acid solution and cleaved with pepsin (Sutton & Ferguson, 1985b) and the resulting mixture was separated by HPLC. About 94% of the radioactivity in the original sample was recovered in the eluate. The chromatogram in Figure 3 shows that there are only two major radioactive fractions in the eluate, containing 28% and 23%, respectively, of the initial total radioactivity. Fraction a, which was collected manually and hence was of higher purity, was hydrolyzed in a vacuum by HCl at 150 °C and analyzed for amino acid composition as described under Methods. The results are given in the inset of Figure 3. The chromatogram of its hydrolysate shows only eight major fluorescent peaks due to known amino acids. By comparison with standard amino acid solutions the sample was found to have the composition shown in Table I. The conspicuous peak on the right of that for isoleucine in the inset of Figure 3 does not correspond to any known amino acid. Since proline does not yield fluorescent product with *o*-phthaldialdehyde, we concluded after scanning the amino acid sequence of the β subunit of bovine heart mitochondrial F_1-ATPase (Runswick & Walker, 1983) that the isolated radioactive peptide in fraction a is SVQAIYVPADDL. The expected molar ratios for the individual amino acids in this peptide to isoleucine are also listed in Table I for comparison. These data show that the radioactive label in *O*-β''-NBD-F_1 is also attached to Tyr-β311, as it was in *O*-β'-NBD-F_1 according to the work of Sutton and Ferguson (1985b), which we have also confirmed in the present study (data not shown). The radioactive peptide in fraction b was isolated in purer form from a duplicate separation with a steeper linear gradient by manual collection. It contained 15% of the total original radioactivity and was found by amino acid analysis to be the tetrapeptide KTVL previously isolated by Sutton and Ferguson (1985a). Therefore, *O*-β'-NBD-F_1 and *O*-β''-NBD-F_1 are geometric isomers with contrasting properties. This simple fact invalidates models for F_1-ATPase with three equivalent catalytic sites.

Table II: Labeling of F_1 Followed by Scrambling of the β Subunits and Subsequent Additional Labeling[a]

sample	reaction time with NBD-Cl (min)	ATPase act. (μmol of ATP mg^{-1} min^{-1})	n	r
0	0 (control)	54.4	0	1
1	11	38.4	0.34	0.71
2	21	27.2	0.50	0.50
3	44	14.1	0.76	0.26
4	71	5.55	0.90	0.10
5	90	3.08	0.94	0.057
6	105	2.55 (−DTT) 51.2 (+DTT)	1.08	0.047
R6	sample 6 rearranged with LiCl	17.7 (−DTT) 39.6 (+DTT)	1.12	0.45[b]

sample	additional reaction time with NBD-Cl (min)	ATPase act. (μmol of ATP mg^{-1} min^{-1})	n	r
7	17	8.10	1.34	0.20[b]
8	28	4.53	1.51	0.11[b]
9	49	1.39	1.62	0.035[b]

[a] The labeling was conducted in a darkened room at 25 °C in Hepes–NaOH buffer, pH 7.0, containing 2 mM EDTA, 5 mM ATP, and 25% glycerol. r = ratio of the specific activity of the labeled enzyme to that of the unlabeled control; n = molar ratio of label to F_1. [b] Because of the partial denaturation of F_1 by LiCl treatment, the r values of the rearranged samples were computed by using 39.6 μmol of ATP mg^{-1} min^{-1} as the specific activity of the control.

According to the model with nonequivalent sites, a switch of the catalytic role of the NBD-suppressed *O*-NBD-β' subunit with the auxiliary role of an unlabeled β'' subunit would be expected to change the highly inhibited ATPase *O*-β'-NBD-F_1 to the almost fully active enzyme *O*-β''-NBD-F_1.

Further Labeling of O-β',β''-NBD-F_1 with [^{14}C]NBD-Cl. In order to reach a decisive conclusion, the possibility should also be examined that the observed reactivation of *O*-β'-NBD-F_1 by LiCl treatment may be due to the changing of the NBD label to a nonintefering conformation while remaining covalently attached to Tyr-β'311. If that is indeed the case, the regenerated ATPase activity will not be inhibited again by additional labeling with NBD-Cl under the same conditions. On the other hand, if the regenerated activity is due to the switching of roles of a labeled β' subunit with an unlabeled β'' subunit, the regenerated activity will again be inhibited by a calculated amount of additonal labeling with NBD-Cl. The diagnostic experiment has been performed and the results are summarized in Table II.

In order to gain a clearer insight into the above question, the observed values of n and r are plotted in Figure 4, which reveals the following information: For direct labeling of F_1 by NBD-Cl, the linear plot of r vs. n extrapolates precisely

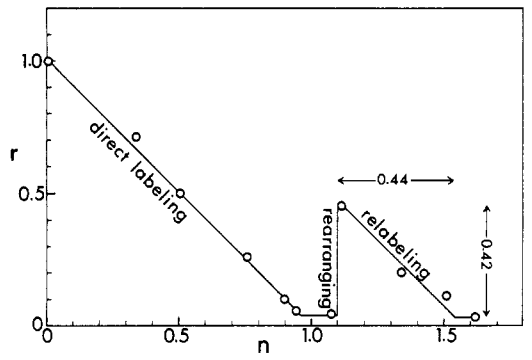


FIGURE 4: Changes in n and r due to direct labeling of F_1-ATPase by NBD-Cl, LiCl-induced rearrangement, and subsequent relabeling. Experimental conditions are given in footnote *a* of Table II.

to $n = 1$ for complete inhibition, indicating strict specificity of labeling from $n = 0$ to $n = 0.95$. Further labeling increases n without changing r. Forced rearrangement of the β subunits of labeled F_1 by LiCl treatment changes its r from 0.047 to 0.45 without significant change in n. Further labeling brings r again down to 0.035. The difference between $\Delta n = 0.44$ and $-\Delta r = 0.42$ could be due to either experimental error or 5% nonspecific labeling. Therefore, the reactivation of *O*-β'-NBD-F_1 by LiCl treatment cannot be simply due to conformation change in the labeled β' subunit.

Different O → N Transfer Rates in O-β′-NBD-F_1 and O-β″-NBD-F_1. The spontaneous transfer of NBD label from Tyr-β311 to Lys-β162 in the labeled enzyme is accompanied by the appearance of fluorescence (Ferguson et al., 1975; Wu & Wang, 1986). The progress of this O → N transfer reaction in the dark can be monitored by measuring the fluorescence intensity at 520 nm. Figure 5A shows that this O → N transfer in *O*-β'-NBD-F_1 was almost complete in 2 h at pH 9 and 25 °C. If we use the fluorescence intensity f at 2.1 h as the approximate limiting value f_∞ of the fluorescence intensity at $t = \infty$ and plot log $(f_\infty - f)$ vs. time, we obtain a half-time of about $^1/_2$ h for this transfer reaction.

On the other hand, similar measurements with *O*-β''-NBD-F_1 gave completely different results. Figure 5B shows that after *O*-β''-NBD-F_1 had been incubated at pH 9 for 2 h in the dark there was still no convincing evidence of any increase in its fluorescence due to the O → N transfer, even though cleavage of this labeled enzyme with pepsin at $t = 0$ and subsequent analysis showed that most of the [^{14}C]NBD label was still attached tto Tyr-β311.

The contrastingly different O → N transfer rates in the above two isomeric forms of labeled F_1 would be very difficult to understand if we had adhered to the notion of equivalent β subunits, for the transfer to take place either within the same β subunit or between two β subunits. But the data in Figure 4 could be reasonably accounted for if we accept the model with nonequivalent β subunits. The failure to observe a similar transfer in *O*-β''-NBD-F_1 could be simply due to the fact that the labeled Tyr-β''311 is not sufficiently near Lys-β'162 or the other Lys-β''162 for intersubunit transfer and that Tyr-311 in either β'' subunit is too far from its own Lys-162 for intrasubunit transfer.

In order to get an order of magnitude estimate of the intrasubunit O → N transfer rate, *O*-[^{14}C]NBD-β was prepared as previously described (Wang, 1985) and used over a 100-fold concentration range for similar fluorescence measurements. The results (not shown) are indistinguishable from those in Figure 5B. Therefore, we may conclude that intrasubunit transfer of the label does not take place in *O*-NBD-β at appreciable rates.

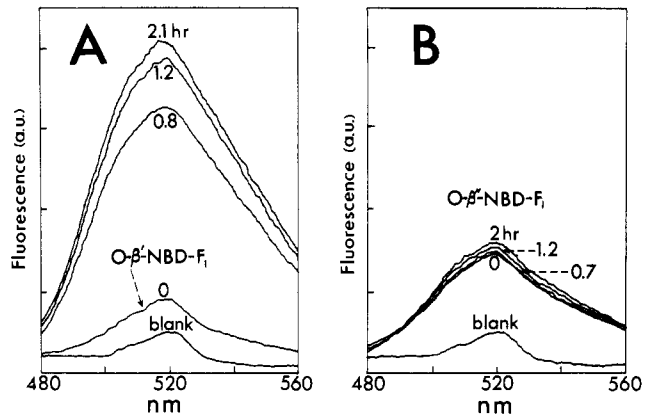


FIGURE 5: Contrasting O → N transfer rates for the *O*-NBD label in *O*-β'-NBD-F_1 and in *O*-β''-NBD-F_1. The extent of the transfer reaction was followed by monitoring the increased intensity of fluorescence emission due to the *N*-NBD label, with 440-nm exciting light of fixed intensity. The transfer reaction was started by adding a calculated amount of 1 M triethanolamine solution to a solution of *O*-β'-NBD-F_1 or *O*-β''-NBD-F_1 in buffer A at $t = 0$ to raise the pH of the mixture to 8.95. Fluorescence emission spectra of the mixtures were taken at the indicated time intervals. Composition of the solutions: (A) 0.1 μM *O*-β'-NBD-F_1 ($n = 1.00$) in buffer A containing 0.167 M triethanolamine, pH = 8.95 [total label] = 0.1 μM; (B) 0.27 μM *O*-β''-NBD-F_1 ($n = 0.36$) in the same medium as (A), [total label] = 0.1 μM.

Discussion

The existence of two geometric isomers of *O*-NBD-F_1 with different properties, where one isomer is fully active and the other isomer is completely inactive in catalyzing the hydrolysis of ATP, leaves little room for doubt that the β subunits in F_1 are functionally not equivalent. Experimental data show that the reactivation of *O*-NBD-F_1 by LiCl treatment is neither due to migration of the NBD label to a nonessential Tyr residue (Figure 3) nor due to changes in secondary and tertiary protein structure (Figure 4), but is probably indeed due to a salt-induced change in the quaternary structure of the enzyme which causes the labeled catalytic subunit and the unlabeled auxiliary subunit to switch their roles. The initial inactivation of F_1 by a single *O*-NBD label, the subsequent reactivation of the inhibited enzyme by LiCl treatment with the label remaining attached to Tyr-β311, and the reinhibition of the reactivated enzyme by further titration with NBD-Cl can all be accounted for quantitatively by assuming that Tyr-β'311 is an essential functional group at a single site in F_1 for ATP hydrolysis.

Energy-transfer measurements reported in the preceding paper (Wu & Wang, 1986) show that when the ADP bound to β'' in F_1 is replaced by ATP, the energy-transfer efficiency is increased by 11%. The observation gives and indication of the direction of structural change at the catalytic site. This conformation change caused by the binding of additional ATP could be responsible for its striking promotional effect on the hydrolytic efficiency of F_1 during the transition from unisite to multisite catalysis (Grubmeyer et al., 1982; Cross et al., 1982) as well as for the regulatory effect of ATP or ADP bound to β'' subunit(s) in steady-state hydrolysis of ATP (Di Pietro et al., 1980).

Recently Harris and Co-workers (1985) isolated the β subunit from *Rhodospirillum rubrum* F_1 (RF_1), which has a V_{max} for the hydrolysis of MgATP similar to that of RF_1 and a V_{max} for the hydrolysis of CaATP only 5000-fold lower than that for RF_1. Their discovery also suggests the possibility that the intrinsic ATPase activity of mitochondrial F_1 (MF_1) could be suppressed for unisite catalysis but enhanced by a

factor of 10^6 (Cross et al., 1982) for promoted catalysis due to ligand-dependent interaction between the subunits, which could alter the rates of product release. This interaction could be weakened and consequently the functional differentiation between the hydrolytic and the regulatory sites partially removed if the ATP and/or ADP are replaced by less precisely fit substrates such as TNP-ATP (Grubmeyer & Penefsky, 1981).

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