Chemical Evidence for Probably Nonequivalent β Subunits in F_1 Adenosinetriphosphatase†

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ABSTRACT: Mitochondrial F_1 adenosinetriphosphatase (MF_1) was allowed to react with 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl) until its NBD label to MF_1 molar ratio (n) reached approximately 2.6. The labeled enzyme was then separated and subsequently allowed to react with dithiothreitol (DTT) in a controlled way to have its covalent label partially removed. At various stages of removal of its covalent label, the enzyme was separated and assayed for its value of n and the ratio (r) of specific ATPase activity of the DTT-treated enzyme to that of the unlabeled control sample. Most of the experimental values of r turned out to be significantly higher than the theoretical maximum values for models of the enzyme with three equivalent β subunits, which have been shown to be equal to $(1 - n/3)^3$ for three alternating sites and $(1 - n/3)^2$ for two alternating sites. On the other hand, the observed values of n and r are consistent with a model of the enzyme based on nonequivalent β subunits, with one active catalytic site and two latent catalytic sites that normally have only regulatory function. Possible complication in the interpretation of data due to a significant amount of nonspecific labeling by NBD-Cl has also been examined and discussed. In addition, the NBD label has been used, after its transfer from the essential Tyr to the essential Lys, as an internal fluorescent probe to monitor protein conformation change at the active site of MF_1. Experimental data show that the binding of adenine nucleotide at the latent site(s) can cause conformational change at the active site and presumably in this way regulate the catalytic property of the active site.

All the F_1-ATPases that have been studied conclusively seem to have the subunit stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$, where α, β, γ, δ, and ϵ represent subunits of decreasing molecular weight (Lambeth & Lardy, 1971; Senior & Brooks, 1971; Pedersen, 1975; Kagawa et al., 1976; Yoshida et al., 1978; Bragg & Hou, 1980; Foster & Fillingame, 1982). Because the three β subunits in each F_1 molecule have identical amino acid sequence (Saraste et al., 1981; Kanazawa et al., 1982; Runswick & Walker, 1983), it is often assumed that they are also functionally equivalent in the enzyme. This assumption is supported by ligand binding measurements that suggest that there is a nucleotide binding site on each of the three β subunits (Esch & Allison, 1979; Cross & Nalin, 1982; Satre et al., 1982). Kinetic studies showed strong interaction between the active sites (Grubmeyer et al., 1982; Cross et al., 1982).

Each F_1-ATPase molecule is completely inactivated when one of its three β subunits is labeled by 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl)[1] (Ferguson et al., 1975a) or *N*,*N*′-dicyclohexylcarbodiimide (Pougeois et al., 1979; Satre et al., 1979). This observation can be readily accounted for by the widely accepted model of F_1-ATPase based on equivalent β subunits with three alternating and interacting sites (Gresser et al., 1982). In this model, the three equivalent and interacting catalytic sites are assumed to participate in sequence with each site going through three major reaction stages in the catalytic cycle. Accordingly, inactivation of any one of the three sites by covalent labeling is expected to block the whole catalytic cycle. Strong subunit interaction could make the rate of labeling of the first β subunit in each F_1 molecule much faster than that for the other two. Consequently, during the inactivation reaction the labels tend to be covalently attached to separate F_1 molecules and block their catalysis until the overall molar ratio of label to F_1 approaches 1. Further labeling will not have much effect on the observed ATPase activity, because the catalytic cycles of most F_1 molecules are already blocked.

On the other hand, the same observation can also be accounted for by models of F_1-ATPase based on interacting but nonequivalent β subunits (Soong & Wang, 1984; Williams et al., 1984). The nonequivalence of intrinsically identical β subunits in an F_1 molecule could arise from the unique way in which one of the β subunits, denoted by β', interacts with the single γ, δ, or ϵ subunit or with the three α subunits (Amzel et al., 1982). In one of such models, it is assumed that β' has an active catalytic site with enhanced reactivity and that each of the other two β subunits, denoted by β'', has an interacting latent catalytic site that binds ATP or ADP and regulates the reactivity of the active catalytic site on β' (Soong & Wang, 1984). Because of its enhanced reactivity, the essential Tyr in β' is expected to be labeled faster than the corresponding residues in the two latent β'' when F_1-ATPase is treated with NBD-Cl. Accordingly, it is also possible to have almost complete inactivation of the enzyme when the overall molar ratio of label to F_1 is equal to 1. Further labeling also will not have much effect on the observed ATPase activity, because the two β'' subunits are latent and normally do not catalyze the hydrolysis of ATP directly.

For the above reasons, it has not been possible to decide between the two types of models for F_1-ATPase from the observed degree of inhibition of ATPase activity when the enzyme is labeled directly with NBD-Cl or DCCD. However, when O-NBD-labeled F_1 with the molar ratio of label to F_1

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[1] Abbreviations: DCCD, *N*,*N*′-dicyclohexylcarbodiimide; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; LDH, L-lactic dehydrogenase; Mes, 2-(*N*-morpholino)ethanesulfonic acid; MF_1, mitochondrial F_1 adenosinetriphosphatase; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole (also named 4-chloro-7-nitrobenzofurazan); N-NBD-MF_1, MF_1 labeled by NBD-Cl at an amino group; O-NBD-MF_1, MF_1 labeled by NBD-Cl at a Tyr residue; n, the molar ratio of NBD label to MF_1; PEP, phosphoenolpyruvate; PK, pyruvate kinase; PMB, *p*-(hydroxymercuri)benzoate; r, ratio of the specific activity of the labeled enzyme to that of the unlabeled control sample.

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approximately equal to 3 is treated with dithiothreitol (DTT) in a controlled way so that about one-third of all the labels are removed, the fractional recovery of original ATPase activity predicted by the two types of models may be quite different. According to the models based on equivalent β subunits, the maximum fraction of original ATPase activity recoverable by this procedure can be calculated in appropriate ways, depending on whether the catalysis requires three alternating sites, two alternating sites, or nonalternating sites. According to the above model with nonequivalent β subunits, the O-NBD label on the more reactive β' subunit could be removed at a faster rate than those on the two latent β'' subunits. If so, the fractional recovery of original ATPase activity can also be estimated from the fraction of NBD label removed by DTT and the fraction of O-NBD label that has been transferred to the essential Lys in the same subunit and consequently is not removable by DTT. Experiments designed with this rationale have been conducted, and the results are presented in this paper.

Experimental Procedures

Materials

ATP, ADP, NADH, phosphoenolpyruvate, pyruvate kinase, lactic dehydrogenase, Hepes, Mes, EDTA, *p*-(hydroxymercuri)benzoate, NBD-Cl, DTT, and Sephadex G-50, 80 mesh, were purchased from Sigma Chemical Co., and [^{14}C]NBD-Cl was from Research Products International Corp.

Mitochondria were prepared from fresh bovine heart (Low & Vallin, 1963). Mitochondrial F_1-ATPase (MF_1) was prepared and stored as described by Knowles & Penefsky (1972).

Methods

Preparation of O-[^{14}C]NBD-MF_1. In order to minimize the spontaneous transfer of NBD label from the essential Tyr to the nearby essential Lys (Ferguson et al., 1975b), the labeling reaction was conducted at pH 7.0 in the dark. In a typical experiment, 0.3 mL of MF_1 in ammonium sulfate suspension (21 mg/mL) was centrifuged. The pellet was dissolved in 0.35 mL of buffer A (50 mM Hepes–NaOH, pH 7.0, 2 mM EDTA, 25% glycerol) and centrifuge-filtered through Sephadex G-50, 80 mesh (Penefsky, 1977), that had been preequilibrated with the same buffer. A 50-μL aliquot of [^{14}C]-NBD-Cl solution in acetone (specific radioactivity 109 mCi/mmol, 2.3 mM) was evaporated to dryness in a polypropylene vial by a stream of nitrogen. The residue was allowed to react with the desalted MF_1 solution in the dark at 25 °C. The progress of reaction was monitored by taking 20-μL aliquots of the mixture at intervals, centrifuge filtration of each sample through Sephadex G-80, 50 mesh, to remove free [^{14}C]NBD-Cl, and determination of the radioactivity, protein concentration, and ATPase activity of the enzyme in the filtrate. The specific activity of this particular batch of unlabeled MF_1-ATPase was 79.3 units/mg (79.3 μmol of ATP hydrolyzed min^{-1} mg^{-1}) when dissolved in 50 mM Hepes–NaOH buffer, pH 8.0, containing 0.25 M sucrose, 2 mM EDTA, and 5 mM ATP. When dissolved in buffer A, its initial specific activity was 53.1 units/mg, which steadily dropped to 46.3 units/mg during the first 20 h but then remained constant at this value for the next 90 h when stored in a polypropylene vial at room temperature.

At 1.3-h reaction time, one-third of the above reaction mixture was centrifuge filtered through preequilibrated a Sephadex G-50, 80 mesh, column, and the labeled enzyme in the filtrate was assayed for ATPase activity, protein concentration, and radioactivity. The labeling reaction in the remainder of the mixture was allowed to proceed in the dark and was terminated by gel filtration after 8 h of reaction time when the observed value of n = label to MF_1 molar ratio was between 2.5 and 3.

Preparation of N-[^{14}C]NBD-MF_1. The above solution of labeled enzyme, which was separated by centrifuge filtration through Sephadex G-50, 80 mesh, at 1.3-h reaction time, was mixed with half its volume of 0.5 M Hepes–NaOH buffer (pH 9.3), and the final mixture (pH 9) was incubated in the dark for 2 h at 34 °C to facilitate the transfer of the O-NBD label to the nearby Lys. After 2 h of incubation, the mixture was again centrifuge filtered through Sephadex G-50, 80 mesh, that had been preequilibrated with buffer A. The product, with a label to MF_1 molar ratio of 0.75, was kept in the dark for later fluorescence studies.

Enzymatic Assays. Protein concentrations were determined by the Coomassie Blue binding method (Bradford, 1976). Radioactivity was assayed by liquid scintillation counting. The molar ratio of label to MF_1 was calculated by using a molecular weight of 3.5×10^5 for MF_1 and the known specific radioactivity of the label.

The ATPase activity was assayed by an ATP-regenerating system coupled to the oxidation of NADH. The assay medium contained 50 mM Hepes–NaOH, pH 8.0, 3 mM $MgCl_2$, 50 mM KCl, 2 mM ATP, 2 mM PEP, 0.4 mM NADH, 21 units/mL PK, and 11 units/mL LDH. The rate of ATP hydrolysis was computed from the linear decrease of A_{340} due to the oxidation of NADH.

Reaction of Labeled Enzyme with DTT. The reaction for the partial removal of O-NBD label was started by injecting a calculated amount of DTT solution into a solution of the labeled enzyme in buffer A and terminated by the injection of *p*-(hydroxymercuri)benzoate (PMB, final concentration 1.0 or 2.5 mM). Preliminary experiments showed that 1 mM PMB has no effect on the specific activity of either MF_1 or N-NBD-MF_1. But the samples were still centrifuge-filtered before being assayed.

Fluorescence Measurements. The strongly fluorescent N-NBD label can be used as an internal probe for monitoring conformation change at the active site. A Hitachi Perkin-Elmer fluorescence spectrophotometer, Model MPF-2A, was used to measure the fluorescence emission of N-NBD-MF_1 with 440-nm excitation light at a slit width of 6 nm and a protein concentration of 0.05 mg/mL. Calibration measurements show that the intensity of fluorescence increases linearly with the concentration of N-NBD-MF_1 in the range from 0 to 0.05 mg of protein/mL.

Results and Discussion

First NBD Label in MF_1-ATPase. The labeled enzyme obtained after 1.3 h of reaction of MF_1-ATPase with [^{14}C]-NBD-Cl as described under Methods was found to have the following characteristics: label to MF_1 molar ratio (n) = 0.85; sp act. of the ATPase = 7.92 units/mg when assayed in the absence of DTT and 40.7 units/mg when assayed in the presence of 2.5 mM DTT; the ratio (r) of the specific activity of labeled enzyme to that of the control MF_1 = 0.15 when assayed in the absence of DTT and 0.88 when assayed in the presence of DTT.

The observed value of r = 0.88 in the presence of DTT suggests that 12% of the NBD label may be already on the essential Lys at this stage of reaction. The observed value n = 0.85 suggests that 15% of the enzyme is still unlabeled at this stage of the reaction. Therefore, the directly observed value of r = 0.15 when assayed in the absence of DTT supports the previous conclusion that each MF_1-ATPase molecule is completely inactivated by a single NBD label.

Table I: Removal of Radioactive Label from ([^{14}C]NBD)$_{2.6}$MF$_1$ by DTT as a Function of Reaction Time[a]

reaction time (min)	n[b]	sp act. (μmol of ATP min^{-1} mg^{-1})	r[c]	$(1 - n/3)^3$	$(1 - n/3)^2$	$1 - n/3$
0	2.62	0.643	0.0144	0.002	0.016	0.127
0.5	1.22	31.6	0.71	0.21	0.35	0.59
1.5	1.20	31.5	0.71	0.22	0.36	0.60
2	1.17	30.1	0.67	0.23	0.37	0.61
3	1.06	31.8	0.71	0.27	0.42	0.65
5	0.99	30.9	0.69	0.30	0.45	0.67

[a] The reaction was started by mixing 400 μL of a solution of the labeled enzyme (6.94 mg/mL) in 50 mM Hepes–NaOH buffer (pH 7.0) containing 2 mM EDTA and 25% glycerol with equal volume of 1.0 mM DTT solution in the same buffer in the dark at 25 °C. At programmed intervals, 80-μL aliquots of the reaction mixture were taken out and mixed with equal volume of 1.0 mM *p*-(hydroxymercuri)benzoate solution in the same buffer to stop the reaction. The resulting mixtures were centrifuge filtered through a 3-mL Sephadex G-50, 80 mesh, column that had been preequilibrated with the same buffer and assayed for protein concentration, radioactivity, and ATPase specific activity. [b] n = ratio of moles of NBD label to mole of MF$_1$. [c] r = ratio of specific activity of NBD-labeled MF$_1$ to specific activity of control MF$_1$.

Table II: Removal of Radioactive Label from ([^{14}C]NBD)$_{2.6}$MF$_1$ by 1-min Reaction with DTT as a Function of Reagent Concentration[a]

[DTT] (mM)	n[b]	sp act. (μmol of ATP min^{-1} mg^{-1})	r[c]	$(1 - n/3)^3$	$(1 - n/3)^2$	$1 - n/3$
0	2.59	0.477	0.011	0.0026	0.019	0.14
0.1	2.04	18.9	0.44	0.033	0.10	0.32
0.2	1.89	25.5	0.59	0.049	0.14	0.37
0.3	1.82	30.3	0.70	0.061	0.16	0.39
0.5	1.66	33.2	0.77	0.089	0.20	0.45
1.0	1.38	33.7	0.78	0.16	0.29	0.54
3.0	1.14	30.7	0.71	0.24	0.38	0.62
10.0	1.03	33.0	0.76	0.28	0.43	0.66

[a] Each reaction was started by mixing 40 μL of a solution of the labeled enzyme (4.68 mg/mL) in 50 mM Hepes–NaOH buffer (pH 7.0) containing 2 mM EDTA and 25% glycerol with equal volume of a DTT solution in the same buffer at 25 °C in the dark. After 1 min, the reaction was terminated by the addition of 80 μL of 5 mM *p*-(hydroxymercuri)benzoate solution in the same buffer. The resulting mixture was centrifuge filtered immediately through a 3-mL Sephadex G-50, 80 mesh, column that had been preequilibrated with the same buffer. Each filtrate was assayed for protein concentration, radioactivity, and ATPase specific activity. [b] n = ratio of moles of NBD label to mole of MF$_1$. [c] r = ratio of specific activity of NBD-labeled MF$_1$ to specific activity of control MF$_1$.

Table III: Removal of Radioactive Label from ([^{14}C]NBD)$_{3.67}$MF$_1$ and ([^{14}C]NBD)$_{4.27}$MF$_1$ by 1-min Reaction with DTT[a]

[DTT] (mM)	n_0	n	sp act. (μmol of ATP min^{-1} mg^{-1})	r	$[(n_0 - n)/3]^3$	$[(n_0 - n)/3]^2$
0	3.67		0.75	0.018		
0.20	3.67	3.12	10.8	0.25	0.006	0.034
0.50	3.67	2.61	13.7	0.32	0.044	0.12
0	4.27		0.61	0.014		
0.20	4.27	3.41	9.3	0.22	0.024	0.082
0.50	4.27	2.81	13.8	0.32	0.12	0.24

[a] The reaction conditions and notation are same as those in Table II. n_0 represents the number of labels per enzyme molecule before the DTT treatment.

Controlled Removal of O-NBD Label from (NBD)$_{2.6}$MF$_1$ by DTT. In order to avoid the nonspecific labeling, the reaction between [^{14}C]NBD-Cl and MF$_1$ was terminated when the molar ratio n reached 2.6. Analytical and kinetic data on the controlled removal of O-[^{14}C]NBD label from ([^{14}C]-NBD)$_{2.6}$MF$_1$ by 0.5 mM DTT as a function of reaction time are summarized in Table I. Similar data on the removal of the label by 1-min reaction with DTT as a function of concentration of the reagent are summarized in Table II.

Models Based on Equivalent β Subunits. The theoretical maximum values of specific activity ratio r can be deduced as functions of n according to the number of equivalent sites as follows.

(A) With Three Alternating Sites. Let us arbitrarily designate the three β subunits in each F$_1$ molecule as 1st, 2nd, and 3rd β subunit. The order of designation is immaterial, since they are equivalent. Let x and y represent the average numbers of label on the 1st and 2nd β subunits, respectively, of each MF$_1$ molecule. Then, the average number of label on the 3rd β subunit is given by $n - x - y$. Therefore, the fraction of the 1st β subunits that is not labeled is equal to $1 - x$, and those of the 2nd and 3rd β subunits are equal to $1 - y$ and $1 - (n - x - y)$, respectively.

Since all three sites must participate in the catalysis, the ratio r of the specific activity of the labeled enzyme to that of the control MF$_1$ is given by

$$r = (1 - x)(1 - y)(1 - n + x + y) \quad (1)$$

Although the number of possible values of r is infinite due to the continuous ranges of possible values of x and y, there is a theoretical maximum value r_{max} that can be obtained by setting

$$(\partial r/\partial x)_y = n - 2x - y - ny + 2xy + y^2 = 0 \quad (2)$$

and

$$(\partial r/\partial y)_x = n - 2y - x - nx + 2xy + x^2 = 0 \quad (3)$$

Simulatenous solution of eq 2 and 3 gives

$$x = n/3 \qquad y = n/3$$

and, hence

$$r_{max} = (1 - n/3)^3 \quad (4)$$

(B) With Two Alternating Sites. Similar consideration gives

$$r = (1/3)[(1 - x)(1 - y) + (1 - x) \times (1 - n + x + y) + (1 - y)(1 - n + x + y)] \quad (5)$$

To obtain r_{max}, we set

$$(\partial r/\partial x)_y = n - 2x - y = 0 \quad (6)$$

and

$$(\partial r/\partial y)_x = n - 2y - x = 0 \quad (7)$$

Simultaneous solution of eq 6 and 7 also gives

$$x = n/3 \qquad y = n/3$$

and, hence

$$r_{max} = 1 - 2n/3 + n^2/9 = (1 - n/3)^2 \quad (8)$$

(*C*) *With Nonalternating Sites.* Similar consideration gives only one theoretical value for a given n:

$$r = (1/3)[(1 - x) + (1 - y) + (1 - n + x + y)] = 1 - n/3 \quad (9)$$

Theoretical values of r_{max} calculated from eq 4 and 8 as well as the theoretical values of r calculated from eq 9 are also listed in Tables I and II for comparison. Since most of the observed values are significantly higher than the corresponding theoretical values in all three cases, we may conclude that the three β subunits in MF_1-ATPase are probably not all equivalent.

Model with Nonequivalent β Subunits. The model with one active subunit β' and two latent subunits β'' is consistent with the observed values of n and r listed in Tables I and II. Because of the enhanced reactivity of its active site in this model, β' is expected to be labeled before the two β'' subunits when MF_1 is treated with NBD-Cl, and r is expected to be approximately equal to $1 - n$ during the early phase of the reaction. For the same reason, the O-NBD label on β' may be removed first, with commensurate restoration of ATPase activity, during the early phase of the reaction when $([^{14}C]NBD)_{2.6}MF_1$ is treated with DTT. The data show that DTT removes the first radioactive label from $([^{14}C]NBD)_{2.6}MF_1$ rapidly and that the removal of the last 1.2 labels is much slower. Table II shows that from 70 to 75% of the original ATPase activity can be restored by removing, presumably mainly from β', an average of 0.77 to 0.93 radioactive label from each $([^{14}C]NBD)_{2.6}MF_1$ and that the ATPase activity of the enzyme remains essentially constant when additional radioactive labels are removed, presumably mainly from the β'' subunits, which do not have active catalytic sites.

The data also show that no more than 76% of the original ATPase activity can be restored to $([^{14}C]NBD)_{2.6}MF_1$ by treatment with DTT. This limitation can probably be attributed to the transfer of NBD label from the essential Tyr to the essential Lys in β'. Although the enzyme was labeled in the dark at pH 7.0 in order to minimize the production of N-NBD-labeled enzyme, the O → N transfer of NBD label probably had taken place to a certain extent during the preparation of the sample. N-NBD-MF_1 is known to exhibit a fluorescence emission maximum near 522 nm (Ferguson et al., 1975b). By measurement of the fluorescence emission at 519 nm stimulated by 440 ± 3 nm exciting light, it was found that about 10% of the labeled enzyme molecules were already in the form of N-NBD-MF_1 at 1.3-h reaction time before the pH of medium was raised from 7 to 9.

Effect of Nonspecific Labeling. Although during the preparation of $([^{14}C]NBD)_{2.6}MF_1$ the labeling reaction was terminated before n exceeded 3 in order to minimize nonspecific labeling, a small amount of nonspecific labeling could still have taken place. It is therefore necessary to reexamine the validity of the above conclusion in the presence of a limited amount of nonspecific labeling. For this purpose, a concentrated MF_1 solution (14.2 mg/mL) was allowed to react with an N_2-dried [^{14}C]NBD-Cl film until the number of covalent labels per enzyme molecule exceeded 3 substantially to saturate the specific sites as far as possible but did not exceed 3 too much to cause irrepairable damage to the enzyme. In one experiment, two samples with 4.98 and 5.56 labels, respectively, per enzyme molecule were obtained. After removal of the S-[^{14}C]NBD labels with PMB, these numbers decreased to 3.67 and 4.27, respectively, which obviously still included substantial amounts of nonspecific labeling. Each of these samples was then allowed to react with 0.2 and 0.5 mM DTT solution for 1 min as in the experiment of Table II. The results are summarized in Table III.

Since the observed values of r before DTT treatment (in Tables I and II) are much smaller than the theoretical values predicted by eq 9, the model with three equivalent nonalternating sites has already been ruled out. But it is necessary to reconsider the models with alternating sites. Let n_0 and n represent the respective numbers of labels per enzyme molecule before and after the DTT treatment. If the $n_0 - n$ labels removed by DTT are all from specific sites, the theoretical maximum values of r should be

$$r_{max} = [(n_0 - n)/3]^3 \quad (10)$$

for three alternating sites and

$$r_{max} = [(n_0 - n)/3]^2 \quad (11)$$

for two alternating sites.

If some of the $n_0 - n$ labels are removed from nonspecific sites that do not contribute to ATPase activity, the expected value of r will be even lower, and hence, eq 10 and 11 should still hold if the models with compulsory alternating sites are correct for the catalytic hydrolysis of ATP by MF_1.

Table III shows that in the presence of nonspecific labeling the observed values of r are still much higher than the theoretical maximum values predicted by eq 10 and 11. Actually, the case of two out of three equivalent sites alternating has already been ruled out by the observation that $r = 0.15$ when $n = 0.85$, mentioned at the beginning of this section, for such a model would predict a value of $r = (1/3)[(0.15(1) + (0.15)(1) + (1)(1)] = 0.43$. Therefore, we may still conclude that the three β subunits probably do not alternate during the steady-state hydrolysis of ATP by F_1-ATPase.

N-NBD Label as a Probe for Conformational Change at Catalytic Site. Figure 1 shows that ADP causes a large decrease in the fluorescence of $(N\text{-}NBD)_{0.75}MF_1$. This decrease is presumably not due to the quenching effect of ADP but due to ADP-triggered protein conformation change (Ferguson et al., 1975) at the labeled active site, because at the same ligand concentration ATP causes a slight increase in the fluorescence of a duplicate sample of the same labeled enzyme. This observation also indicates that the inhibition of ATPase activity by the N-NBD label is probably not due to steric prevention of necessary protein conformation change by the label but due to the labeling of an essential amino group that is required for catalysis.

The effect of varying the concentration of ADP or ATP on the ratio of fluorescence intensity in the presence of adenine nucleotide (F) to that in the absence of adenine nucleotide (F_0) is illustrated in Figure 2. Since according to the above discussion the initial labeling of MF_1 by NBD-Cl is almost entirely on the β'-subunit, the stoichiometric formula of this particular N-NBD-labeled enzyme may be more explicitly written as $\alpha_3\beta''_2[(N\text{-}NBD)_{0.75}\beta']\gamma\delta\epsilon$. Although the cause of continued slow decrease of fluorescence at ADP concentrations above 1 mM is still unknown, most of the decrease occurs in the range from 0.05 to 1 mM, which corresponds to an approximate dissociation constant $K_{ADP} \approx 0.2$ mM for the

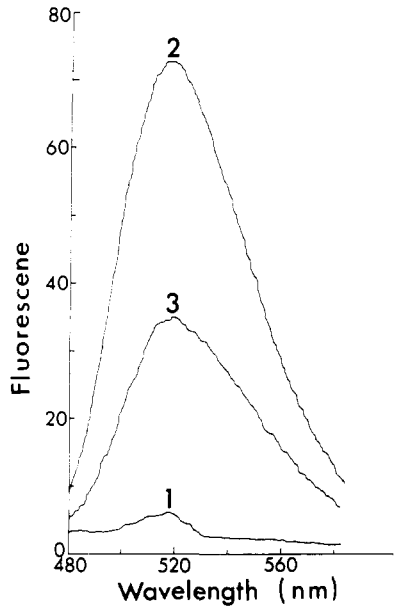

FIGURE 1: Effect of ADP on the fluorescence emission of $(N\text{-}NBD)_{0.75}MF_1$. The emission spectra were taken with 440 ± 3 nm excitation light of fixed intensity. Composition of solutions was as follows: (1) 0.05 mg of MF_1/mL in buffer A (50 mM Hepes–NaOH, 2 mM EDTA, 25% glycerol, pH 7.0); (2) 0.05 mg of $(N\text{-}NBD)_{0.75}MF_1$/mL in buffer A; (3) 0.05 mg of $(N\text{-}NBD)_{0.75}MF_1$/mL in buffer A + 4 mM ADP. The fluorescence emission spectrum of buffer A is the same as (1), which is due to Raman scattering by water.

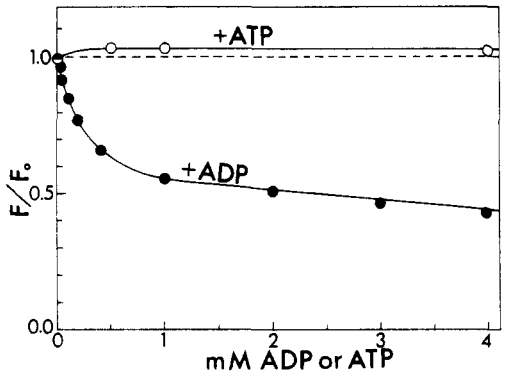

FIGURE 2: Dependence of the fluorescence of $(N\text{-}NBD)_{0.75}MF_1$ on concentrations of ADP and ATP. F is fluorescence intensity of the labeled enzyme in the presence of ADP or ATP; F_0 is fluorescence intensity of the labeled enzyme in the absence of ADP and ATP. Composition of solutions was 0.05 mg of labeled enzyme/mL in buffer A containing ADP or ATP at the indicated concentrations.

complex between ADP and the labeled enzyme in 50 mM Hepes–NaOH buffer (25% glycerol) at pH 7. Since this value is 2–3 orders of magnitude higher than that for the first ADP binding site of MF_1 determined by kinetic studies (Grubmeyer et al., 1982), we may conclude that the ADP that is responsible for the major portion of the observed fluorescence decrease in $\alpha_3\beta''_2[(N\text{-}NBD)_{0.75}\beta']\gamma\delta\epsilon$ is not bound to the active catalytic site on β' that carries the fluorescent probe but to the second and/or third site(s) on the two β'' subunits that do not carry the probe. Therefore, the present results not only are consistent with the concept of strong site–site interaction in MF_1 (Kayalar et al., 1977;Grubmeyer et al., 1982; Cross et al., 1982) but also show for the first time that the binding of adenine nucleotide to the regulatory β'' subunit(s) can cause a protein conformation change in the catalytic subunit β' and presumably in this way regulate the property of the catalytic site.

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Registry No. NBD-C, 10199-89-0; ADP, 58-64-0; ATP, 56-65-5; MF_1, 9000-83-3.

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