Specific Tryptophan Substitution in Catalytic Sites of Escherichia coli F1-ATPase Allows Differentiation between Bound Substrate ATP and Product ADP in Steady-state Catalysis*

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Tryphtophan was specifically inserted as the residue immediately preceding the P-loop sequence in F1-ATPase catalytic sites. The mutant enzyme (βF148W) showed normal enzymatic characteristics. The fluorescence responses of β-tryptophan 148 enabled us to differentiate between nucleoside di- and triphosphate bound in catalytic sites; MgADP quenched at 350 nm, whereas MgAMPPNP and MgADP-BeF\textsubscript{2} complex enhanced the fluorescence at 325 nm. With MgATP, both effects were seen simultaneously. This allowed analysis of bound catalytic site nucleotides directly under steady-state MgATP hydrolysis conditions. At mM concentration of MgATP (V\textsubscript{max} conditions) one of the three catalytic sites was filled with substrate MgATP and the other two sites were filled with product MgADP. A model for F1-ATPase steady-state turnover is presented that encompasses these findings.

Given the structural similarity of the P-loop in nucleotide-binding proteins, this approach may prove widely useful.

Bacteria, mitochondria, and chloroplasts contain a membrane-bound ATP synthase that is responsible for ATP synthesis coupled to oxidation or light capture reactions. In bacteria this enzyme may also act as an ATPase to generate the transmembrane proton gradient. Understanding the mechanism of ATP synthesis and ATP-driven proton pumping is the goal of many laboratories. While useful hypotheses have been generated (1, 2), none is yet widely accepted, because catalysis has not yet been described in sufficient experimental detail.

ATP synthases are composed of two sectors, the F0 which forms a trans-membrane proton conduction path, and the F1 which contains three catalytic nucleotide-binding sites, one on each of the three β-subunits. F1 may be isolated in soluble form and has proved valuable for experimental studies. A detailed structure of bovine mitochondrial F1 was recently resolved by x-ray crystallography (3). Ease of genetic manipulation has meant that the Escherichia coli enzyme has also been extensively studied (for reviews see Refs. 4–6), and because the sequences of the β-subunits are strongly conserved, we may conclude that their structure will be very similar from whatever source.

The three catalytic sites of E. coli F1 show widely different affinities for substrate MgATP (7). Substoichiometric amounts of MgATP bind very tightly to catalytic site one (K\textsubscript{d} = 10\textsuperscript{-10} M, Ref. 8). MgATP hydrolysis is very slow when only this first site is occupied ("unisite catalysis"). Because of the low dissociation rates of substrate and products, kinetic and thermodynamic parameters for unisite catalysis can be measured by using the centrifuge column technique to separate enzyme-bound from free ligands (9), and this approach has yielded considerable functional insights into catalysis, particularly when combined with mutational or environmental modification of the catalytic sites (10–13).

To achieve physiological catalysis rates, however, additional catalytic sites must be filled with MgATP ("multisite catalysis"; for reviews see Refs. 14, 15). Analysis of substrate binding parameters under multisite catalysis conditions required the development of a rapid, true equilibrium, analytical technique. Recently, we designed and applied such a technique (7, 16, 17). By specifically introducing a tryptophan residue into the adenine-binding subdomain of E. coli F1 catalytic sites, at position β331, and using its fluorescence to monitor catalytic site occupancy, we were able to show that (i) all three catalytic sites must be filled to obtain physiological MgATP hydrolysis rates; (ii) the rate of hydrolysis of MgATP when only two sites are filled is slow (0–2% of V\textsubscript{max}); and (iii) in absence of Mg\textsuperscript{2+} (conditions under which there is no hydrolysis) the pronounced binding cooperativity observed with MgATP is absent and all three sites bind ATP with equal affinity.

The next logical step in analysis of multisite catalysis is to determine, under steady-state MgATP hydrolysis conditions, what fraction of the three catalytic sites is filled with MgATP and what fraction with MgADP. A prerequisite would be a probe that can differentiate between bound MgATP and bound MgADP. Due to its location in the adenine-binding subdomain of the catalytic sites, residue βTrp-331 responds to binding of the base moiety, and its fluorescence is virtually completely quenched upon addition of MgATP, MgADP, MgAMPPNP, MgTNP-ATP, free ATP, free ADP, or free TNP-ATP (7, 16, 17). Thus, no differentiation between bound nucleoside di- or triphosphate is possible on the basis of the βTrp-331 fluorescence signal.

Model experiments have suggested that tryptophan fluorescence is susceptible to the electric field of a charge in the immediate environment (18). Thus, a tryptophan residue located close to the γ-phosphate might respond differently to bound MgATP versus MgADP. As shown by comparison of several known protein structures (19), and confirmed in the x-ray structure of bovine mitochondrial F1 (3), the "Homology A" or "P-loop" motif (20) surrounds the phosphates of a bound nucleotide molecule. Therefore, to obtain an optical probe with the desired properties, we decided to introduce tryptophan residues close to the P-loop in the catalytic sites of F1.

At first glance, none of the amino acid residues within the P-loop in E. coli F1 catalytic sites (β148GGAGVGKT\textsuperscript{154}) appeared to be a promising candidate, i.e. where replacement by Trp would preserve enzyme function. One possible exception was βVal-153, since it was shown that substitution of this Val by Tyr in yeast F1 gave partially functional enzyme (21). Other
βTrp-148 in F1 Catalytic Sites Distinguishes ATP from ADP

candidates, not within but very close to the P-loop, were βPhe-148 and βAsn-158. The latter is Leu in mitochondrial and chloroplast F1. Therefore, we generated β148W, βV153W, and βN158W mutants, and to increase the signal-to-noise ratio for fluorescence experiments the mutations were expressed from plasmid pOW1, in which all of the nine natural tryptophan residues present in wild-type F1, have been replaced (22). In this paper we describe the effects of the mutations in whole cells and the characteristics of the isolated mutant F1 enzymes, particularly in regard to their fluorescence properties.

EXPERIMENTAL PROCEDURES

Construction of Mutant Strains—Site-directed mutagenesis was performed by the method of Vandeber et al. (23) using the T7-GEN mutagenesis kit (U.S. Biochemical Corp.). The template was the HindII-KpnI (uncG DC) fragment taken from plasmid pOW1 (22) and ligated into M13mp18. The mutagenic oligonucleotides were (i) AGTGGTTCTGTGGGTTGCAG to create the mutation β148W (changes from wild-type are indicated in boldface), (ii) GTGGCGGCTGTTGGTAAAA for βV153W, and (iii) GCTAAACGGCATGATGATGGA for βN158W. Phage recombinative forms containing the desired mutations were identified by DNA sequencing. The resulting plasmids pCB1 (containing the mutation β148W), pCB2 (βV153W), and pCB3 (βN158W) were transformed into strain An888 (24), to give strains CB1, CB2, and CB3, respectively.

Enzyme Purification and Characterization—Membrane vesicles were prepared according to Ref. 25, and F1 was purified as described in Ref. 26. Purity and subunit composition of the F1 preparations were checked by SDS-gel electrophoresis (27). Protein concentration of membrane preparations was determined according to Ref. 28 and that of F1 solutions using the Bio-Rad protein assay (29). The molecular mass of F1 was taken to be 382,000 Da.

Assays of Enzyme Function—Growth yield analyses in limiting glucose (3 mM) were performed as described in Ref. 30. pH gradient formation in membrane vesicles was measured using acridine orange fluorescence quenching (31). ATPase activities were assayed in 50 mM Tris/H2SO4, pH 8.5, at 30°C. Km values for MgATP were determined in 50 mM Tris/H2SO4, pH 8.0, at 23°C, by fluorescence measurements carried out at 23°C in a SPEX Fluorolog 2 spectrophotometer equipped with DATAMAX software. The buffer was 50 mM Tris/H2SO4, pH 8.0. The enzyme was pre-equilibrated in this buffer by consecutive passage through two 1-ml Sephadex G-50 columns.

In all experiments, the excitation wavelength was 295 nm; background signals (buffer, Raman scatter) were subtracted. Inner filter and volume effects were corrected for by conducting parallel titrations with wild-type F1. The emission spectra shown in Fig. 1 were corrected as described (34); all other spectra are uncorrected. The concentration of MgATP and MgAMPPNP bound to β148W F1, after MgATP addition was determined as follows. The fluorescence (F) was measured at 325 and 350 nm. At these wavelengths the relative fluorescence of MgATP-saturated enzyme (F<sub>MgATP</sub>) was 0.967 and 0.882, respectively, setting the fluorescence of the enzyme before MgATP addition to 1.000 at each wavelength. For MgATP-saturated F1, the respective values (F<sub>MgATP</sub>) were assumed to be 1.000 and 1.000 (from fluorescence data obtained with MgAMPPNP and MgADP·Be<sup>2+</sup>). The number of catalytic sites filled with MgATP (N<sub>MgATP</sub>) or MgADP (N<sub>MgADP</sub>) was then calculated by solving Equation 1:

\[
F = (N_{\text{MgATP}} \cdot F_{\text{MgATP}}) + (N_{\text{MgADP}} \cdot F_{\text{MgADP}}) + (3 - N_{\text{MgATP}} - N_{\text{MgADP}}) / 3 \quad (\text{Eq. 1})
\]

for the fluorescence data obtained at both wavelengths. This equation makes the following assumptions. 1) The fluorescence values at saturation reflect nucleotide binding to all three catalytic sites. This assumption is strongly supported by our previous work with the β331W mutant F1 (7). 2) All three βTrp-148 residues show the same response on binding of a given nucleotide. As noted under "Results," this assumption was found to be supported for MgATP but may cause underestimation of MgADP binding stoichiometry.

RESULTS

Effects of the Mutations β148W, βV153W, and βN158W on the Function of ATP Synthase in Vivo—Each of the mutations was generated by site-directed mutagenesis as described under "Experimental Procedures" and then transferred to plasmid pOW1 and expressed in strain An888. Plasmid pOW1, which was constructed previously (22), generates tryptophan-free F1 when expressed in strain An888, thus for each of the new mutant strains the introduced Trp is the only Trp residue in F1. Effects of the mutations on ATP synthase by oxidative phosphorylation in vivo were assessed from growth of the mutant strains on succinate plates and from growth yields in medium containing limiting (3 mM) glucose. As can be seen from Table I (columns 2 and 3), only the mutation β148W gave normal growth. The mutation βV153W resulted in partial impairment of growth, and βN158W in almost complete impairment.

Effects of the Mutations on ATPase Activity in Membranes—Membrane vesicles were prepared and membrane ATPase activities were assayed (Table I, column 4). In agreement with the growth characteristics, the ATPase activity of membrane vesicles from β148W mutant was identical to that of membranes containing the parental tryptophan-free enzyme (pOW1/An888) and actually higher than that of wild-type. The mutation βV153W gave ~80% of wild-type ATPase activity, and the mutation βN158W gave less than 10% assay of ATP-driven proton pumping by measurement of quenching of acridine orange fluorescence indicated that the βV153W and βN158W mutations impaired this function significantly, whereas ββ148W mutant was similar to wild-type (data not shown).

Purified soluble F1 was prepared from each mutant. Oligomeric stability of all three enzymes, as judged from pH dependence of ATPase activity (36), appeared normal up to at least pH 8.5. On SDS gels, all three enzymes showed a high degree of purity and normal subunit composition (with the "heavier" γ-subunit observed previously in tryptophan-free F1; see Ref. 22). The V<sub>max</sub> ATPase activities together with the K<sub>m</sub> and k<sub>cat</k> values are given in Table I, and it is evident that β148W F1 has catalytic characteristics fairly similar to wild-type, whereas both βV153W and βN158W F1 are significantly impaired.

| Mutation | Growth on succinate | Growth yield in 3 mM glucose | Membrane ATPase |
|----------|---------------------|-----------------------------|-----------------|
| Wild-type | ++ + + +             | 100                         | 3.6             |
| Unc<sup>a</sup> | –                 | 55                           | 0.01            |
| βF148W<sup>b</sup> | ++ + +             | 94                          | 4.8             |
| βV153W<sup>c</sup> | + +               | 72                          | 2.9             |
| βN158W<sup>c</sup> | – + +              | 59                          | 0.34            |
| pOW1/AN888<sup>c</sup> | ++ + +            | 96                          | 4.8             |

<sup>a</sup>The wild-type strain was pDP34/AN888 (35).
<sup>b</sup>The Unc<sup>−</sup> strain was pUC118/AN888.
<sup>c</sup>Each of the mutations was carried on plasmid pOW1 in strain AN888. In plasmid pOW1 all the natural tryptophans in F1 have been replaced (22). Thus, strain pOW1/AN888 expresses tryptophan-free F1, and in the mutant strains the introduced Trp is the only Trp in F1.
Fluorescence Properties of βV153W and βN158WF1—The corrected tryptophan fluorescence spectra (λexc = 295 nm) of βV153W and βN158WF1 are shown in Fig. 1, A and B. The emission maximum of βV153WF1 is at 325 nm and that of βN158WF1 is at 322 nm. These wavelength positions indicate that the environment of both introduced tryptophan residues is highly nonpolar. Addition of MgADP to βV153WF1 resulted in quenching of fluorescence (Fig. 2A), reaching about 15% at saturation, without noticeable shift in the spectrum. The fluorescence response was the same with MgATP and MgAMP-PNP. The fluorescence of βN158WF1 was quenched maximally by about 20% upon MgADP binding (Fig. 2B), and the quenching was accompanied by a slight red-shift (~2 nm). Again, MgATP and MgAMP-PNP elicited the same response. Although these signals could be used to measure nucleotide binding parameters, a far superior probe is already available for this purpose, namely the βTrp-331 fluorescence, which undergoes virtually complete quenching upon nucleotide binding (7, 16). Therefore the βV153W and βN158W mutants were not further pursued.

Fluorescence Properties of βF148WF1—The corrected tryptophan fluorescence spectrum of βF148WF1 is shown in Fig. 1C. The maximum is at 328 nm, indicating a nonpolar environment for residue βTrp-148. The fluorescence was quenched upon binding of MgADP (Fig. 3A), accompanied by a small blue-shift of the emission maximum (3 nm). The maximal quench with MgADP occurred at 350 nm. In contrast, binding of MgAMP-PNP resulted in a fluorescence increase (Fig. 3B), together with a pronounced blue-shift of the emission maximum (6–7 nm). The maximal fluorescence increase with MgAMP-PNP occurred at 325 nm. Addition of MgATP resulted in an increase of fluorescence at 325 nm and quenching at 350 nm (Fig. 3C). Thus, the spectrum seen with MgATP contained characteristics of the responses obtained with both MgAMP-PNP and MgADP. This is readily explicable, since a fraction of the bound MgATP will be hydrolyzed to MgADP in the catalytic sites of the enzyme. Thus, the βTrp-148 enzyme potentially provides a tool to determine the relative fraction of catalytic sites occupied by MgATP and MgADP during multisite, steady-state hydrolysis.

It seemed necessary, however, to corroborate the implicit assumption that the fluorescence spectrum seen in presence of MgAMP-PNP is truly representative of that of F1 containing bound, unhydrolyzed MgATP. Recently, x-ray crystallographic analysis of the structure of Dictyostelium discoideum myosin complexed with MgADP and beryllium fluoride (BeF2) has shown that the myosin-MgADP-BeF2 complex mimics closely the prehydrolysis MgATP-bound state, with berylhum in the position normally occupied by the γ-phosphorus, fluorine (or hydroxyl) in positions of the γ-phosphate oxygens, and the β- oxygen to beryllium distance indistinguishable from the bridge oxygen distances in ATP (38). Beryllium fluoride in combination with MgADP has been shown to potentiate inhibiting MgATP hydrolysis by F1 (39). Here we generated the βF148WF1-MgADP-BeF2 complex by adding BeSO4 and NaF to F1-MgADP as described under “Experimental Procedures.” The fluorescence spectrum of the F1-MgADP-BeF2 complex is shown in Fig. 3D; it is virtually identical to that obtained with MgAMP-PNP (Fig. 3B). Control experiments showed that, in absence of MgADP, addition of beryllium fluoride had no significant effect on fluorescence. Thus, it appeared justified to assume that the spectrum seen in Fig. 3, B and D, is representative of the MgATP-bound state.

It was found that 5 mM P, did not elicit a response of the βTrp-148 fluorescence, either in presence or absence of MgADP.

βF148WF1: Further Examination of the Fluorescence Responses Obtained on Binding of MgADP and MgAMP-PNP—In order to quantitatively evaluate the spectra obtained on binding MgATP (Fig. 3C), it was first necessary to analyze the fluorescence responses obtained on binding of MgADP and MgAMP-PNP in detail. We have previously established, using the βY331 enzyme, that binding of MgADP and MgAMP-PNP to the three F1 catalytic sites is best described by a model with one site of higher affinity and two sites of lower affinity (7). Binding of MgADP to βF148WF1 was measured using the fluorescence decrease at 350 nm as the signal, and the data are shown in Fig. 4A. The solid line in Fig. 4A represents a fit of a theoretical curve to the data using a model with two classes of binding sites (see Fig. 4A legend for equation). A reasonable fit was obtained which indicated that the fluorescence quenching at saturation is 12% and that binding of MgADP to the site of higher affinity accounted for 50% of the total fluorescence quenching, whereas binding to the two sites of lower affinity accounted for the other 50%. The calculated Kd values were as follows: site 1, Kd1 = 0.7 μM; sites 2 and 3, Kd2,3 = 340 μM. These values should be regarded as approximate only, because of the small fluorescence responses; nevertheless, they parallel previous data obtained with the βY331 enzyme in that Kd2,3 is similar to K(MgATP), whereas Kd1 is approximately 3 orders of magnitude lower.

Binding of MgAMP-PNP to βF148WF1 was measured using fluorescence enhancement at 325 nm as the signal, and the data are shown in Fig. 4B. The solid line in Fig. 4B is a fit of a theoretical curve to the data, assuming a model with one site of higher affinity and two sites of lower affinity, as found previously (7). Again, a reasonable fit was obtained, with Kd1 = 3 μM and Kd2,3 = 64 μM. The fluorescence increase at saturation was 14%, and the data indicated that binding of MgAMP-PNP to each of the three sites contributed equally to the fluorescence enhancement.

βF148WF1: Quantitation of Fluorescence Responses Obtained on Binding of MgATP, and Occupancy of Catalytic Sites by MgADP and MgATP during Multisite, Steady-State Hydrolysis—As described above, addition of MgATP to βF148WF1 caused a fluorescence increase at 325 nm and a quenching at 350 nm, indicating that both unhydrolyzed MgATP and product MgADP were bound to catalytic sites and being sensed by the βTrp-148 fluorescence. The relative fraction of sites occupied by MgATP or MgADP was calculated using Equation 1 under “Experimental Procedures.” Fig. 5A shows the calculated total occupancy of the catalytic sites by MgATP plus MgADP, over a wide range of added MgATP concentration. As expected from previous work (7, 16, 17), the total occupancy of catalytic sites approached three at mM concentration. Fig. 5B shows the calculated fraction of sites occupied by MgATP, and Fig. 5C shows the fraction of sites occupied by MgADP. With MgATP

$^{1}$ C. Bowman and J. Weber, unpublished results.
concentration sufficient for \( V_{\text{max}} \) hydrolysis rates (mM range) the enzyme-bound MgATP was 1 mol/mol F1 (Fig. 5B) and enzyme-bound MgADP approached 2 mol/mol F1 (Fig. 5C). The ratio of bound MgADP/bound MgATP (Fig. 6) was constant at concentrations of MgATP added from 50 mM up to 2 mM, and the average of all data points at >50 mM MgATP was 1.8.

Six different enzyme preparations were used for the experiments in Fig. 5, A–C, and Fig. 6, and the scatter of the points is likely due to variable contamination by tryptophan-containing proteins. As discussed previously in Ref. 22, even 1–2% (w/w) contamination of the F1 can make a significant contribution to the overall tryptophan fluorescence signal. It should also be noted that the MgADP binding stoichiometries in Fig. 5C are likely to be underestimates. One assumption made in calculating occupancy of the catalytic sites by MgATP and MgADP was that all three \( \beta \text{Trp-148} \) residues showed an identical response to binding of MgATP or MgADP. The data indicated that this assumption was valid for MgAMPPNP binding (Fig. 4B), and therefore by extrapolation for MgATP. However, in the case of MgADP (Fig. 4A) this appeared to be an oversimplification because the data indicated that the fluorescence response associated with binding of MgADP to site one was larger than that for sites two and three. Underestimation of MgADP binding stoichiometry in Fig. 5C would also cause the total occupancy (Fig. 5A) and the bound MgADP/MgATP ratio (Fig. 6) to be underestimated.

\( K_{\text{m}} \) (MgATP) for the \( \beta \text{Trp-148} \) mutant enzyme is 195 mM (Table II), and \( V_{\text{max}} \) hydrolysis rate was achieved at mM concentrations. The data of Fig. 5A show that filling of the third catalytic site occurred in this same concentration range. Therefore, the results indicate that at \( V_{\text{max}} \), all three F1 catalytic sites are filled, consistent with previous work, and that under steady-state hydrolysis conditions, one-third of the catalytic sites are filled with unhydrolyzed MgATP and two-thirds with product MgADP.

It should be emphasized that data points in Fig. 5A were taken when steady-state had been reached. At the lowest substrate concentration used, this took less than 2 min. At higher MgATP concentrations, the response was complete within mixing time (15 s). Typically, both 325- and 350-nm fluorescence signals remained stable for several minutes, then decreased very slowly. At no MgATP concentration was an initial "spike" concentration sufficient for \( V_{\text{max}} \) hydrolysis rates (mM range)
at 325 nm observed, which would have been indicative of a transiently higher stoichiometry of bound MgATP. Thus, even at low substrate concentrations, hydrolysis of bound MgATP appeared to be comparatively fast.

The nucleotide analog ATP\(_{g}\) is hydrolyzed only slowly by F\(_1\)-ATPase (40). We found in titration experiments that the total occupancy of catalytic sites in the \(\beta F_{148W}\) mutant enzyme by MgATP\(_{g}\) was similar to that found with MgATP. However, with MgATP\(_{g}\) an initial spike at 325 nm was observed after addition, indicative of transiently higher stoichiometry of bound nucleoside triphosphate. From the initial spikesignal we calculated the following parameters. At 4.2, 25, and 190 \(\mu\)M concentrations of MgATP\(_{g}\) respectively, 1.1, 1.4, and 1.8 catalytic sites were occupied by MgATP\(_{g}\). These values are significantly higher than corresponding values for MgATP (Fig. 5B). These results are consistent with, and support, our interpretation of the fluorescence spectra obtained with MgATP.

FIG. 3. Effect of nucleotides on tryptophan fluorescence spectra of purified F\(_1\) from \(\beta F_{148W}\) mutant. Conditions were as described under "Experimental Procedures." Uncorrected spectra are shown. Each spectrum shown is the average of at least two independent measurements. A, addition of 2 mm MgADP; B, addition of 2 mm MgAMPPNP; C, addition of 2 mm MgATP; D, addition of 0.5 mm MgADP plus beryllium fluoride (BeF\(_x\)).

DISCUSSION

The first goal of this study was to generate a tryptophan fluorescence probe able to differentiate between an empty catalytic site, an MgATP-filled site, and an MgADP-filled site, in E. coli F\(_1\)-ATPase. A further goal was to use the probe to establish fractional occupancy of the three catalytic sites by MgATP and MgADP during steady-state hydrolysis. We introduced Trp in three different positions within or close to the P-loop (20) by generating the mutations \(\beta F_{148W}, \beta V_{153W}, \) and \(\beta N_{158W}\), and we expressed each mutant in "tryptophan-free F\(_1\)" (22) so that the introduced Trp was the sole Trp residue. Two of the Trp residues, \(\beta Trp-153\) and \(\beta Trp-158\), were able to distinguish between an empty and a nucleotide-occupied site but gave the same response (quenching of fluorescence) on binding of MgATP, MgAMPPNP, or MgADP. The third, \(\beta Trp-148\), fulfilled the requirements, because it gave quenching of fluorescence with MgADP (at 350 nm) and an enhancement of fluorescence with MgAMPPNP and MgADP-BeF\(_x\) (at 325 nm). Moreover, with MgATP both quenching of fluorescence at 350 nm and an enhancement at 325 nm occurred simultaneously.

FIG. 4. Titration of \(\beta F_{148W} F_1\) with MgADP and MgAMPPNP. Conditions were as described under "Experimental Procedures." Typically, in a single experiment three data points were obtained by adding increasing concentrations of nucleotide. A, MgADP. The symbols represent data points; the solid line is a theoretical curve fitted to the data. The underlying model assumed two classes of binding site, each class contributing a distinct fraction of the total fluorescence response. The equation used was:

\[
\Delta F = (\Delta F_1 \cdot ([\text{MgADP}]/([\text{MgADP}] + K_{\text{a1}})) + (\Delta F_2 \cdot [\text{MgADP}]) / ([\text{MgADP}] + K_{\text{a2}})), \quad (\text{Eq. 2})
\]

where \(\Delta F\) is the fluorescence quench at 350 nm, and \(\Delta F_1\) and \(\Delta F_2\) are the components of fluorescence quench at the two classes of site. B, MgAMPPNP. The symbols represent data points; the solid line is a theoretical curve fitted to the data. The underlying model assumed one site of higher affinity and two sites of lower affinity, each site contributing identically to the total fluorescence response. The equation used was:

\[
\Delta F = (\Delta F_1 \cdot ([\text{MgAMPPNP}]/([\text{MgAMPPNP}] + K_{\text{a1}})) + (2\Delta F_1 \cdot ([\text{MgAMPPNP}]/([\text{MgAMPPNP}] + K_{\text{a2}}))), \quad (\text{Eq. 3})
\]

where \(\Delta F\) is the fluorescence increase at 325 nm, and \(\Delta F_1\) is the component of fluorescence increase at each of the three sites.
showing that βTrp-148 differentially senses bound unhydrolyzed MgATP and bound product MgADP in catalytic sites under steady-state hydrolysis conditions. The βF148W mutant strain showed normal growth characteristics under oxidative phosphorylation conditions, and enzymatic characteristics of purified βF148WF1 were similar to those of wild-type enzyme.

The fluorescence spectra of residues βTrp-153 and βTrp-158, in absence of nucleotides in the binding site, indicated that their environments are highly nonpolar and that of βTrp-148 indicated a moderately nonpolar environment. Previous work with the βY331W mutant enzyme has shown that in absence of bound nucleotide, the catalytic sites are highly polar and likely to be filled with water molecules (7). Thus all three Trp residues studied here are probably buried in the protein matrix, pointing away from the nucleotide.

Upon binding of MgADP, MgAMPPNP, and MgATP the fluorescence of βTrp-153 and βTrp-158 was quenched. The simplest explanation is that nucleotide binding leads to a change in the protein conformation in the direct environment of either Trp residue. The same explanation can be offered for the quenching of βTrp-148 fluorescence by MgADP. In contrast, binding of MgAMPPNP led to enhancement of βTrp-148 fluorescence, combined with a pronounced blue-shift of the spectrum. The MgADP·BeF₆ complex (which is known to closely mimic the prehydrolysis MgATP-bound state in myosin, Ref. 37) gave the same spectrum as MgAMPPNP. This response might also be explained as due to a conformational rearrangement of the protein, albeit different from that observed with MgADP. An attractive alternative explanation, however, is that the electric field of the negative charges introduced with the nucleotide γ-phosphate affects the Trp fluorescence (see Introduction). The fact that the MgADP·BeF₆ complex caused the same response as MgAMPPNP would be in agreement with either interpretation, as such a complex is isoelectronic and potentially isosteric with MgAMPPNP (and MgATP) (37).

Titration experiments were carried out with βF148WF1 in order to analyze the fluorescence responses with MgADP and MgAMPPNP (Fig. 4, A and B). Quantitative evaluation of the results was affected by the small size of the responses; nevertheless, several conclusions could be drawn with confidence. With either nucleotide, binding cooperativity was evident, and in both cases the data fit reasonably well to a model assuming one site of higher affinity and two sites of lower affinity. This model was established from previous work with the βY331W enzyme (7), which displays a far superior tryptophan fluorescence signal and is representative of wild-type in enzymatic properties. Thus, for MgADP and MgAMPPNP the lower K_d corresponds to binding to catalytic site one and the higher K_d corresponds to binding to catalytic sites two and three. The fluorescence signals reached saturation at mM ligand concentrations, i.e. 1 order of magnitude above K_m(MgATP), as was also seen with the βY331W enzyme (7), and under these conditions all three catalytic sites would therefore be filled with ligand.

We next studied the fluorescence responses of βTrp-148 upon titration of βF148WF1 with MgATP concentrations ranging from 0.2 μM up to mM. The data were described in Fig. 5, A–C, and show several important features. At MgATP concentrations sufficient to achieve V_max rates of hydrolysis, all three catalytic sites became occupied by nucleotide, consistent with our previous work (7). At the lowest concentration of MgATP of 0.2 μM (where [MgATP] ~ [F₁]) around 0.6 mol total nucleotide was bound per mol F₁, corresponding to binding to the highest affinity catalytic site one. When the concentration of MgATP in

![Fig. 5. Titration of βF148WF1 with MgATP. Conditions were as under "Experimental Procedures." NaATP and MgSO₄ were added at constant 2.5:1 ratio, and MgATP concentration in the medium (abscissa) was calculated as in Ref. 33. Each data point represents a single experiment. From the resulting fluorescence changes at 325 and 350 nm, the stoichiometries of enzyme-bound MgADP and MgATP were determined as described under "Experimental Procedures." A, total bound Mg-nucleotide (MgADP plus MgATP); B, bound MgATP; C, bound MgADP.](image1)

![Fig. 6. Titration of βF148WF1 with MgATP; ratio of bound MgADP/bound MgATP. From the data presented in Fig. 5, B and C, the ratio of bound MgADP to MgATP was calculated.](image2)
the medium was raised to 5 μM and above, the average nucleotide occupancy rose, as catalytic site two began to fill. However, even immediately after mixing the amount of bound MgATP never exceeded 1 mol/mol, which indicates that upon binding of MgATP to site two, hydrolysis of MgATP bound at site one was a fast reaction, and release of MgADP from site one was slow, consistent with the slow net turnover rate under these conditions. Further increase of the MgATP concentration up to mM concentrations was sufficient to fill all three catalytic sites and to achieve V_max rates of hydrolysis. Under steady-state turnover conditions one-third of the catalytic sites contained MgATP and two-thirds contained MgADP (Fig. 6).

A model for steady-state, "multisite" MgATP hydrolysis based on these findings is presented in Fig. 7. In the model, the catalytic site with highest affinity for MgATP is designated as H, the site with intermediate affinity is designated as M, and the site with lowest affinity for MgATP is designated as L. After release of MgADP from site L in the previous reaction cycle, site H contains MgATP, site M contains MgADP, and site L is transiently unoccupied (State D in Fig. 7). Then site L fills rapidly with MgATP from the medium (k_cat/K_M = 10^9 M^-1 s^-1) to yield State A in Fig. 7. Instantaneously, MgATP at site H is hydrolyzed to MgADP + P_i; this process triggers a synchronized switch in affinity at all three catalytic sites, which is shown by the arrows in State B, Fig. 7. In another fast reaction, P_i is released from the (now) site M, resulting in State C, Fig. 7. Subsequent MgADP release from site L is the rate-limiting step in the cycle. Consequently, State C is the most populous state of all the enzyme molecules present under steady-state MgATP hydrolysis conditions, and the fluorescence response obtained reflects State C. The model incorporates information from previous models in that it involves three catalytic sites, of widely differing affinity for MgATP, with positive catalytic cooperativity observed on binding of MgATP to the lowest affinity site, and a synchronized change in affinity of the sites at one step of the catalytic cycle (1, 2). The essential feature of this model is that it accounts for the new finding from the fluorescence experiments that two of the sites are occupied by MgADP and only one by MgATP in the form of enzyme that predominates in time-average during the catalytic cycle in steady-state MgATP hydrolysis. It may be inferred that this is a necessary feature for the reverse reaction, namely steady-state ΔpH₁-driven MgATP synthesis.

In the structure of mitochondrial F₁ obtained by x-ray crystallography (3), one catalytic site is occupied by MgAMPPNP and one site by MgADP, whereas the other site is empty. In our model (Fig. 7) this corresponds to State D. Our data show that this form of the enzyme is present as only a small fraction of the molecules during steady-state catalysis; nevertheless, it is not unreasonable to suppose that under the crystallization conditions used (3) this form of the enzyme may have been selectively sequestered into the crystals.

It is relevant to point out that during catalysis there are potentially at least two states through which the catalytic site passes in addition to the MgATP- and the MgADP-bound states, namely the catalytic transition state and the "MgADP + P_i"-bound state. X-ray crystallographic analysis of other ATP-or GTP-hydrolyzing enzymes has suggested that the catalytic transition state is mimicked by bound MgADP-AlF₄⁻ complex (37, 39). We generated the βF₁48W F₁-MgADP-AlF₄⁻ complex (see "Experimental Procedures") and found that its fluorescence spectrum resembled that seen on addition of mM concentration of MgATP (as in Fig. 3C), that is to say it contained characteristics of both MgADP-bound and MgAMPPNP-bound forms. One interpretation of this result is that this spectrum truly represents that of the catalytic transition state. However, the experiment is ambiguous because it is also possible that under these conditions a fraction of the catalytic sites is in the MgADP-bound state. This would be the case, for example, if the F₁ mechanism mandates that only one of the catalytic sites can be in the catalytic transition state at any given time, as appears to be the case for P-glycoprotein (41). Since the time spent in the catalytic transition state is very short in comparison to the time spent in the ground states, the contribution to the overall fluorescence signal during steady-state catalysis should be small. It would be valuable to obtain a probe that sensed the transition state specifically.

We also attempted to determine the contribution of the "MgADP + P_i"-bound state to the overall fluorescence signal. It was found that addition of P_i together with MgADP had no effect on the βTrp-148 fluorescence response (see "Results"). In all likelihood, however, this is due to the fact that an F₁-MgADP + P_i complex was not generated. Previous work has shown that P_i, by itself has very weak binding affinity at catalytic sites in soluble F₁, and addition of P_i does not affect the binding affinity of MgADP (7, 42). Therefore, there are limitations as to how far we can dissect the catalytic cycle in respect to differential rates of release of P_i versus MgADP using the current data. However, it should be pointed out that the model of Fig. 7 must be essentially correct in that, since a state with all three catalytic sites occupied with nucleotide (one MgATP and two MgADP) is the predominant long-lived species during the catalytic cycle, there is clearly no possibility that release of MgADP precedes that of P_i.

Given the small size of the underlying fluorescence responses and other considerations discussed above, we stress that the model of Fig. 7 is a working hypothesis. It is hoped that in the future different Trp probes can be developed to test and extend the model, for example by specifically reporting the catalytic
transition state or the presence or absence of P. The most important contribution of the current work is to demonstrate for the first time that reaction events occurring at the catalytic sites of F$_1$-ATPase during steady-state catalysis are amenable to analysis using direct optical probes.

Finally it may be noted that, given the general similarity of structure of the P-loop sequence in many nucleotide-binding proteins, and the fact that βTrp-148 was specifically inserted in the position immediately preceding the first residue of the P-loop, there seems to be a good chance that insertion of Trp at the equivalent position might provide a valuable probe to distinguish bound nucleoside triphosphate from diphosphate in a range of different proteins and enzymes.

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