Specific Placement of Tryptophan in the Catalytic Sites of Escherichia coli F₁-ATPase Provides a Direct Probe of Nucleotide Binding: Maximal ATP Hydrolysis Occurs with Three Sites Occupied*

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Residue βY331 of Escherichia coli F₁-ATPase is known from previous affinity labeling, mutagenesis, and lin-benzo-ADP binding experiments to interact directly with the adenine moiety of substrates bound in catalytic sites. Here we mutagenized βY331 to tryptophan. Mutant cells grew well on succinate or limiting glucose; purified mutant F₁ had $k_{cat}$, $K_m$, and $lin$-benzo-ADP binding characteristics similar to wild type. Fluorescence from βY331 residues exhibited a maximum at 349 nm, indicating a polar environment in unoccupied sites. ATP, ADP, or AMPPNP caused virtually complete quenching of βW331 fluorescence, so that the fluorescence of mutant F₁, with occupied catalytic sites resembled that of wild-type enzyme. Therefore the βW331 fluorescence provided a direct probe of nucleotide binding to catalytic sites under true equilibrium conditions.

We measured ATP binding and hydrolysis in parallel experiments and found that occupancy of one or two catalytic sites per F₁ molecule did not yield significant rates of hydrolysis while occupancy of all three sites yielded $V_{max}$ rates. $K_m$(ATP) was similar to $K_d$, the $K_d$ for ATP binding to the third catalytic site. We also measured AMPPNP and ADP binding parameters. For ADP, the "on" rate at the first catalytic site was much faster ($≥ 5 × 10^5 \text{ M}^{-1} \text{s}^{-1}$) than seen previously by centrifuge column procedures, although the $K_d$ was not much changed. For AMPPNP, the "on" rate at the first site was 2 orders of magnitude less than for ADP or ATP, and the $K_d$ was similar to that for ADP.

ATP synthesis by oxidative phosphorylation or by photophosphorylation, in mitochondria, chloroplasts, or bacteria, occurs on the enzyme ATP synthase. The catalytic sites for conversion of ADP and P₃ to ATP are located on the β-subunits of the F₁-sector of this enzyme (reviewed in Duncan and Cross, 1992; Senior, 1992; Allison et al., 1992). Affinity and photoaffinity labeling studies demonstrated that residue βY331 (using Escherichia coli numeration) lies within the catalytic site (Bulough and Allison, 1986; Garin et al., 1986; Cross et al., 1987; Wise et al., 1987; Admon and Hames, 1987), and because residue βY331 reacted substantially with 2-azido-ATP, there was the indication that it might abut on the adenine ring of bound substrate.

Mutagenesis experiments using E. coli ATP synthase (Wise, 1990) demonstrated that substitution of F for residue βY331 did not strongly affect catalysis, whereas the substituents C, S, A, or G all caused strongly attenuated catalysis, both in vivo and in vitro. Our laboratory subsequently showed that substitution of the nonpolar L, like F, could sustain catalytic activity, whereas the polar E was detrimental (Weber et al., 1992a). We also found that the adenine nucleotide analog $lin$-benzo-ATP was a good substrate for E. coli F₁-ATPase activity and that on binding to wild-type F₁, the fluorescence of $lin$-benzo-ADP was strongly quenched. Analyses of the fluorescence properties of $lin$-benzo-ADP bound in catalytic sites of wild-type and mutant enzymes then demonstrated that residue βY331 in wild-type enzyme interacts directly with the base moiety of the bound nucleotide analog and that the environment around the bound adenine moiety in the catalytic site is nonpolar (Weber et al., 1992a). Therefore, direct interaction of βY331 with the adenine ring of ATP in a hydrophobic subdomain of the catalytic site appears to facilitate catalysis in wild-type enzyme.

Tryptophan residues can be valuable as "reporter probes" in proteins, and one can conceive several possible applications in F₁. For example, in a recent study, we placed a tryptophan residue specifically in the binding site for the antibiotic aurovertin, by making the mutation βR398W. The mutation did not adversely affect catalytic activity, and we were able to use the fluorescence properties of the mutant enzyme to study the properties of the aurovertin-binding site. When $lin$-benzo-ADP was bound in the catalytic sites, fluorescence resonance energy transfer occurred between the βR398W residue and bound $lin$-benzo-ADP, allowing calculation of the distance between the catalytic site and the aurovertin site on each β-subunit (Weber et al., 1992b). In a second example, the intrinsic tryptophan fluorescence of the wild-type Schizosaccharomyces pombe mitochondrial F₁ was used to probe binding of nucleotides and conformational interactions (Divita et al., 1991, 1992, 1993).

Bordo and Argos (1991) showed by statistical analyses of sequence comparisons that, in naturally occurring proteins, the residues that substitute most commonly for tryptophan are Y, F, and L. Since it was already established that Y, F, and L all support catalysis in F₁ when present at residue β331, it seemed possible that the mutation βY331W would yield active enzyme, with the substituted tryptophan being strategically placed within the catalytic site. Here, we report studies of βY331W mutant F₁, showing that the enzyme is active and that the

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The abbreviations used are: $lin$-benzo-ADP, 8-amino-3-(β-D-ribofuranosyl)imidazo[4,5-g]-quinazoline 5'-diphosphate (it has formally a benzene ring inserted between pyrimidine and imidazole rings of adenine, linearly extending the ring system). AMPPNP, adenyl-5'-imidodiphosphate.
fluorescence specifically from the βW331 residues may readily be observed. Almost complete quenching of this fluorescence signal occurred on binding of the natural ligands ATP and ADP and of the analog AMPPNP. Therefore, the βW331 fluorescence provides a direct probe of nucleotide binding at catalytic sites. This has enabled us to measure, under true equilibrium conditions, thermodynamic and kinetic binding constants for nucleotides. Additionally, we were able to determine the dependence of the rate of ATP hydrolysis on the degree of filling of the three catalytic sites by ATP.

MATERIALS AND METHODS

Construction of the βY331W Mutation

Insertion of New NheI Restriction Site in γ-Subunit Gene—In order to facilitate transfers of β-subunit mutations we constructed a plasmid (pDP34N) which expresses all of the structural genes of ATP synthase and contains a unique NheI site in codon 271 of the γ-subunit gene. The mutagenic oligonucleotide used was 5'-CTCTGTCAGGCTAGCATTACT, where the underlined T converts y271-codon GCC·GCT, retaining alanine as residue y270. Briefly, the method was as follows. The template was single-stranded phage M13 mp18 containing a HindIII-KpnI insert encoding the 3' segment of the γ-subunit gene and the entire j- and κ-subunit genes (Parsonage et al., 1987), and the mutagenesis method was that of Taylor et al. (1985) as described previously by Lee et al. (1991). Phage replicative form containing the new NheI site was identified by restriction digestion, and the new restriction site was then moved into plasmid pDP34 (Massey et al., 1988) yielding plasmid pDP34N. In growth tests, and assays of ATPase and ATP-driven proton pumping in membrane vesicles, it was found that plasmid pDP34N gave the same results as pDP34, showing that the introduced silent mutation had no inhibitory effects on ATP synthesis. Using essentially the same procedure we also introduced the NheI site into plasmid pDP31 (Parsonage et al., 1987), yielding plasmid pDP31N, and we confirmed that no inhibitory effects of the silent mutation were seen with this plasmid either.

Generation of the βY331W Mutation—M13 mp18 containing the HindIII-KpnI insert with the introduced NheI site (above) was the template for a second round of mutagenesis. The mutagenic oligonucleotide was 5'-CTCTGGGTATCT=CCGGCCGTT, where the underlined bases change j-subunit codon 332 from TAC→TGG, generating the βY331W mutation, and also introducing a new NaeI site. Phage replicative form containing the new NaeI site was identified by restriction digestion, and the new restriction site was then moved into plasmid pDP34N, generating plasmid pDP34N. In growth tests, and assays of ATPase and ATP-driven proton pumping in membrane vesicles, it was found that plasmid pDP34N gave the same results as pDP34, showing that the introduced silent mutation had no inhibitory effects on ATP synthesis. Using essentially the same procedure we also introduced the NheI site into plasmid pDP31 (Parsonage et al., 1987), yielding plasmid pDP31N, and we confirmed that no inhibitory effects of the silent mutation were seen with this plasmid either.

Enzyme Purification and Characterization

Membrane vesicles were prepared according to Senior et al. (1979); soluble F1 was purified as described by Al-Shawi and Senior (1992) and, when indicated, depleted of endogenous nucleotides as described by Senior et al. (1992). Purity and subunit composition of F1 preparations was checked by SDS-gel electrophoresis (Laemmli, 1970). Protein concentration of F1-solutions was determined using the Bio-Rad protein assay (Bradford, 1976). The molecular mass of F1 was taken to be 382,000 Da (Senior and Wise, 1983).

Assays of Enzyme Function

Growth yield analyses in limiting glucose (3 mM) were performed as described by Senior et al. (1984). ATPase assays were performed as described in Weber et al. (1992a) except that the ATPase activities shown in Fig. 4 were measured at 23°C and pH 8.0.

Fluorescence Measurements

A SPEX Fluorolog 2 spectrofluorometer was used, and all experiments were carried out at 23°C. Before use, F1 was equilibrated in 50 mM Tris- SO4, 2.5 mM MgSO4, pH 8.0, by passage through 1-ml Sephadex G-50 centrifuge columns, except that when the enzyme was intended for the MgATP binding measurements (Fig. 4), MgSO4 was omitted from the centrifuge column buffer. The lin-benzo-ADP binding experiments were performed as described by Weber et al. (1992b) (λex = 332 nm; λem = 388 nm). Correction of the protein fluorescence spectra (Fig. 2) was carried out as described by Weber et al. (1992b). For titration of the tryptophan fluorescence with nucleotides, the emission wavelength (λem) was 360 nm; at this wavelength, the technical (uncorrected) βW331 fluorescence spectrum exhibited its maximum.

Materials

lin-benzo-ADP was synthesized as described by Leonard et al. (1976, 1978). Concentration determinations were based on an extinction coefficient of 9750 μM/cm at 331 nm for lin-benzo-ADP (Leonard et al., 1976).

RESULTS

Functional Effects of the βY331W Mutation in Cells—Strain pSWM4/JP17 (carrying the mutation βY331W) grew well on plates containing succinate as sole carbon source, and its growth yield in limiting glucose medium was 80–90% of that of the corresponding wild-type (pDP34N/JP17) strain. These results show that oxidative phosphorylation in the mutant strain is not impaired to any major degree.

General Properties of Purified βY331W Mutant F1—As judged from the elution profile of the Sephacryl S300 gel filtration column used in the final purification step, a stable F1-ATPase of normal molecular size was formed in the mutant strain. On SDS-gels, purified βY331W mutant F1 showed a normal subunit composition. When ATPase activity was measured under standard conditions (30°C, pH 8.5) as a function of MgATP concentration, the βY331W mutant enzyme reached a Vmax of 13 units/mg, which was about 50% of the value for wild-type F1 (25–30 units/mg). On the other hand, Kapp was also reduced in the mutant F1 (0.04 mM as compared with 0.08 mM for wild type); thus kcat/Km was approximately 2 × 108 M−1 s−1 for both enzymes.

Binding of lin-Benzo-ADP to βY331W Mutant F1—Previously we showed that the fluorescent ADP analog lin-benzo-ADP binds to the three catalytic sites on native E. coli F1. The fluorescence intensity of bound analog and the wavelength positions of excitation and emission spectra were highly dependent on the nature of the amino acid residue present at position β331. Of the β331 amino acid substitutions investigated (F, L, A, E), none caused the same strong quenching of the fluorescence of the analog as was observed with wild type (Weber et al., 1992a). Here we found that binding of lin-benzo-ADP to βY331W mutant F1 resulted in a fluorescence response which was indistinguishable from that observed with wild-type enzyme; there was strong quenching (see Table I) and no change in wavelength position of the fluorescence spectra as compared with those for free lin-benzo-ADP.

Using the quenching of lin-benzo-ADP fluorescence as signal, the parameters for lin-benzo-ADP binding to βY331W mutant
F₁ were determined in titration experiments as described previously by Weber et al. (1992a). None of these experiments gave any indication of participation of binding sites causing a lin-benzo-ADP fluorescence signal deviating from the data described above. As can be seen from Fig. 1, the binding curve reached a plateau at a stoichiometry of 3 mol of lin-benzo-ADP bound per mol of F₁. From the general shape of the curve, the affinity of the mutant F₁ for the analog appeared to be somewhat higher than that of the wild-type enzyme. Dissociation constants were determined by fitting of theoretical binding curves to the experimental data by non-linear least squares regression analysis; the results are given in Table I. In previous work with wild-type and mutant enzymes (Weber et al., 1992a, 1992b), the fit obtained assuming a model with three independent binding sites, two of them being identical, could not be improved by assuming a model with three different sites. Here, in contrast, with βY331W F₁ the fit assuming a model with K₁ = K₃ (Table I, line 2) was slightly inferior to that based on a model with three different binding sites (Table I, line 3). However, as can be seen from Fig. 1, the scatter of the data points is such that the former model cannot be ruled out. Using this model (which allows a ready comparison of Kᵢ values to the data previously published by Weber et al., 1992a, 1992b), the increase in affinity for lin-benzo-ADP due to the substitution of βY331 by W amounted to a factor of 2-3 (compare Table I, lines 1 and 2). This is in agreement with the decrease in Kₐ for ATP noted above. A further point to note is that for the βY331W mutant F₁, at each of the three lin-benzo-ADP binding sites, the interaction between residue βW331 and the fluorophore was the same.

Fluorescence Properties of βY331W Mutant F₁—The addition of 3 genetically engineered tryptophan residues in position 331 of the three β-subunits to the 9 tryptophan residues already present in wild-type F₁, was expected to have an impact on the fluorescence of the enzyme. At identical protein concentrations, native βY331W mutant F₁ gave a significantly higher fluorescence intensity than wild-type enzyme, and upon excitation at 278 nm as well as at 295 nm, the fluorescence emission spectrum was red-shifted. However, the extent of the fluorescence increase and the exact wavelength position of the emission spectrum was somewhat variable (an example is given in Fig. 2, A and B). As will be described below in detail, the fluorescence of the βW331 residues is quenched by nucleotides bound to the catalytic sites. Such bound nucleotides are not completely removed by passage of native F₁ through a centrifuge column (see e.g. Senior et al., 1992), and this was the likely cause of the variation seen. Therefore, in order to measure the “true” fluorescence spectrum of βY331 mutant F₁, we prepared and investigated nucleotide-depleted enzyme (which contains zero nucleotides per mol of F₁, bound to the catalytic sites; Senior et al., 1992). In wild-type F₁, depletion of endogenous nucleotides had no effect on the wavelength position of the tryptophan fluorescence spectrum, only the fluorescence intensity was slightly (≤5%) decreased. In nucleotide-depleted βY331W mutant F₁, however, the emission spectrum was further red-shifted, and, most prominently, the fluorescence intensity was further increased. Upon excitation at 278 nm (where the emission spectrum shows the contributions of tyrosine and tryptophan residues), nucleotide-depleted βY331W mutant F₁ exhibited an emission maximum at about 333 nm, as compared with 322 nm for the wild-type enzyme (Fig. 2A). Upon excitation at 295 nm (which is selective for tryptophan residues), the 3 added tryptophan residues shifted the maximum from about 339 to 357 nm (Fig. 2B). As can be seen from the difference spectrum given in Fig. 2B, the reason for the red-shift was the fact that the introduced βW331 residues had an emission maximum of about 349 nm (the same result was obtained whether the dif-
ference was calculated from the nucleotide-depleted or the native enzyme). These data indicate that the environment of the βW331 residues is highly polar when the catalytic sites are not occupied by nucleotide (see Efthik, 1991).

Effects of Ligands on the βW331 Fluorescence—It had been shown previously that residue β331 is located in the immediate environment of the base moiety of catalytic site-bound nucleotides (Weber et al., 1992a); therefore binding of nucleotides was expected to have an effect on the βW331 fluorescence. And indeed, binding of ATP to nucleotide-depleted βY331W mutant F1 resulted in virtually complete quenching of the βW331 fluorescence, thus making the fluorescence spectrum of mutant F1 very similar to that of wild-type enzyme (Fig. 3). The same effect of ATP was seen in native mutant F1, and ADP and AMPPNP exhibited similar effects to ATP on the βW331 fluorescence (not shown). Binding of nucleotide to wild-type enzyme (native or nucleotide-depleted) had no effect on the tryptophan fluorescence signal.

Addition of F1 had very little effect on the fluorescence of nucleotide-depleted or native βY331W mutant F1; 5 mM F1 decreased the fluorescence by about 2%. Furthermore, a careful reexamination showed a similar decrease in the wild-type enzyme (native as well as nucleotide-depleted) which had not been detected previously (Weber et al., 1992b). A detectable effect of pyrophosphate (3 mM) was observed with the native βY331W mutant F1, but not in nucleotide-depleted enzyme. In native enzyme, the fluorescence increased (by 5–10%), and the emission spectrum was slightly red-shifted. The obvious explanation is that pyrophosphate displaced some of the catalytic site-bound nucleotides. However, even at 3 mM pyrophosphate the displacement did not appear to be complete (data not shown).

ATP Binding: ATPase Activity versus Catalytic Site Occupancy—The fluorescence response on binding of nucleotides to the catalytic sites in βY331W mutant F1 allowed a direct approach to the problem of how many sites of this type are filled during steady-state catalysis. We measured ATPase activity and fluorescence intensity as a function of the MgATP concentration in parallel experiments under identical conditions (50 mM Tris sulfate, pH 8.0, 23°C; ATP/MgSO4 ratio of 10/4; protein concentration 40–55 nM). The uncorrected fluorescence emission spectrum of the βW331 residues (λex = 295 nm) exhibited a maximum at 390 nm, and this signal was used to assess nucleotide binding. Both native and nucleotide-depleted forms of the βY331W mutant F1 were investigated. The enzymatic activity was determined from the amount of P1 liberated at 30 s after addition of MgATP. The occupancy of the catalytic sites was calculated as the average occupancy over the first 30 s after addition of MgATP. Examples of the fluorescence quenching obtained on addition of various concentrations of MgATP are shown in Fig. 4A.

ATPase activity and catalytic site occupancy were plotted versus MgATP concentration (Fig. 4B). Kd and Km values for MgATP were calculated by fitting theoretical curves to the experimental data. For both enzyme preparations, a model assuming one Km value could describe the substrate concentration dependence of the ATPase activity satisfactorily. For the nucleotide-depleted enzyme, a Km value of 38 μM was obtained, for native F1, Km = 45 μM. In the very low substrate concentration range the measured ATPase activities were actually lower than expected from the respective Km values. This discrepancy may be explained by the fact that at these concentrations it took a few minutes for the binding reaction to reach equilibrium (data not shown). Increased the ATPase assay time in these cases from 30 s to 2 min resulted in higher calculated ATPase activities which, however, were not substantial enough to require the introduction of an additional Km value in the 1 μM range.

For evaluation of the MgATP binding curves (Fig. 4B) a model was used in which each of the three sites was assigned an independent Km value. Also it was taken into account that, even at the highest substrate concentrations, during the first 30 s after addition of substrate the degree of saturation of catalytic sites was only about 95% (see Fig. 4A). The maximal value of N determined by iteration was 2.85 mol/mol of F1. For the nucleotide-depleted enzyme, we obtained N(Vmax) = 0.1 μM, Kd1 = 5 μM, and Km = 25 μM; for native F1, Kd1 was <0.1 μM, Kd2 = 3 μM, and Kd3 = 29 μM. These values are apparent ones only. At low substrate concentrations the binding stoichiometries given in Fig. 4B are expected to diverge from the true equilibrium values, because binding is less complete than at the higher substrate concentrations, leading to overestimation of Kd1 and Kd3. When binding curves were constructed using the equilibrium binding data (i.e. data taken at times when binding of the MgATP had reached steady state), Kd1 was in both cases <0.1 μM, Kd2 = 0.9 μM for the nucleotide-depleted and 0.8 μM for the native enzyme; Kd3 remained unchanged (26 and 33 μM, respectively).

A comparison of the ATPase and ATP binding data shows that only the Km is of the same magnitude as Kd. This means that all three catalytic binding sites have to be filled for Vmax rates of ATP hydrolysis. Fig. 4C shows a plot of ATPase activity versus catalytic site occupancy. The circles represent data points for the measured ATPase activity (left axis) at different measured values of catalytic site occupancy (plotted on the horizontal axis). It is clear that at maximal ATPase activity three sites are occupied, as concluded above, and that when only one site is occupied catalytic activity is very low, in agreement with previous work (Penefsky and Cross, 1991). The activity of an enzyme molecule with two sites filled is not immediately obvious from such data, however, because at a measured (average) catalytic site occupancy of two, a certain proportion of the enzyme molecules will actually have three sites filled. From the Km(1)ATP values given above it is possible

2 For these experiments the native F1 was pre-equilibrated in buffer containing no Mg2+ and was found to contain around 0.2 mol of nucleotide/mol of F1 bound at catalytic sites. Previous work indicates this would be predominantly ADP (Senior et al. 1992). It did not interfere with the MgATP binding experiments.
to calculate what this proportion is (see Fig. 4C legend). The dotted and solid lines in Fig. 4C are plots of the calculated percent of molecules having three sites filled (right vertical axis) versus the (average) catalytic site occupancy (horizontal axis). The fact that the lines coincide well with the data points (circles) suggests that virtually all of the ATPase activity seen is due to enzyme molecules with three sites filled.

Binding of ADP to βY331W Mutant F1—The ADP binding parameters for nucleotide-depleted mutant F1 were determined from the binding curves resulting from a number of titration experiments in which increasing concentrations of ADP were added to an F1 solution (an example is given in Fig. 5, open circles). A model assuming two types of site gave a satisfactory fit. One binding site had higher affinity and 1.4 sites had lower affinity (see Table 11). In presence of 5 mM Pi, (Fig. 5, closed circles), the affinity of the first site was slightly increased and that of the remaining sites slightly reduced (see Fig. 5 and Table II). Although small, these effects of Pi were reproducible.

When ADP titrations were performed with native βY331W mutant F1, it was evident that there was already some degree of quenching occurring at the start of the titration experiment, before the first addition of “exogenous” ADP (as in Fig. 2B, curve 2). Obviously, in the native enzyme pre-equilibrated in magnesium-containing buffer (see “Materials and Methods”), a fraction of the catalytic sites was already occupied by nucleotides. The binding stoichiometry of these nucleotides, as judged by the fluorescence signal, was 0.4–0.8 mol/mole of F1.
for the initial part, the ADP titration curves obtained with native βY331W mutant F₁ were the same as those for the nucleotide-depleted enzyme, and at higher concentrations of free ADP, the resulting binding curves were superimposable (Fig. 5, squares). Therefore the binding parameters for ADP in Table II apply equally to nucleotide-depleted and native enzyme.

Investigation of the ADP binding kinetics by following the quenching of βW331 fluorescence was hampered by the fact that the experimental device (standard cuvette with stirrer) did not give the time resolution necessary to monitor the binding step(s). For example, in an attempt to determine the association rate constant of the first site in nucleotide-depleted βY331W mutant F₁, even when using ADP concentrations as low as 0.10 mM, the major part of the fluorescent decrease was complete within the mixing time (10 s). However from these data it could be estimated that the ADP association rate constant at the first site is at least $5 \times 10^3$ s$^{-1}$, in the presence or absence of 5 mM P$_i$. Using for calculation a $K_D$ of 0.1 μM (Table II), this would imply that the corresponding ADP dissociation rate constant is $\approx 0.05$ s$^{-1}$.

**Binding of AMPPNP to βY331W Mutant F₁** —The titration curves and the resulting binding curves (Fig. 6A) for the interaction of AMPPNP with nucleotide-depleted mutant enzyme were not substantially different from those for ADP. Again, one higher affinity site was found together with 1.4 sites of lower affinity (Table II). Inclusion of 5 mM P$_i$ in the buffer did not cause significant changes in AMPPNP binding properties (Table II).

In contrast to the thermodynamic binding parameters, the AMPPNP binding kinetics differed markedly from those for ADP. As can be seen from Fig. 6B, upon addition of 0.44 μM AMPPNP to 73 nM nucleotide-depleted βY331W mutant F₁, the βW331 fluorescence decrease occurred over a period of several minutes. At these concentrations binding is expected to occur mainly at the first site. Assuming a reversible second-order reaction, the association rate constant was calculated to be $6.0 \times 10^3$ s$^{-1}$. Taking the $K_D$ at this site to be 0.14 μM (Table II), this yields a dissociation rate constant of $8.4 \times 10^{-4}$ s$^{-1}$.

**DISCUSSION**

Substitution of residue βY331 in the β-subunit of E. coli F₁-ATPase by tryptophan gave a functional enzyme with an important new feature. The fluorescence of the βW331 residues was strongly quenched on binding of nucleotides to the catalytic sites, providing a direct probe of catalytic site occupancy. Using this signal we have determined thermodynamic and kinetic binding parameters for ADP and AMPPNP binding to catalytic sites under true equilibrium conditions. We demonstrate also that $V_{max}$ for ATP hydrolysis is attained only when three catalytic sites are occupied by MgATP. Each of these aspects is discussed in detail below.

The extent of the quenching of βW331 fluorescence by ATP confirmed that the residue in position β331 is part of the base-binding subdomain of the catalytic site, as was deduced earlier (Weber et al., 1992a). In that previous work it was suggested that large hydrophobic amino acid side chains were required at this position to maintain the nonpolar character of the subdomain necessary for normal or close-to-normal catalytic activity. In agreement with this suggestion we showed here that with tryptophan in position β331, ATP synthesis by oxidative phosphorylation in vivo was close to normal, and in purified βY331W mutant F₁, $k_{cat}/K_m$ was similar to that for wild-type F₁. The mutant F₁ showed a normal binding stoichiometry for lin-benzo-ADP (3 mol/mol of F₁), with slightly increased affinity as compared with wild type. This was also reflected in a slightly lower $K_m$ for ATP. Overall, however, the properties of βY331W mutant F₁ appeared quite similar to wild type.

The large degree of quenching of the lin-benzo-ADP fluorescence observed when the analog was bound to the catalytic sites of wild-type F₁, but not when bound to the βY331W mutant enzyme, led previously to the proposal that the phenolic hydroxyl group of tyrosine β331 makes direct interaction with the base moiety of a bound nucleotide molecule (Weber et al., 1992a). The results described here suggest that a similar interaction occurs between tryptophan in position β331 and the...
fluorophore. Our previous work (Weber et al., 1992a) had also indicated that this region of the catalytic site is quite hydrophobic when ligand is bound and that increase in the polarity caused, e.g. by the mutations βY331A and βY331E, produced parallel detrimental effects on enzyme activity. Here we found that, as indicated by the wavelength position of the βW331 fluorescence spectrum, this region of the catalytic sites is actually highly polar when the sites are unoccupied. Thus on substrate binding this region of the catalytic sites changes from being highly polar to nonpolar. The polar nature of unoccupied sites presumably allows for rapid substrate association rates and the hypodrophic nature of the substrate-catalytic site complex facilitates catalysis.

The βW331 fluorescence provided an ideal tool with which to study binding of nucleotides to the catalytic sites of F1. Until now, the application of fluorescence spectroscopy for this purpose has relied mainly on the use of fluorescent nucleotide analogs (see e.g. Garboczi et al., 1990; Thomas et al., 1992; Mills and Richter, 1991; Rao et al., 1988; Kögner et al., 1986; Tiedge and Richter, 1986; Weber et al., 1989, 1990, 1992a, for reviews of earlier work see Vignais and Lunardi, 1985). Protein fluorophore was only rarely used, mostly because of lack of an appropriate signal. For example in wild-type E. coli F1, upon addition of ADP or ATP, no significant change in either overall (tryptophan plus tyrosine) or tryptophan fluorescence was detectable (Weber et al., 1992b). In beef heart mitochondrial F1, the overall (essentially tyrosine) fluorescence decreases by less than 10% upon interaction with ADP (Tiedge et al., 1992). A better fluorescence signal is given by the two naturally occurring tryptophan residues in the α-subunit of Schizosaccharomyces pombe mitochondrial F1 (Divita et al., 1991, 1992, 1993). However, in the latter case the extent of the signal was significantly lower than that obtained here by specific placement of a tryptophan residue into the catalytic site.

Most importantly, the mutant enzyme allowed us to compare $K_d$ and $K_m$ values for MgATP directly, and the results obtained are of considerable relevance for the mechanism of the enzyme (for reviews on this topic, see Senior, 1988; Penevsky and Cross, 1991; Cross, 1992; Boyer, 1993). Although there is substantial evidence that the catalytic sites show pronounced cooperativity, i.e. substrate binding at one or possibly two sites promotes product release from another interacting site during both ATP synthesis and hydrolysis, it has not yet been established how many of the three potential catalytic sites must be filled to bring about maximal steady-state catalysis rates. Based on $K_m$ measurements for ATP hydrolysis, it was suggested that the mitochondrial enzyme might work in two different cooperative modes, described as "bi-site" catalysis (already with substantial little physiological significance.

The βW331 fluorescence signal was also used to reinvestigate parameters for binding of ADP and AMPPNP. In both cases the maximal binding stoichiometry seen was 2.4–2.5 mol of nucleotide/mol of F1. A likely explanation is that at the F1 concentration of 50–150 nM used, some dissociation of enzyme into subunits occurred during the rather long time course of the titration experiments, which reduced the apparent binding capacity. Although this could be overcome by performing a series of separate experiments at each nucleotide concentration, rather than by adding nucleotide incrementally, this would have required much larger amounts of enzyme.

Previously AMPPNP binding parameters in wild-type E. coli F1 were estimated by the centrifuge column procedure (Wise et al., 1983). Three exchangeable binding sites were found, with the first site having $K_d = 0.3 \mu M$ and the third site having $K_d = 20 \mu M$. The results found here for the βY331W mutant enzyme (Table II) are in agreement with the earlier work.

In contrast, the ADP binding parameters found here in βY331W mutant F1 differ in some respects from ADP binding parameters determined previously for the wild-type enzyme using the centrifuge column technique. The major difference is that the ADP association rate constant at the first catalytic site was calculated here to be $5 \times 10^6 M^{-1} s^{-1}$, i.e. 2–3 orders of magnitude faster than reported previously (Al-Shawi and Senior, 1992; Senior and Al-Shawi, 1992). One possible reason why the centrifuge column method failed to give the true "on" rate for ADP became obvious from analysis of the binding kinetics. From the association rate constant and the observed $K_d$ for binding of ADP to the first catalytic site, the calculated dissociation rate constant value is $0.05 s^{-1}$, which might lead to substantial loss of bound ligand on the centrifuge column and consequently an underestimation of the association rate. It should also be noted that the rate of dissociation of ADP from the first catalytic site of wild-type F1, as determined previously by the centrifuge column method, was also slower than the currently calculated rate (e.g. Al-Shawi and Senior, 1992) found a rate of $1.2 \times 10^{-3} s^{-1}$ in the absence of P$_i$ and $3.4 \times 10^{-4} s^{-1}$ with P$_i$, present, at pH 8.5). In these experiments dilution of the F1,ADP complex into buffer was used to initiate release of bound ATP, and it may be that the combination of the fast "on" rate and insufficient dilution caused significant retention and/or re-binding of ADP. A similar phenomenon was noted with mitochondrial F1 (Cunningham and Cross, 1988). This explanation is not fully satisfactory, however, because the presence of excess nonradioactive ADP in the dilution buffer did not affect the apparent ADP dissociation rate constant. An alternative explanation, worthy of future exploration, could be that subsequent to initial rapid binding of ADP to the first catalytic site, a slower conformational change to a new F1,ADP conformation occurs. The new F1,ADP complex would have the slower ADP dissociation rate seen in the centrifuge column experiments. Also, the ADP association rate constant measured previously by the centrifuge column procedure might reflect the rate of conformational change that ensues subsequent to the rapid initial phase of ADP binding.

In addition to the points raised by Boyer (1993), we would add that a further source of error in analyzing reaction velocity measurements is that linearization methods weigh individual data points differently, tending to overemphasize those obtained at very low substrate concentrations where the signal to noise ratio is the highest.
The $K_d$ for ADP binding at the first catalytic site in the $\beta$Y331W mutant was calculated here to be around 0.1 $\mu$m (Table II), with the presence of P$_i$ causing only a small effect. Taking into account that the $\beta$Y331W mutant enzyme appeared to have about 2–3-fold higher affinity than wild-type enzyme for nucleotides, this would suggest that wild-type enzyme has $K_d$ (ADP) of 0.2–0.3 $\mu$m at the first catalytic site. This is similar to previously calculated values using the centrifuge column technique (e.g. Al-Shawi and Senior (1992) reported values of 0.69 $\mu$m (with P$_i$) or 1.7 $\mu$m (no P$_i$) at pH 8.5, and Senior and Al-Shawi (1992) reported a value of 0.26 $\mu$m (with P$_i$)).

Because of limitations of time resolution in our fluorescence measurements, we could set only a minimal value ($\geq 5 \times 10^5$ M$^{-1}$ s$^{-1}$) on the ADP association rate at the first catalytic site. Possibly the true rate is similar to that measured for ATP binding ($7 \times 10^5$ M$^{-1}$ s$^{-1}$; Senior et al., 1992). This would mean that E. coli F$_1$ resembles mitochondrial F$_1$ in having similar ADP and ATP association rate constants at the first catalytic site (Cunningham and Cross, 1988). The $K_d$ for ADP at the first site is not significantly altered by the new data; it remains much higher than that observed for the mitochondrial enzyme (1 nM; Cunningham and Cross, 1988). The possible physiological importance of this difference is discussed by Senior (1992).

In summary, we demonstrate that specific placement of tryptophan in E. coli F$_1$ catalytic sites provides a direct fluorimetric probe of catalytic site environment and occupancy by substrate. In future studies with the mutant enzyme, using more rapid fluorescence spectroscopy techniques, it should be possible to achieve a complete resolution of individual ligand and substrate binding steps under a variety of conditions.

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