Three critical residues, \(\beta\)-Lys-155, \(\beta\)-Asp-242, and \(\beta\)-Glu-181, situated close to the \(\gamma\)-phosphate of MgATP in F1-ATPase catalytic sites, were investigated. The mutations \(\beta\)K155Q, \(\beta\)D242N, and \(\beta\)E181Q were each combined with the \(\beta\)Y331W mutation; the fluorescence signal of \(\beta\)-Trp-331 was used to determine MgATP, MgADP, ATP, and ADP binding parameters for the three catalytic sites of the enzyme. The quantitative contribution of side chains to binding energy at all three catalytic sites was calculated. The following conclusions were made. The major functional interaction of \(\beta\)-Lys-155 is with the \(\gamma\)-phosphate of MgATP and is of primary importance at site 1 (the site of highest affinity) and site 2. Release of MgATP during oxidative phosphorylation requires conformational re-positioning of this residue. The major functional interaction of \(\beta\)-Asp-242 is with the magnesium of the magnesium nucleotide at site 1; it has little or no influence at site 2 or 3. In steady-state turnover, the MgATP hydrolysis reaction occurs at site 1. \(\beta\)-Glu-181 contributes little to nucleotide binding; its major catalytic effect derives apparently from a role in reaction chemistry per se. This work also emphasizes that nucleotide binding cooperativity shown by the three catalytic sites toward MgATP and MgADP is absolutely dependent on the presence of magnesium.

The final step in oxidative phosphorylation, the synthesis of ATP driven by a proton gradient, is carried out by the enzyme ATP synthase. It is abundant in mitochondria, chloroplasts, and bacteria; in bacteria, it is also used to build a transmembrane proton gradient, using ATP hydrolysis as the energy source. The enzyme consists of the membrane-embedded \(F_0\)-sector, containing the proton-conducting pathway, and the membrane-extrinsic \(F_1\)-sector, containing the nucleotide-binding sites. Purified, soluble \(F_1\) retains ATPase activity and is extensively used as an experimental model. It shows the subunit composition \(\alpha_3\beta_3\gamma\delta\epsilon\) (for reviews, see Refs. 1–3). X-ray crystallography of bovine mitochondrial \(F_1\) demonstrated that the three \(\alpha\)- and three \(\beta\)-subunits are alternately arranged in a hexagon, with \(\gamma\) in the center. The three catalytic sites are made up predominantly by \(\beta\)-subunit residues, with two side chains from \(\alpha\)-subunits also in close proximity to the bound nucleotide (4).

\(F_1\) shows strong positive catalytic cooperativity. If just one of the three catalytic sites becomes occupied by MgATP ("unsite catalysis"), hydrolysis proceeds slowly, and the products MgADP and P\(_i\) are released very slowly (5, 6). In a molecule in which two sites have bound MgATP ("bisite catalysis"), hydrolysis proceeds, but release of products is slow, so that net turnover remains slow (7). Only when all three catalytic sites in each molecule bind MgATP does rapid, physiological ATPase turnover ensue (8). Under conditions of rapid turnover, the vast majority of molecules in the solution exist at any given point in time in a state in which all three catalytic sites are occupied, two by MgADP and one by MgATP (7).

Deduction of the catalytic properties of \(F_1\) has relied heavily on development of a true equilibrium binding technique for analysis of MgATP binding. It had been shown that in active, soluble, purified \(F_1\), all three catalytic sites bind the nonhydrolyzable nucleotide analogs MgAMP-PNP\(^1\) and Mg-linbenzo-ADP (9, 10), and these reports demonstrated pronounced binding cooperativity among the three sites. For analysis of MgAMP-PNP binding, it was possible to use radioactive nucleotide in conjunction with the centrifuge column technique because significant loss did not occur during centrifugation. For analysis of Mg-lin-benzo-ADP binding, the intrinsic fluorescence signal of the analog itself could be used. Neither of these approaches was viable for measurement of MgATP binding. For MgATP binding, the method of choice has proved to be tryptophan fluorescence spectroscopy using specifically engineered tryptophan residues placed strategically in the catalytic sites as reporter probes. This technique has demonstrated that all three sites bind MgATP, with marked binding cooperativity being evident (8, 11). Parallel results were obtained with the slowly hydrolyzed analog Mg-TNP-ATP (12). Magnesium is critical for catalytic site binding cooperativity, and in its absence, all three sites bind ATP with the same affinity (9). That is to say, the catalytic sites behave symmetrically toward nucleotides in the absence of magnesium.

Site-directed mutagenesis is a valuable approach for studies of enzyme mechanism and has already been applied advantageously to studies of \(F_1\), catalytic sites (1–3). Three residues that have proved to be critical for catalysis are \(\beta\)-Lys-155, \(\beta\)-Asp-242, and \(\beta\)-Glu-181 (13–18). \(\beta\)-Lys-155 is located in the Homology A (P-loop) motif (19), and previous mutagenesis experiments indicated that \(\beta\)-Lys-155 interacts with the \(\gamma\)-phosphate of MgATP, contributing necessary binding energy to drive catalysis (13, 14). \(\beta\)-Asp-242 is located in the Homology B motif (19), and mutagenesis suggested that the catalytic activity of this residue derives from its role as a ligand for magnesium in MgATP (13). Several laboratories have studied \(\beta\)-Glu-181, and there is general agreement that a carboxyl group at this position is critical (13, 16–18). Our laboratory reported that the mutation \(\beta\)E181Q had a dramatic effect on the reaction equilibrium and rate constants in unsite catalysis by destabilizing the reaction transition state (13). Thus, \(\beta\)-Glu-181 has been considered to be involved in the catalytic reaction step.

The x-ray structure of bovine mitochondrial \(F_1\) (4) revealed that previous proposals regarding the locations of \(\beta\)-Lys-155, \(\beta\)-Asp-242, and \(\beta\)-Glu-181 were correct and that previous de

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\(^1\)The abbreviations used are: MgAMP-PNP, Mg-5'-adenylyl \(\beta,\gamma\)-imidodiphosphate; Mg-TNP-ATP, Mg-2'(3')-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate.

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* This work was supported by National Institutes of Health Grant GM22934 (to A. E. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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F1-ATPase, Roles of Three Catalytic Site Residues*

(Received for publication, October 1, 1996, and in revised form, November 7, 1996)

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The JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 272, No. 6, Issue of February 7, pp. 3648–3656, 1997

Printed in U.S.A.
Productions about the roles of these residues in catalysis were well founded. Fig. 1A shows the position of each in the catalytic site. In addition, it may be noted that the discovery of a water molecule situated between $\beta$-Glu-181 and the $\gamma$-phosphate of MgATP (see Fig. 1A) led to the suggestion (4) that $\beta$-Glu-181 might act as a catalytic base, activating the water for nucleophilic attack on the $\gamma$-phosphate.

In previous mutagenesis studies of $\beta$-Lys-155, $\beta$-Asp-242, and $\beta$-Glu-181, the catalytic and nucleotide binding parameters reported were restricted to catalytic site 1 (the site of highest affinity) because only "unisite" techniques could be used. The tryptophan fluorescence technique had not yet been developed, and so nucleotide binding parameters for catalytic sites 2 and 3 were not accessible. Therefore, to further investigate the molecular role of these three critical residues, we combined each of the inhibitory mutations, $\beta$K155Q, $\beta$D242N, and $\beta$E181Q, with the $\beta$Y331W mutation and determined nucleotide binding parameters at all three catalytic sites. As shown in Fig. 1B, in wild-type enzyme, $\beta$-Tyr-331 makes van der Waals contact with the adenine ring of bound nucleotide in the catalytic sites. When Trp is substituted at residue $\beta$331, it shows a large fluorescence signal that is completely quenched on binding of nucleotide (8). Purified F1 was obtained from each of the mutants $\beta$K155Q/$\beta$Y331W, $\beta$D242N/$\beta$Y331W, and $\beta$E181Q/$\beta$Y331W, and binding parameters for MgATP, MgADP, ATP, and ADP at each of the three catalytic sites were determined using the $\beta$-Trp-331 fluorescence signal.

EXPERIMENTAL PROCEDURES

Construction of Mutant Escherichia coli Strain $\beta$K155Q/$\beta$Y331W—The $\beta$K155Q mutation was moved from plasmid pDP33 (20) into plasmid pSWM4 (which contains the $\beta$Y331W mutation (8)) by replacing the 1.0-kilobase Bst1107I fragment in pSWM4 with the corresponding fragment from pDP33. The resultant plasmid was named pSWM31. The presence of both mutations in pSWM31 was confirmed by DNA sequencing. Strain JP17 (21), which contains a large deletion in the chromosome $\beta$-subunit gene, was transformed with plasmid pSWM31 to yield strain SWM31.

Construction of Mutant E. coli Strain $\beta$E181Q/$\beta$Y331W—The same procedure as described above was followed, with the exception that the 1.0-kilobase $\beta$1107I fragment carrying the $\beta$E181Q mutation originated from plasmid pDP36 (20). The mutant strain (pSWM32/JP17) was named SWM32.

Construction of Mutant E. coli Strain $\beta$D242N/$\beta$Y331W—The 2.5-kilobase HindIII-KpnI fragment from pSWM4, containing the entire $\beta$-subunit gene with the $\beta$Y331W mutation, was ligated into M13mp18, and mutagenesis was performed as described previously (10). The mutagenic oligonucleotide was TGCTGTTCGTTAACAACATC, the underlined base changing residue $\beta$242 from Asp to Asn (GAC to AAC) and introducing a new HpaI site. The 2.2-kilobase NheI-KpnI fragment was transferred from mutant phage into pSWM4, generating the new plasmid pSWM36. DNA sequencing was performed to show that the $\beta$-subunit gene in pSWM36 contained both $\beta$D242N and $\beta$Y331W mu-
TABLE I
Characterization of E. coli strains and purified F1

| Mutation          | Growth on succinate | Growth yield in limiting glucose | ATPase of purified F1 |
|-------------------|---------------------|----------------------------------|----------------------|
| Wild type         | ++                  | 100                              | 28                   |
| Unc-              | −                   | 53                               | 0.008                |
| βY331W            | ++                  | 90                               | 0.010                |
| βK155Q/βY331W     | +                   | 48                               | 0.008                |
| βE181Q/βY331W     | +                   | 49                               | <0.001               |
| βD242N/βY331W     | −                   | 53                               | 0.010                |
| βK155Q/βE181Q     | −                   | 53                               | 0.008                |
| βD242N           | −                   | 53                               | 0.014                |

a The wild type was pDP34/JP17 (8).
b Unc- was pUC118/JP17.
c βY331W data are from Ref. 8.
d Data are from this work.

e Data are from Ref. 13.


tations and no undesired mutations. pSWM36 was introduced into strain JP17, yielding strain SVM36.

Characterization of Mutant Strains—Growth yield analysis in limiting (3 mM) glucose liquid medium and growth tests on solid succinate medium were performed as described (22).

Enzyme Purification and Characterization—F1 was purified from each of the mutant strains described above and from wild-type strain SWM1 (23) according to Weber et al. (10). ATPase assays were performed as described by Weber et al. (11), except that the ATPase activities shown in Fig. 8 were measured at 23 °C and pH 8.0. Procedures for SDS-gel electrophoresis and protein assay were as described previously (10).

Fluorescence Measurements—For studies of binding of nucleotides, F1 was pre-equilibrated in buffer (50 mM Tris/SCN, pH 8.0) by dilution to ~8 mg/ml and passage of 100-μl aliquots consecutively through two 1-ml Sephadex G-50 centrifuge columns. In previous work (8, 11), this procedure was seen to effectively remove catalytic site-bound nucleotide. Fluorescence experiments were carried out using a Spex Fluorolog 2 spectrophotometer at 23 °C. Final enzyme concentration in the cuvette was 100–200 nM. For MgATP binding experiments, the buffer (50 mM Tris/SCN, pH 8.0) contained 2.5 mM MgSO4, and ADP was added as indicated. Titrations with MgATP were performed by adding ATP and MgSO4 in a constant ratio of 2.5:1. MgATP and MgADP concentrations were calculated according to Ref. 24. For ADP and ATP binding experiments, the 50 mM Tris/SCN, pH 8.0 buffer contained 0.5 mM EDTA, ADP or ATP was added as indicated. Excitation and emission wavelengths were 295 and 390 nm, respectively. Background signals (buffer, Raman scatter) were subtracted; inner filter and volume effects were corrected for by parallel titrations with wild-type F1. Evaluation of the data was achieved using DATA-MAX software; calculation of nucleotide binding parameters was performed according to Ref. 8 using the equations given in the figure legends.

RESULTS

Functional Effects of the Mutations βK155Q/βY331W, βE181Q/βY331W, and βD242N/βY331W in Cells—In Table I, the effects of the double mutations are presented and compared with data for each of the mutations when present singly. In accordance with the results obtained for the single mutations βK155Q, βE181Q, and βD242N (13), none of the double mutants was able to grow on succinate plates, nor did the growth yield in medium containing limiting (3 mM) glucose exceed that of the negative Unc− control. Thus, all three mutations, βK155Q, βE181Q, and βD242N, abolish ATP synthase activity in vivo when present in combination with the βY331W mutation. As reported previously (8), the βY331W mutation alone had only a minor effect on ATP synthase activity in vivo.

Properties of Purified F1 from the Mutant Strains—As judged from the Sephacryl S300 elution profile in the final step of purification and from SDS-gel electrophoresis, purified F1 from all three double mutants showed the same molecular size and subunit composition as wild-type F1. The specific ATPase activity of purified F1 was determined and in each case was very low indeed (Table I, fourth column). In the case of the βK155Q/βY331W and βD242N/βY331W enzymes, the activities were similar to those seen earlier (13) for the βK155Q and βD242N single mutant enzymes. For the βE181Q/βY331W double mutant enzyme, no ATPase activity was observed (limit of detection was ≤0.001 units/mg), whereas the βE181Q single mutant F1 preparation showed very low but detectable activity in previous work (13). The reason for this difference is not clear; nevertheless, the data of Table I show clearly that each of the mutations under study retains its potent inhibitory effect on catalysis in purified enzyme when present with the βY331W mutation. It may be recalled that the βY331W mutation alone reduces kcat by ~50%, but has no effect on kcat/Km in purified F1 (8).

Tryptophan Fluorescence Signals of Purified F1 from the Mutant Strains—Tryptophan fluorescence spectra of purified F1 from all three double mutants were very similar to that of the βY331W mutant F1 (see Fig. 2 in Ref. 8), and in each case, the βW331 fluorescence signal was quenched virtually completely upon addition of saturating nucleotide, providing an ideal tool to determine effects of each of the three inhibitory mutations on catalytic site nucleotide binding parameters. The similarity of the fluorescence spectra indicates normal folding of the β-subunits in the mutant enzymes.

Nucleotide Binding to βK155Q/βY331W Purified F1—Titrination curves for MgATP and MgADP binding are presented in Fig. 2 (A and B, respectively). Dissociation constants were determined by fitting theoretical binding curves to the experimental data by nonlinear least-squares regression analysis. The lines in Fig. 2A represent fits to the MgATP binding data using a model assuming three independent binding sites per F1 (8, 13, 14). As determined previously (8, 11), this model provides the optimal fit with the βY331W enzyme. The calculated dissociation constants are given in Table II. Two conclusions are immediately apparent: (i) instead of three catalytic sites each of very widely different affinity, as in βY331W F1, the βK155Q/βY331W enzyme has Kd very similar to Kcat; and (ii) the βK155Q mutation strongly reduces binding affinity for MgATP at catalytic sites 1 and 2, but has a much smaller effect on site 3. It should be noted that in βY331W F1, only a limit value of ≤50 nM can be assigned to Kd from the fluorescence data because catalytic site 1 was already filled at the lowest concentration of MgATP used (170 nM). Experiments with wild-type F1 using radioactive MgATP and unisite techniques yielded a much lower value for Kd of 0.2–0.4 nM (13, 25). Therefore, the effects of the βK155Q mutation on Kd are much larger than is apparent from the data in Table II, amounting to a reduction of 3 orders of magnitude. Previous determination of Kd,MgATP for the βK155Q single mutant enzyme using unisite techniques gave values of 0.3–0.4 μM (13, 14), in good agreement with the value of 0.5 μM for the βK155Q/βY331W mutant reported in Table II.

Fig. 2B shows titration curves for MgADP binding. The lines represent fits to a model assuming two classes of binding sites (see Fig. 2B legend). As noted previously, this model provides the optimal fit for MgADP binding to βY331W F1 (8). As Table II shows, the βK155Q mutation had no significant effect on calculated MgADP binding affinity at any of the catalytic sites. The difference between the two curves in Fig. 2B is due to different N values for the two classes of binding sites. Whereas for βY331W F1, the best fit is obtained with 1.1 sites of higher affinity and 1.4 sites of lower affinity, for βK155Q/βY331W F1, the corresponding N values are 0.4 and 2.3, respectively. An explanation for the occurrence of non-integer binding stoichiometries is not apparent. However, even if the model assumed for calculation of binding parameters is not fully correct, still it is apparent from Fig. 2B that, at most, the βK155Q mutation
perturbs MgADP binding only at site 1, and then not to a major degree.

Titrination curves for ATP and ADP (i.e. absence of magnesium and presence of 0.5 mM EDTA) are presented in Fig. 3 (A and B, respectively). In all cases, a model assuming a single class of binding site (solid lines) gave an adequate fit (see Fig. 3 legend). For the βY331W enzyme, the number of sites (N) = 2.9 for ATP and 2.7 for ADP; for βK155Q/βY331W F₁, N = 3.0 for ATP and 2.9 for ADP. The calculated binding affinities are given in Table II. It is apparent that the binding affinity for ATP is decreased considerably by the βK155Q mutation and that the binding affinity for ADP is reduced, but to lesser extent.

Together, the data presented in Figs. 2 and 3 show that the lysine residue in position β155 of F₁ catalytic sites interacts primarily with the γ-phosphate of the nucleotide, as has been proposed earlier (14). The βK155Q mutation strongly reduced binding affinities for MgATP and ATP, but affected ADP and MgADP binding much less. Moreover, the three catalytic sites are not the same: β-Lys-155 contributes far more to binding of MgATP at sites 1 and 2 than it does at site 3. This difference is important for the release of MgATP during oxidative phosphorylation, as discussed below.

Nucleotide Binding to βE181Q/βY331W Purified F₁—Titrination curves for binding of MgATP and MgADP to βE181Q/βY331W F₁ are displayed in Fig. 4 (A and B, respectively). As described above, the MgATP data were fitted assuming a model with three independent binding sites (Fig. 4A), and calculated Kᵦ values are given in Table II. It is evident that the βE181Q mutation has no significant influence on binding of MgATP at catalytic sites 2 and 3. Kᵦ for βE181Q/βY331W F₁ could not be accurately calculated because catalytic site 1 was already filled at the lowest concentration of MgATP used (170 nM). Inspection of the binding curves in Fig. 4A indicates little effect of the βE181Q mutation on Kᵦ. However, it should be noted that previous unisite experiments indicated a Kᵦ of 40 nM for the βE181Q single mutant enzyme (Ref. 13; see also Ref. 16).

For MgADP binding in Fig. 4B, a model assuming two classes of binding sites (as described above) gave a good fit to the βE181Q/βY331W data, with 0.5 sites of higher affinity and 1.9 sites of lower affinity. The calculated Kᵦ(MgADP) values (Table II) indicate that there was no effect of the βE181Q mutation on MgADP binding affinity at any of the catalytic sites. The difference between the βE181Q/βY331W and βY331W curves in Fig. 4B is again due to differences in binding stoichiometry (N values) at site 1 as compared with sites 2 and 3 (see above), and the same comments apply.
Curves for binding of ATP and ADP to the \(\beta\)K155Q/\(\beta\)Y331W mutant \(F_1\) in the absence of magnesium are reported in Fig. 5 (A and B, respectively). ATP bound to 2.6 sites, each with a \(K_d\) of 20 \(\mu M\), and ADP bound to 2.8 sites, each with a \(K_d\) of 30 \(\mu M\). Thus, both nucleotides were bound with slightly higher affinity in the \(\beta\)E181Q/\(\beta\)Y331W mutant than in the parental \(\beta\)Y331W enzyme (Table II). Elimination of the negative charge of the \(\beta\)-Glu-181 residue could remove a repulsion toward the phosphates when no magnesium is present for compensation of their negative charge.

Overall, the data show that \(\beta\)-Glu-181 is not primarily involved in nucleotide binding, especially at catalytic sites 2 and 3. At catalytic site 1, it makes no contribution to MgADP binding and, at most, a small contribution to MgATP binding.

**Nucleotide Binding to \(\beta\)D242N/\(\beta\)Y331W Purified \(F_1\)**—Binding curves obtained by titration of \(\beta\)D242N/\(\beta\)Y331W \(F_1\) with MgATP and MgADP are shown in Fig. 6 (A and B, respectively). For MgATP, the model assuming three independent binding sites gave a good fit to the data, and calculated values for dissociation constants are given in Table II. The highest affinity catalytic site 1 was most affected by the \(\beta\)D242N mutation, with \(K_{d1}\) being increased to 0.3 \(\mu M\). Previous work using unisite techniques had given a similar value of 0.28 \(\mu M\) for \(K_{d1}\) at pH 8.0 in \(\beta\)D242N single mutant \(F_1\). In contrast, at sites 2 and 3, significant changes in \(K_{d(MgADP)}\) did not occur (Table II). A similar result was obtained from the MgADP binding curves (Fig. 6B). Here, \(K_{d(MgADP)} = 12 \mu M\) at all sites (Table II) \((N = 2.5)\). Thus, the MgADP-binding site of higher affinity had effectively disappeared in the \(\beta\)D242N/\(\beta\)Y331W enzyme.

Binding of ATP and ADP to \(\beta\)D242N/\(\beta\)Y331W \(F_1\) in the

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\(\text{[S]}([E]) = N \cdot ([S]_f([S]_f + K_d) \quad (\text{Eq. 3})\)

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\(^2\) A. L. Hazard and A. E. Senior, unpublished data.
The absence of magnesium is shown in Fig. 7 (A and B, respectively). In both cases, a model assuming a single class of binding site gave a satisfactory fit, and calculated dissociation constants are shown in Table II. ATP and ADP were both bound with slightly higher affinity in the \( \beta D242N/\beta Y331W \) mutant than in the \( \beta Y331W \) parent enzyme. In \( \beta D242N/\beta Y331W \) F1, \( N = 2.7 \) with ATP and 2.7 with ADP.

From its location in the catalytic site, as revealed in the x-ray structure (Fig. 1A), \( \beta \)-Asp-242 is positioned to be one of the coordinating ligands for the magnesium ion in MgATP (4). Such a role was adumbrated by biochemical studies of the \( \beta D242N \) mutant (13) and has been proposed for the analogous Asp residue in the Homology B sequence in other enzymes. Data in this paper confirm that the carboxyl side chain of \( \beta \)-Asp-242 does bind the magnesium of MgATP and MgADP; moreover, they show that this role is limited to catalytic site 1. MgATP and MgADP binding at catalytic sites 2 and 3 was not affected by the \( \beta D242N \) mutation. Thus, \( \beta \)-Asp-242 is an important contributor to the high binding affinity of catalytic site 1 for MgATP, and it is very reasonable to propose that release of MgATP during oxidative phosphorylation involves disengagement of the bond between the Asp-242 side chain and the magnesium ion. \( \beta \)-Asp-242 also contributes to binding of MgADP at catalytic site 1, to a lesser extent than with MgATP. The behavior seen with ADP and ATP in the \( \beta D242N/\beta Y331W \) mutant supports these conclusions. With both nucleotides, all three catalytic sites show the same affinity, with no cooperativity evident, and the dissociation constant is similar to that of sites 2 and 3 for MgATP or to that of sites 1–3 for MgADP (Table II). Binding of ATP and ADP in the \( \beta D242N/\beta Y331W \) mutant is actually slightly tighter than in the \( \beta Y331W \) parental enzyme (22 \( \mu \)M versus 71 \( \mu \)M for ATP and 11 \( \mu \)M versus 83 \( \mu \)M for ADP). The missing negative charge of \( \beta \)-Asp-242 may lessen repulsion toward the negative phosphates of the nucleotides in the absence of magnesium.

Magnesium Dependence of Steady-state ATPase Activity in the \( \beta D242N/\beta Y331W \) Double Mutant—The results described above showed that \( \beta \)-Asp-242 plays a critical role in binding of
MgATP specifically at catalytic site 1, and we wished to find whether this role is correlated with effects on steady-state catalysis. Excess magnesium ion inhibits ATPase activity in E. coli F₁ (9), and we used this property to investigate this question. ATPase activity of βD242N/βY331W double mutant F₁ was assayed at a constant ATP concentration (5 mM) in the presence of increasing magnesium ion concentration (Fig. 8). In the wild type and βY331W, an ATP/magnesium ratio of 2.5:1 proved optimal for activity as previously established, and increasing concentrations of magnesium ion led to significant inhibition. In contrast, ATPase activity in the βD242N/βY331W mutant was not inhibited by magnesium even at 25 mM. Thus, the mutant had lower affinity for inhibitory magnesium cation. Control experiments showed that binding of MgATP to all three sites was retained under saturating conditions in the presence of 25 mM magnesium ion in both βD242N/βY331W and βY331W enzymes. These results confirm the role of β-Asp-242 in catalysis through its role in liganding magnesium of the substrate MgATP, and they emphasize that catalytic site 1 is the catalytic site at which steady-state catalysis occurs.

**DISCUSSION**

**General**—The aim of this work was to study the molecular role of β-Lys-155, β-Asp-242, and β-Glu-181 in catalytic sites of F₁-ATPase. The first two are located in the P-loop and Homology B sequences, respectively. All three are located close to the γ-phosphate of bound MgATP (4), and previous mutagenesis experiments from this and other laboratories have shown that all three are critical for both ATP synthesis and ATP hydrolysis. Application of unisite techniques has revealed a wealth of information regarding the molecular role of these residues at catalytic site 1 (highest affinity site), but there is not yet comparable information regarding catalytic sites 2 and 3. We recently introduced the technique of site-directed tryptophan fluorescence spectroscopy to determine nucleotide occupancy and binding parameters in catalytic sites of F₁ under true equilibrium conditions (8,11). Here, we applied this technique to determine functional effects of the mutations βK155Q, βD242N, and βE181Q at all three catalytic sites.

**β-Lys-155**—We found that β-Lys-155 is particularly important for MgATP binding: it has its major effects at catalytic sites 1 and 2 and a small effect at catalytic site 3 (the site of lowest affinity) (Figs. 2 and 3 and Table II). The contributions of β-Lys-155 to overall MgATP binding energy are as follows: site 1, 3.9 kcal/mol (calculated from data in Refs. 13, 14, and 25); site 2, 3.4 kcal/mol (this work); and site 3, 1.1 kcal/mol (this work). Lack of strong interaction between β-Lys-155 and MgATP at catalytic site 3 appears to be the major reason for the relatively low affinity of this site. On the other hand, β-Lys-155 did not provide binding energy for MgADP binding at any site (Table II). The primary functional interaction of β-Lys-155 is therefore with the γ-phosphate of bound MgATP.

In the x-ray structure of F₁ (4), only two catalytic sites are filled, one with MgAMP-PNP and one with MgADP. It is not known which corresponds to site 1 (highest affinity) and which to site 2. However, it is valuable to note the spatial relationship of β-Lys-155 to the nucleotide in both cases. In the MgAMP-PNP-containing site (Fig. 1A), the distance from the ε-amino group of β-Lys-155 to the nearest γ-phosphate oxygen of the nucleoside triphosphate is 2.7 Å, and that to the nearest β-phosphate oxygen is 3.3 Å, whereas the distance to the mag-

![FIG. 7. ATP and ADP binding to catalytic sites of βD242N/βY331W mutant F₁ in the absence of magnesium. Details are the same as described in the legend to Fig. 3. A, ATP; B, ADP. ●, βK155Q/βY331W; ○, βY331W.](image)

![FIG. 8. Mg²⁺ dependence of ATPase activity under steady-state conditions in βD242N/βY331W mutant F₁. ATPase activity was assayed at 23 °C in 50 mM Tris/SO₄, pH 8.0, with 5 mM NaATP and MgCl₂ as indicated. ●, βK155Q/βY331W; ○, βY331W; □, wild type.](image)
nesium ion is 5.4 Å. In the MgADP-containing site, corresponding distances are 2.7 Å (to the nearest β-phosphate oxygen) and 5.2 Å (to magnesium).

It has been proposed that in oxidative phosphorylation, the proton gradient provides necessary energy to release MgATP from F1, by overcoming the barrier for changing binding affinity at the site where MgATP is synthesized from tight to loose (26). Table II shows that upon introduction of the βK155Q mutation, the binding affinity for MgATP at catalytic site 1 was drastically reduced. Conformational changes necessary for proton gradient-induced release of MgATP during ATP synthesis must therefore involve movement of β-Lys-155 away from the γ-phosphate. For MgATP hydrolysis, the critical role of β-Lys-155 must derive from its immobilization and orientation of the nucleotide, at least in part, and other mechanisms may also be operative.

Our studies on β-Lys-155 are also germane to the question of what mechanism determines binding cooperativity among the three catalytic sites. It was recently suggested that structural asymmetry of F1, caused by the γ-subunit is responsible for the generation of the highest affinity catalytic site (27). Since, in intact F1, binding of ATP or TNP-ATP (in the absence of magnesium) occurs with equal or very similar affinity at all three catalytic sites (11, 12), and we show further in Table II of this paper that binding of ADP (in the absence of magnesium) also occurs with equal affinity at all three sites, it is evident that while the γ-subunit certainly is necessary for binding cooperativity to be displayed, it is not sufficient. Data in this paper actually substantiate this argument at the molecular level. When MgATP occupies the catalytic sites, β-Lys-155 must occupy a different position in relation to the nucleotide in each of site 1, site 2, and site 3 because the βK155Q mutation affects the three sites differentially (Table II). In contrast, when ATP occupies the catalytic sites, β-Lys-155 appears to occupy the same position in relation to the nucleotide in all three sites since the βK155Q mutation affects all three sites equally.

β-Asp-242—From its location in the catalytic site (Fig. 1A) and by analogy with other enzymes, one of the coordinating ligands for the magnesium of MgATP is predicted to be β-Asp-242, through an intervening hydrogen-bonded water. This work establishes that β-Asp-242 is one of the magnesium ligands and, moreover, that this role is realized predominantly at catalytic site 1. The binding energy contributed by β-Asp-242 to MgATP binding at site 1 is 3.0 kcal/mol, and the values at sites 2 and 3 are 1 and 0 kcal/mol, respectively. The result for site 3 was not unexpected, as there is no significant difference between the binding affinity for MgATP at site 3 and for free ATP at any of the three sites in βY331W F1 (Table II). The binding energy contributed by β-Asp-242 to MgADP binding at sites 1–3 is 2.7, 0, and 0 kcal/mol, respectively. Distances between the magnesium and β-Asp-242 carboxyl oxygens in the x-ray structure of the MgAMP-PNP-containing site (Fig. 1A) are 3.9 and 4.1 Å. The closest contacts of magnesium are with oxygens of the γ-phosphate (2.2 Å) and β-phosphate (2.5 Å) and the hydroxyl of β-Thr-156 (2.3 Å). In the MgADP-containing site, the two β-Asp-242 carboxyl oxygens are 4.3 Å from the magnesium. The closest contacts of magnesium are with oxygens of the β-phosphate (2.3 Å) and the hydroxyl of β-Thr-156 (2.3 Å).

Removal of the carboxyl side chain at position β242 not only decreased the affinity at site 1 for both MgATP and MgADP, it also abolished the inhibitory effect of magnesium ion on ATPase activity (Fig. 8). Taken together with the fact that the βD242N mutation reduced nucleotide binding affinity primarily at site 1, this emphasizes that site 1 is where hydrolysis occurs in steady-state catalysis.

β-Glu-181—As described in the Introduction, site-directed mutagenesis studies have shown that β-Glu-181 plays a critical role in catalysis in F1-ATPase and that the carboxyl group is of paramount importance. Abrahams et al. (4) suggested that β-Glu-181 could act as a catalytic base by activating a water molecule to perform in-line nucleophilic attack on the γ-phosphate of MgATP. In the x-ray structure of the MgAMP-PNP-containing site (Fig. 1A), this water is situated 4.1 Å from the γ-phosphorus atom; the nearest carboxyl oxygen of β-Glu-181 is 5.1 Å from the γ-phosphorus atom. In the MgADP-containing site, a similarly positioned water is absent, and the nearest carboxyl oxygen of β-Glu-181 is 7.0 Å from the β-phosphorus atom. The results presented in this study establish that the mutation βE181Q had no influence on the binding affinity for MgATP at catalytic sites 2 and 3 or on the binding of MgADP, ATP, or ADP at site 1, 2, or 3. There may be some effect on MgATP binding at site 1 (see “Results”), but taken overall, β-Glu-181, although located in the phosphate-binding pocket, clearly contributes less to nucleotide binding or positioning than either of the other two residues studied here. Thus, its critical role in catalysis most likely derives from a different source.

Information obtained so far from mutagenesis of F1, in several laboratories implicates β-Glu-181 in reaction chemistry, but does not yet point to any specific mechanism. Within different nucleoside triphosphate-hydrolyzing enzymes, a variety of answers to the question of whether a general base residue is present have been proposed. For p21ras, it was suggested initially that Gln-61 is the catalytic base, but more recent work suggests that in this protein, the γ-phosphate of GTP itself acts as the base, leaving a stabilizing and orienting role for Gln-61 (28–31). Sato et al. (32) suggest that Asp-133 is the catalytic carboxylate that activates a water molecule for the attack of the γ-phosphate in SecA protein of E. coli. Lys-71 plays an important role in bovine Hsc70 protein, and ATPase activity was abolished in mutants K71E, K71M, and K71A. Crystals obtained from these mutants contain bound ATP, and participation of Lys-71 in catalysis as a proton acceptor has been proposed (33). In myosin, where no obvious catalytic base candidate is present in the catalytic site, a highly conserved serine residue associated with the γ-phosphate-binding pocket may function as an exchanger of protons between the phosphate and the attacking water molecule (34). Thus, it would seem that the presence of a catalytic base side chain is not a sine qua non for rapid nucleoside triphosphatase activity, and before concluding that β-Glu-181 is acting as catalytic base, further work will be needed. It may be pertinent that the unisite MgATP hydrolysis rate remained unchanged at pH 5.5–9.5 (35). β-Glu-181 may stereochemically orient and polarize the attacking water without net proton abstraction.

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3 It is also relevant that the βE181Q mutation had no effect on K(MgATP), whereas βK155Q and βD242N increased it by 3 orders of magnitude (13).
F$_1$-ATPase Catalytic Site Mutants

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