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Differentiation of Catalytic Sites on *Escherichia coli* F_1ATPase by Laser Photoactivated Labeling with [^{3}H]-2-Azido-ATP Using the Mutant βGlu381Cys:ϵSer108Cys To Identify Different β Subunits by Their Interactions with γ and ϵ Subunits[†]

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ABSTRACT: The ATP binding affinities of the catalytic sites in the three β subunits of the *Escherichia coli* F_1 ATPase (ECF_1) have been explored in relation to the interaction of these subunits with the small subunits γ and ϵ. ECF_1 from the mutant βE381C:ϵS108C was reacted with different concentrations of [^{3}H]-2-azido-ATP and covalent insertion of the nucleotide analogue induced by photoactivation of the azide group to a nitrene with single-pulse UV laser excitation. The enzyme showed cooperative binding of [^{3}H]-2-azido-ATP in the presence of Mg^{2+}. The highest affinity site was located at β_{free}, the one of the three β subunits in the mutant that does not form disulfide bonds with either the γ or the ϵ subunit. This β subunit is, therefore, the site of unisite catalysis in the enzyme. The second mole of [^{3}H]-2-azido-ATP to bind was located in the β subunit that links to ϵ (β_ϵ), while the lowest affinity binding of the substrate analogue was with the β subunit that links to γ (β_γ). In the absence of Mg^{2+}, all three β subunits bound [^{3}H]-2-azido-ATP with a similar, low affinity. The results show that binding of MgATP is determined by, and/or must determine, the interactions of the different $\alpha-\beta$ subunit pairs with the single-copy subunits, γ, δ, and ϵ of the enzyme.

F_1F_0-type ATPases catalyze oxidative phosphorylation or photophosphorylation by using a transmembrane protonmotive force to drive ATP synthesis [reviewed in Senior (1988), Issartel *et al*. (1992), and Hatefi (1993)]. In the reverse direction, these enzymes use ATP to generate a proton gradient that can be used in ion transport processes. They are multisubunit complexes consisting of a hydrophilic part, F_1, which in the *Escherichia coli* enzyme (ECF_1)[1] is composed of five subunits in the stoichiometry of α_3, β_3, γ, δ, ϵ, and an intrinsic membrane domain, F_0, which in its simplest form, as in the *E. coli* enzyme (ECF_0), contains three subunits, a, b, and c, in the molar ratio of 1:2:9−12 (Senior, 1988; Foster & Fillingame, 1982). The F_1 part is joined to F_0 by a stalk, contributed by γ, δ, ϵ, and the b subunits in ECF_1F_0 (Capaldi *et al*., 1994).

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[1] Abbreviations: AMP·PNP, 5′-adenylyl β,γ-imidodiphosphate; DTT, dithiothreitol; ECF_1, soluble portion of the *Escherichia coli* F_1F_0-ATPase; ECF_1F_0, *Escherichia coli* F_1F_0 ATP synthase; EDTA, ethylendiaminetetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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F_1F_0-type ATPases can bind nucleotides in each of three catalytic sites located on β subunits. In the presence of Mg^{2+}, the first ATP binds very tightly, while the second and third ATP molecules bind with lower affinities. Substrate ATP, bound in the high-affinity site, can be hydrolyzed but only slowly in what is called unisite catalysis (Grubmeyer *et al*., 1982). Binding of ATP at a second catalytic site increases ATP hydrolysis in the first site by as much as 10^6-fold; i.e., the enzyme also shows multisite or cooperative catalysis (Cross *et al*., 1982; Noumi *et al*., 1986; Al-Shawi & Senior, 1992).

The recent high-resolution structure of F_1 from beef-heart mitochondria (MF_1) (Abrahams *et al*., 1994) gives important clues about the mechanism of cooperativity of the enzyme. As expected from the unusual stoichiometry of subunits, the F_1 is intrinsically asymmetric, with the three $\alpha-\beta$ pairs different with respect to their interactions with the single-copy γ subunit and, in the crystal form described, with respect to nucleotide occupancy. In what is probably the ADP plus azide inhibited form, one catalytic site contains ADP (in β subunit β_{DP}) and is tightly closed, a second contains AMP•PNP (β_{TP}) and is partly open, while the third is empty (β_E). Thus, with respect to the states of the catalytic sites, the structure is that expected for an alternating site type of mechanism of cooperativity (Boyer, 1993).

The obvious question raised by the structural data is what determines the states of the different catalytic sites? For example, is it the interactions of the γ, δ, and ϵ subunits with the $\alpha-\beta$ pairs that determine their conformation and thereby their affinities for nucleotide? To examine this issue, we have taken advantage of mutants we have described (Aggeler *et al*., 1995) that allow us to differentiate the three β subunits by their covalent cross-linking to the γ and/or ϵ subunits. These have been used in combination with laser-photolysis-aided covalent binding of [^{3}H]-2-azido-ATP to establish the relationship between nucleotide affinity and the interaction of the different β subunits with the small subunits.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes were obtained from Boehringer Mannheim and New England Biolabs. Sephadex G-50 was purchased from Pharmacia Biotech Inc., and all other chemicals used were of analytical grade and purchased from Sigma.

Construction and Characterization of the βY331W:βE381C:ϵS108C Mutant Strain of ECF_1. A *Nsi*I linker (New England Biolabs) was inserted in the Acc65I site of the plasmid pSWM4 (Weber *et al*., 1993) containing the βY331W mutation, a generous gift of Dr. A. E. Senior (University of Rochester Medical Center, New York). The 4.4 kb *Xho*/*Nsi*I portion of pSWM4 was next inserted into plasmid pRA100 (Aggeler *et al*., 1992) to obtain pRA201. Finally, a 6.7 kb *Eag*I fragment of pRA201 was ligated with a 5.9 kb *Eag*I fragment of pRA134 (containing βE381C:ϵS108C; Aggeler *et al*., 1995). This plasmid, pGG1, contains all of the genes encoding the ECF_1F_0 ATPase. The correct orientation of the fragments was screened by *Nae*I digestion. The resulting plasmids were transformed into strain *AN888* (*unc*B$^+$ Mu::416, *argH, pyrE, entA, nalA recA*; Lightowlers, 1987). Subcloning procedures were carried out using the *E. coli* strain *XL1 Blue* (Davis *et al*., 1986).

Preparation of ECF_1. ECF_1 was isolated from pGG1:AN888 and from pRA134:AN888 by a modification of the method of Wise *et al*. (1981), described in Gogol *et al*. (1989). Purified enzymes were precipitated for 1 h in 70% ammonium sulfate at 4 °C, centrifuged at 10000*g* for 20 min, and dissolved in 50 mM MOPS, pH 7.0, 0.5 mM EDTA, and 10% glycerol (v/v). In order to establish a defined nucleotide condition, the enzyme was passed through two consecutive centrifuge columns (Sephadex G-50 medium) and equilibrated in the same buffer (Aggeler *et al*., 1992).

$CuCl_2$-Induced Cross-Link Formation. Cross-linking was carried out as follows: 0.1 mM EDTA and 2.5 mM $MgCl_2$ were added to the solution of ECF_1 and incubated with 150 μM $CuCl_2$ for 3 h at room temperature (Aggeler *et al*., 1995). In order to analyze the cross-linked products, the samples were dissolved in DTT-free dissociation buffer and applied to an SDS-containing 10−18% polyacrylamide gel (Laemmli, 1970). To quantitate cross-link yields, Coomassie Brilliant Blue-stained gels were scanned with a Microtek flatbed scanner, and the intensity of the protein bands was digitized by the *NIH Image 1.53* processing and analysis program for Macintosh.

Fluorescence Titration of the βY331W:βE381C:ϵS108C ECF_1 Mutant. Fluorescence measurements were performed in a SLM 8000 photon counting spectrofluorometer at room temperature. The emission and excitation wavelengths were 342 and 285 nm, respectively. The nucleotide-depleted F_1 mutants (100 nM) were diluted in a 50 mM MOPS, pH 7.0, and 10% glycerol (v/v) buffer and titrated with MgATP (1:1) to a volume of 400 μL in a 0.4 × 1 cm quartz cuvette. Calculations of kinetic constants were performed using the KaleidaGraph data analysis and graphics program for personal computers.

Photoaffinity Labeling. [^{3}H]-2-Azido-ATP was kindly provided by Dr. W. S. Allison, University of California, San Diego. ECF_1 from the mutant βE381C:ϵS108C (5 μM), from which loosely bound nucleotides had been removed, was mixed with [^{3}H]-2-azido-ATP (2−150 μM) and photolyzed by using a Nd:YAG laser with potassium dideuteriophosphate "doubling" crystals with a pulse duration of ∼8 ns (Hockensmith *et al*., 1986). Samples were irradiated at 266 nm, the wavelength of maximal absorbance of 2-azido-ATP (Czarnecki *et al*., 1984). Each sample was held on ice for the entire experiment, conditions under which the maximum temperature increase from irradiation (with 1.4×10^{17} photons) is no more than 2 °C (Hockensmith *et al*., 1986). After photolysis, the mutant enzyme was cross-linked by $CuCl_2$ treatment as described above, and then radioactivity in the α and β subunits was determined from gel slices. The incorporation was quantitated as moles of reagent bound per mole of enzyme from the specific activity of [^{3}H]-2-azido-ATP of 6 cpm/pmol.

Other Methods. ATPase activity was measured with an ATP regenerating system described by Lötscher *et al*. (1984). Protein concentrations were estimated with the BCA protein assay from Pierce Chemical Co.

RESULTS

The mutant βE381C:ϵS108C has been described previously (Aggeler *et al*., 1995). In this mutant, it is possible to obtain high-yield cross-linking of one β to an ϵ subunit via disulfide bond formation between Cys 381 of the β and Cys 108 of ϵ. A second cross-link is formed between Cys 381 of a second β and the intrinsic Cys 87 of the γ subunit. There

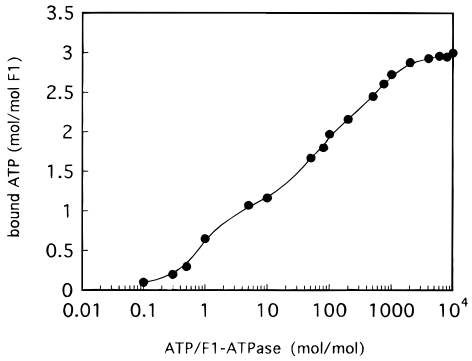

FIGURE 1: MgATP binding to the mutant ECF_1 (βY331W:βE381C:ϵS108C). The mutant ECF_1, βY331W:βE381C:ϵS108C (100 nM), was titrated with MgATP (1:1) in a buffer containing 50 mM MOPS, pH 7.0, and 10% glycerol. The MgATP binding stoichiometries were calculated from the decrease in fluorescence of residue βW331, which was recorded at $\lambda_{em} = 342$ nm with the emission slit at 8 nm.

is also cross-linking of Cys 381 of the third β to the δ and of an α to the δ, both via the intrinsic Cys 140 of the δ subunit. The general approach used in this study was as follows: (i) catalytic sites in ECF_1 from the mutant βE381C:ϵS108C were loaded by addition of various concentrations of the ATP analogue [^{3}H]-2-azido-ATP, (ii) the photoactivatable reagent was bound covalently by laser UV photolysis, and (iii) the incorporation of ATP in the three β subunits was quantitated after cross-linking to the small subunits by $CuCl_2$ treatment of the mutant. Prior to the full experiments, several important controls were performed and conditions were optimized as below.

The Mutations βE381C and ϵS108C Do Not Affect Nucleotide Binding Affinities. The mutant βE381C:ϵS108C has an ATPase activity comparable to wild-type enzyme (Aggeler *et al*., 1995), implying that nucleotide binding is not affected by the introduced mutations. To test this more definitively, a mutant was constructed, βY331W:βE381C:ϵS108C, containing the introduced Cys residues in β and ϵ and, in addition, containing the Trp for Tyr change in the catalytic site, which allows nucleotide binding to be measured directly by fluorescence methods (Weber *et al*., 1993).

In the presence of Mg^{2+}, binding of ATP to this mutant quenched the fluorescence of the introduced Trp residues, resulting in a fluorescence spectrum similar to that of the wild-type enzyme. A titration of the fluorescence quenching showed binding of 3 mol of ATP/mol of ECF_1 with three different dissociation constants (K_d) of <92 nM, 2.1 μM, and 40.3 μM, values close to those reported by Weber *et al*. (1993, 1994) for the mutant βY331W, i.e., <50 nm, 0.5 μM, and 25 μM respectively. These results confirm that introducing the Cys in the DELSEED region of the β subunits has no major effect on nucleotide binding affinities. Figure 1 shows a typical binding curve for the mutant βY331W:βE381C:ϵS108C plotted as a function of the molar ratio of substrate to enzyme.

Optimization of the Covalent Incorporation of [^{3}H]-2-Azido-ATP. It has been shown that 2-azido-ATP binds into the catalytic sites in F_1 with affinities similar to those of ATP and, after photoactivation, inserts predominantly at Tyr 331 (Cross *et al*., 1987; Wise *et al*., 1987). Several conditions were examined for maximizing the activation and covalent insertion of the reagent. The Mineralight hand lamp we had used previously in experiments with the tetrafluorophenyl azide−maleimide series of photoactivatable cross-linkers [e.g., Aggeler *et al*. (1992)] gave yields of covalent incorporation of [^{3}H]-2-azido-ATP into ECF_1 of no more than 25−30% whether irradiating at 254 or 366 nm. This could be increased to 40−45% by using multiple UV lamps. Ultimately, the optimal conditions were obtained by using laser UV photolysis to activate the azido compound following a protocol similar to that used by Hockensmith *et al*. (1986) in their studies of protein−DNA interactions. Thus, photolabeling of the enzyme by [^{3}H]-2-azido-ATP was conducted on ice by adding the reagent over a range of different concentrations to 5 μM ECF_1 in a final volume of 20 μL and then rapidly (within 2 s) activating by UV laser photolysis using a tunable Nd:YAG laser at 266 nm, the wavelength of maximal absorbance of [^{3}H]-2-azido-ATP. A single light pulse of 8 ns was used, which provided sufficient photons (1.4×10^{17}) to convert all of the [^{3}H]-2-azido-ATP molecules to the nitrene. The reaction time, therefore, for covalent modification of ECF_1 by the reagent was the lifetime of the nitrene ($\sim 10^{-4}$ s) [see Staros (1980)]. Thus, the labeling is a single hit experiment, as only reagent already bound into nucleotide binding sites is able to covalently insert into the protein. The yield of labeled products using this procedure was 70−75%. Irradiation of the enzyme under identical conditions, but without the [^{3}H]-2-azido-ATP, gave at most 5% inhibition, presumably due to photo damage.

[^{3}H]-2-Azido-ATP Labeling of ECF_1 in the Presence of Mg^{2+}. For the experiments reported here, cross-linking of the mutant (βE381C:ϵS108C) was catalyzed by adding 150 μM $CuCl_2$. The yield of disulfide bond formation obtained was from 75% to 90% for $\beta-\epsilon$ and from 70% to 80% for the $\beta-\gamma$ products, respectively. A typical cross-linking profile is shown in Figure 2. An amount of 350 μg of protein was loaded onto the gels in order to allow quantitation of the labeling of the subunits.

Figure 3 shows the incorporation of [^{3}H]-2-azido-ATP into the different β subunits in the presence of Mg^{2+} calculated from the distribution of radioactivity in the cross-linked products $\beta-\epsilon$ and $\beta-\gamma$ with correction for the yield of subunit−subunit cross-linking. It can be seen that the first, highest affinity binding site for [^{3}H]-2-azido-ATP is on β_{free}, i.e., that β subunit not cross-linked to either γ or ϵ. The second mole of reagent is bound into β_ϵ (that β which cross-links to the ϵ subunit), while the lowest affinity binding is with β_γ (the β subunit that can be cross-linked to γ). At a 1:1 ratio of [^{3}H]-2-azido-ATP to ECF_1, approximately 0.4 mol of the ATP analogue was bound to β_{free}, which is, therefore, the site of unisite catalysis by ECF_1. At saturation, the covalent insertion of [^{3}H]-2-azido-ATP into β_{free} and β_ϵ was the same, i.e., 0.75−0.76 mol/mol of subunit.

Binding of ATP to ECF_1 in the Absence of Mg^{2+}. The binding of ATP to ECF_1 from the mutant βY331W:βE381C:ϵS108C in EDTA buffer was different from that in the presence of Mg^{2+} as shown in Figure 4. In the absence of Mg^{2+}, the three catalytic sites were equivalent, each with a K_d of around 100 μM, a result similar to that obtained by Weber *et al*. (1994a) with the mutant βY331W. Figure 4 also shows data for the binding of [^{3}H]-2-azido-ATP to the mutant βE381C:ϵS108C in the absence of Mg^{2+}. All three β subunits bound the reagent with a similar affinity, and in the same yields, indicating that the adenine pockets of the catalytic sites are equivalent under these conditions.

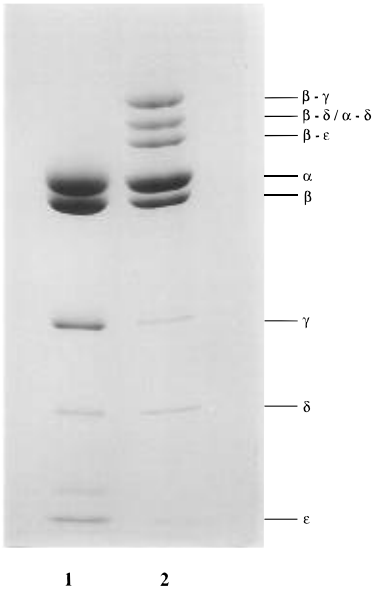

FIGURE 2: Cross-linking of the βE381C:ϵS108C ECF_1 mutant after photolysis. The enzyme (5 μM) and [^{3}H]-2-azido-ATP were irradiated by an UV laser (266 nm) and incubated with 150 μM $CuCl_2$ for 3 h at room temperature (lane 2). The cross-link products were separated with a 10−18% linear gradient of SDS−PAGE (16 × 26 cm) and stained with Coomassie Blue. The gel was scanned with a Mikrotek flat-bed scanner to determine the cross-link amount. The amount of $\alpha-\delta$ and $\beta-\delta$ was calculated on the basis of a 1:1 ratio of the two cross-linked products (M. A. Haughton and R. A. Capaldi, 1995, unpublished results). Aliquots (350 μg) of mutant ECF_1 were incubated in dissociation buffer in the absence of DTT prior to SDS−PAGE. Lane 1 shows untreated enzyme.

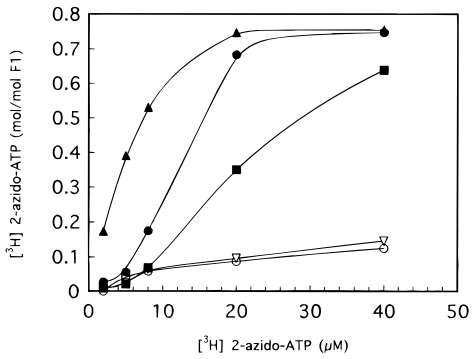

FIGURE 3: Photoaffinity labeling of the βE381C:ϵS108C mutant ECF_1 by [^{3}H]-2-azido-ATP in the presence of Mg^{2+}. The ECF_1 mutant (βE381C:ϵS108C) was incubated with [^{3}H]-2-azido-ATP and $MgCl_2$ (1:1) on ice and illuminated at 266 nm by a UV laser. Cross-linking and separation procedures were carried out as described in Experimental Procedures. The amount of bound [^{3}H]-2-azido-ATP was corrected for the cross-linking yield. Symbols: ▲, free β subunit; ●, β cross-linked with subunit ϵ; ■, β subunit bound to γ, ○, free α subunit; ▽, α cross-linked with subunit δ.

DISCUSSION

The studies described here show that photoaffinity labeling with [^{3}H]-2-azido-ATP can be used to examine the nucleotide binding affinities of the different β subunits when this labeling is performed with ECF_1 from the mutant βE381C:ϵS108C. By laser photoactivation of [^{3}H]-2-azido-ATP at 266 nm, the wavelength of maximal absorbance of the reagent, it proved possible to obtain cross-linking yields of the nucleotide with the protein of close to 75%. Moreover, as the activating light was given in a single short high-intensity flash, all of the reagent was converted to the nitrene at the same instant, and the labeling of the enzyme was thereby a single-hit experiment, because the decay of the active nitrene is faster (10^{-4} s) than exchange of bound for free nucleotide.

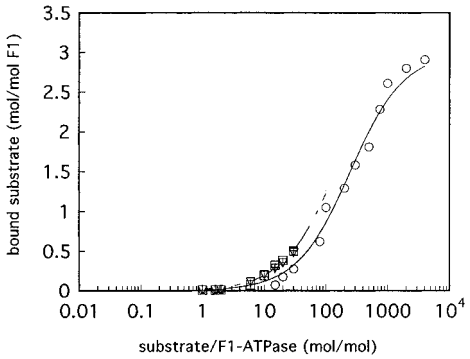

FIGURE 4: Effect of EDTA on binding of ATP and [^{3}H]-2-azido-ATP to the mutant ECF_1 from the mutants βY331W:βE381C:ϵS108C and βE381C:ϵS108C, respectively. The experiments were carried out in the presence of 0.5 mM EDTA and the absence of Mg^{2+}. The experimental procedure is described in Figures 1 and 2, respectively. [^{3}H]-2-Azido-ATP incorporation into the different β subunits of the mutant is presented as follows: ▽, β_{free}; +, β_ϵ; □, β_γ. ATP binding to ECF_1 from the mutant βY331W:βE381C:ϵS108C recorded by quenching of the fluorescence of the βW331 residue is shown by the open circles. The lines are theoretical curves based on the K_d values (real or apparent) given in the text.

A clear cooperativity of binding of [^{3}H]-2-azido-ATP to ECF_1 was observed in the presence of Mg^{2+}, with incorporation of reagent into all three β subunits, identified here as β_{free}, β_ϵ, and β_γ. At substoichiometric amounts of nucleotide to enzyme, the binding of [^{3}H]-2-azido-ATP was almost exclusively in β_{free}, identifying this β subunit as the site of unisite catalysis. Estimates of the K_d for this high-affinity nucleotide binding site are below 40 nM based on equilibrium binding studies as well as fluorescence titration studies (Wise *et al*., 1983; Weber *et al*., 1993). The [^{3}H]-2-azido-ATP labeling results give a binding affinity for this site of below 100 nM based on curve-fitting programs (this is not an equilibrium measurement as the substrate becomes covalently bound). The second mole of [^{3}H]-2-azido-ATP to bind to ECF_1 becomes incorporated into β_ϵ. The K_d for this second mole was around 1−2 μM in equilibrium binding or fluorescence titration experiments (Wise *et al*., 1983; Weber *et al*., 1983; results in Figure 1). The half-maximal concentration for modification of β_ϵ by [^{3}H]-2-azido-ATP was 8−10 μM, and the binding affinity based on curve fitting was around 2 μM (subject to the limitations above). This value is lower than the K_d of binding of ATP (or ADP) in any of the noncatalytic sites, i.e., 24 μM (Weber *et al*., 1994b). Therefore, it can be assumed that the binding affinity being monitored by incorporation of [^{3}H]-2-azido-ATP is that of a catalytic site via insertion into Y331.

The third mole of [^{3}H]-2-azido-ATP to bind is in β_γ, and this occurs with a binding affinity of around 20−30 μM, making it impossible to distinguish whether the reaction is with the catalytic site or noncatalytic site (Tyr 354) without peptide mapping experiments of the labeled subunit. Nevertheless, the clear result is that three β subunits, and at least two catalytic sites, have different nucleotide binding affinities that appear to be fixed with respect to the location of the small subunits, particularly the γ and ϵ subunit. This, in turn, implies that Mg^{2+}ATP binding is determined by and/or must determine the interactions of the different $\alpha-\beta$ pairs with the single-copy subunits.

In confirmation of the recent results of Weber *et al*. (1994), we found that cooperativity of nucleotide binding in catalytic sites requires Mg^{2+}. As reported by these authors, the binding of ATP to ECF_1 in the presence of EDTA occurs with a binding affinity of around 100 μM when measured by the fluorescence technique using the mutant βY331W. The similar binding affinities of the three sites are not a consequence of substituting Trp for Tyr in the adenine pocket. The labeling of all three β subunits with [^{3}H]-2-azido-ATP occurs with a similar apparent binding affinity, understandable if the catalytic sites are in an open conformation, e.g., equivalent to β_E in the structure reported by Abrahams *et al*. (1994), with the binding of ATP directed by interactions of the adenine moiety, and with little or no contribution of the P loop region in the absence of Mg^{2+}.

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