Exploring the Adenine Nucleotide Binding Sites on Mitochondrial F1-ATPase with a New Photoaffinity Probe, 3'-O-(4-Benzoyl)benzoyl Adenosine 5'-Triphosphate*

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Noreen Williams‡ and Peter S. Coleman§
From the Laboratory of Biochemistry, Department of Biology, New York University, New York, New York 10003

3'-O-(4-Benzoyl)benzoyl ATP (BzATP) was synthesized and used as a photoactivatable, covalently binding affinity probe to study site-specific adenine nucleotide binding to the ATPase of submitochondrial particles and the purified soluble F1-ATPase of rat liver mitochondria. In the absence of actinic light, BzATP was a good substrate for enzymatic hydrolysis with both soluble and membrane-bound F1-ATPase. Photolysis of either the membrane-bound or soluble ATPase complex in the presence of BzATP resulted in the covalent incorporation of analog and a concomitant loss of enzyme activity. Yet, 4-benzoylbenzoic acid, the photoreactive analog and a concomitant loss of enzyme activity. Yet, 4-benzoylbenzoic acid, the photoreactive

Resolution via polyacrylamide gel electrophoresis of the component subunit polypeptides of soluble F1-ATPase, photolabeled with [3H]- or [32P]BzATP, showed that all of the radioactivity was incorporated into only the α and β subunits of the enzyme. Radioactivity was found in both the α and β subunits when [2,8-3H]BzATP was photolyzed in the presence of Mg2+, whereas only the β subunit was labeled in the absence of Mg2+. On the other hand, incorporation of label with [γ-32P]BzATP always appeared exclusively in the β subunit whether or not Mg2+ was present.

A mechanism of enzyme action for the F1-ATPase is presented based on these and other recent data. The proposed mechanism suggests that a catalytic site resides on the β subunit. This site binds ATP-(Mg2+) and is closely associated, both topologically and functionally, with a specific ADP binding site situated on an immediately adjoining α subunit.

The membrane-bound F1-ATPase of mitochondria (ATP synthetase) is the terminal enzyme in oxidative phosphorylation catalyzing the phosphorylation of ADP to form ATP.

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‡ Present address, Department of Physiological Chemistry, Johns Hopkins School of Medicine, Baltimore, MD 21205.
§ Recipient of a New York University Research Challenge Fund Grant. To whom correspondence should be addressed.

Upon removal from the mitochondrial inner membrane, the then soluble F1 can only catalyze ATP hydrolysis, a Mg2+-requiring process (1, 2). The F1 enzyme is composed of 9 subunits of 5 different kinds (α, β, γ, δ, and ε) plus an inhibitor peptide, with a total molecular mass in the vicinity of 380,000 and a near spheric diameter of 85–90 Å (2).

A large number of studies employing F1, from a variety of phylogenetically diverse sources (chloroplasts, yeast, bacteria, and vertebrate mitochondria) have suggested that multiple adenine nucleotide binding sites exist on this enzyme (3–9). These adenine nucleotide loci (as many as 4 different enzyme environments have been reported; see Slater et al. (10)) have been proposed to comprise 2 principal and independent functions: catalytic and regulatory (7). The putative regulatory binding site(s) for adenine nucleotides seem to demand purine structures which possess the unmodified adenine ring. On the other hand, the catalytic site appears to be less stringent; various nucleoside triphosphates can serve as substrates for F1-catalyzed hydrolysis (11–13). It would appear that the requirement for a structurally unmodified adenine during coupled ATP synthesis in intact mitochondria derives from the near absolute specificity for ADP and ATP translocation across the inner membrane by the adenine nucleotide carrier (14).

The number and location of adenine nucleotide binding sites on F1 have been studied recently by chemical affinity labeling with 5-[(p-fluorosulfonyl)benzoyl] adenosine (15–17) as well as by photoaffinity labeling with arylazido-β-alanyl ATP (18, 19), arylazido-β-αlanyl ADP (20), and 8-azido ATP and ADP (21–23). The results obtained from these studies have indicated that ADP and ATP bind to both the α and β subunits. Nevertheless, no clear evidence has suggested whether any functional relationship exists between these 2 differentiable adenine nucleotide binding sites.

We have synthesized a new photoaffinity derivative of ATP which possesses an unmodified adenine ring but contains a photoactive benzophenone group. Benzophenone is an α,β unsaturated ketone and is capable of being excited to a diradical triplet state intermediate by low intensity, long wavelength ultraviolet irradiation. This triplet intermediate may then abstract hydrogen from a methylene group on a target molecule and thereby create a covalent bond with the target acceptor (24).

With this new photoaffinity substrate analog, we have demonstrated that two independent yet closely neighboring adenine nucleotide binding sites, presumably at the α/β subunit interface on F1, are responsible for catalysis.

EXPERIMENTAL PROCEDURE

Materials

Animals—Adult, male Long Evans exbreeder rats, obtained from...
Blue Spruce Farms (Altamont, NY) were fed and watered ad libitum then starved for 16 h prior to killing.

Reagents—The following chemicals were purchased from the indicated sources: 4-benzoylbenzoic acid, 1,1'-carbonyldimidazole, and N,N-dimethylformamide from Aldrich; Cellix D, acrylamide, methylene bisacrylamide, ammonium persulfate, Coomassie blue R-250, bromphenol blue, N,N',N'-tetramethylenediamine, and N,N,N',N'-tetramethylethylenediamine, and 1,1'-carbonyldimidazole (0.43 M) and 4-benzoylbenzoic acid (0.14 M) dissolved in 25 ml of anhydrous N,N-dimethylformamide, were stirred 15 min at room temperature. Then, ATP (disodium salt, 0.03 M), in 125 ml of deionized H2O, was added and the reaction was stirred overnight. Solvent was removed by rotary evaporation under vacuum and the reaction products were washed with acetone on a Buchner funnel to remove unreacted 1,1'-carbonyldimidazole and 4-benzoylbenzoic acid. The crude product was purified on a light-shielded column employed as received.

Preparation of BzATP—Adopting the procedure of synthesis from Guilroy and Jeng (19), modified accordingly, we have prepared the ATP photoaffinity analog BzATP, as well as the 2,6-T and γ,γ'-P derivatives. The synthesis was performed as follows. 1,1'-carbonyldimidazole (0.43 M) and 4-benzoylbenzoic acid (0.14 M) dissolved in 25 ml of anhydrous N,N-dimethylformamide, were stirred 15 min at room temperature. Then, ATP (disodium salt, 0.03 M) in 125 ml of deionized H2O, was added and the reaction was stirred overnight. Solvent was removed by rotary evaporation under vacuum and the reaction products were washed with acetone on a Buchner funnel to remove unreacted 1,1'-carbonyldimidazole and 4-benzoylbenzoic acid. The crude product was purified on a light-shielded column containing Sephadex LH-20 (bed volume, 160 ml). The fractions were identified with 0.1 M ammonium formate (pH 7.4). Only material from the first and fourth resolved fractions proved to be substrates for mitochondrial ATPase activity. The first peak (600-ml elution volume), was shown to be unreacted ATP by comparative TLC against an ATP standard, while the fourth peak was identified as the BzATP product (1620-ml elution volume), and comprised an estimated 15–20% yield relative to ATP.

We also prepared [3H]BzATP from [2,8-3H]ATP and γ-32P BzATP from γ-32P ATP. The synthetic [3H]BzATP yielded specific activities of 4 × 10^3 dpm/μmol and 8 × 10^3 dpm/μmol for 2 preparative batches, and the γ-32P BzATP gave a specific activity of 1.2 × 10^4 dpm/μmol. Qualitative identification and the purity of each synthetic batch of BzATP were routinely determined by thin layer chromatography on Avicel microcrystalline TLC plates containing a fluorescent indicator (Analtech). The developing solvent employed was 1-butanol/acetic acid (4:1:1). The Rf values found for BzATP, 4-benzoylbenzoic acid, and ATP were 0.63, 0.81, and 0.12, respectively.

The stability of BzATP to a broad pH range was determined by TLC. BzATP (0.01 M) was incubated at 25 °C in 10 mM Tris-maleate buffer over a pH range of 4 to 10 in unit increments. After 30 min, the BzATP was chromatographed as described above. For every pH examined, only one spot was detected, yielding the normal Rf value and indicating no breakdown of BzATP.

NMR spectral analysis showed a downfield proton shift from 5 (ATP) to 6 ppm (BzATP), indicating a substitution at the 3'-hydroxyl position of the ribose moiety for the BzATP product synthesized (19).

Elemental analysis of BzATP, as the hydrated ammonium salt, was:

\[C_{24}H_{24}N_{4}P_{3}\]

Calculated: C 36.30 H 5.10 N 12.40 P 12.63

Found: C 36.30 H 5.10 N 12.40 P 12.63

Photoactivity of the analog was ascertained preliminarily by long wavelength UV (>340 nm) illumination of a TLC plate, spotted at the origin with BzATP, prior to development according to the chromatographic system described above. After 5 min of photoirradiation the origin with BzATP, prior to development according to the chromatographic system described above. After 5 min of photoirradiation the origin with BzATP, prior to development according to the chromatographic system described above. After 5 min of photoirradiation the origin with BzATP, prior to development according to the chromatographic system described above. After 5 min of photoirradiation the origin with BzATP, prior to development according to the chromatographic system described above. After 5 min of photoirradiation the origin with BzATP, prior to development according to the chromatographic system described above. 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(2836) (Amersham) was added to the individual slices, after treatment with NCS (Amersham) H₂O (8:1, v/v) for 2 h at 30 °C. Radioactivity of each gel slice (or 5 identical combined slices, in the case of slab gel electrophoresis) was determined with a Beckman Model 8100 liquid scintillation counter.

Counting efficiency was routinely determined with internal standards ([³H]toluene; specific activity, 1.127 × 10⁶ dpm/ml) for the [H], and by external standards (H-number or channel ratio method) for both [H] and [γ-³²P]-labeled samples.

Determination of Protein—Submitochondrial particle protein was measured by the biuret method (28) using 0.33% (w/v) deoxycholate. Soluble F₁ protein was determined by the Lowry procedure (33). Bovine serum albumin was employed as a reference standard in both cases.

RESULTS

The effects of BzATP both as substrate analog and photofinity label were studied with the ATPase enzyme in a membrane-integrated (submitochondrial particle-ATPase) as well as a soluble form (F₁-ATPase).

BzATP as a Substrate Analog for Submitochondrial Particle-ATPase Activity—BzATP functioned as a capable substrate for the hydrolysis reaction catalyzed by sonic submitochondrial particle ATPase in the absence of actinic illumination. Although the submitochondrial particle-ATPase displayed a diminished Vₘₐₓ with BzATP compared to ATP, the data in Table I indicate that nearly identical apparent Kₘ values were obtained from submitochondrial particles with either substrate. The specific activity of the enzyme preparation with BzATP was reduced to about 11% of that obtained with ATP.

Table I also indicates that ADP inhibited submitochondrial particle-catalyzed hydrolysis of both BzATP and ATP, although different apparent Kₘ values were obtained as a function of the substrate employed.

Photoinhibition of Submitochondrial Particle-ATPase Activity with BzATP—Upon irradiation with long wave, low intensity UV light for 10 min, BzATP effectively inhibited the ATP hydrolytic activity of submitochondrial particles (Fig. 2). The addition of 10 mM MgCl₂ to the photolysis incubation substantially increased the degree of photoinhibition from 51% without Mg²⁺ to 77% in the presence of Mg²⁺. The unirradiated control containing BzATP, as well as an irradiated control incubation without BzATP, yielded no significant loss of ATPase activity compared with freshly prepared submitochondrial particles (0.2 and 4.3%, respectively).

Fig. 2 also shows data from experiments where submitochondrial particles, prephotolyzed in the presence of BzATP, were assayed for residual ATPase activity employing a range of ATP concentrations suitable for Kₘ determinations. The apparent Kₘ observed was identical with that of control studies without BzATP whether or not the system was illuminated. Upon illumination, the Vₘₐₓ was significantly decreased, yielding 23% of the specific activity of the control preparation. In another series of control studies, the illumination of submitochondrial particles in the presence of 4-benzyloxybenzoic acid, the photoactive moiety of the BzATP analog, showed an insignificant loss of ATPase activity (2.9%) which was due to nonspecific photolabeling.

These data indicate that upon photolysis the substrate analog BzATP probably binds irreversibly to the putative catalytic site of membrane-integrated F₁ and thereby removes a substantial proportion of submitochondrial particle-ATPase from the reaction. In contrast, the photoactive 4-benzyloxybenzoic acid alone is not directed specifically to the catalytic site, but rather ligands randomly to nonselective loci on the enzyme.

The labeling pattern obtained, subsequent to slab PAGE, of the submitochondrial particle-ATPase complex that had been photolabeled with [³H]BzATP showed that the α/β region of the F₁ did contain significant amounts of photoincorporated [³H]BzATP. However, the resolution of these 2 subunits on the radioactive slab gel was not sufficient to allow for further characterization. Other protein components of the submitochondrial particle vesicles were labeled as well with [³H]BzATP, including at least one principal polypeptide with an apparent molecular mass around 30,000, which may indi-
cates probe binding to the adenine nucleotide carrier moiety (14) (not shown).

In view of the complex structural nature of the vesicular submitochondrial particle preparation relative to that of soluble F1, questions arise as to whether the location of the catalytic locus for BzATP is identical with that for ATP, and whether this site (or sites) on the membrane-integrated enzyme corresponds exactly with that of the soluble F1. The fact that BzATP presents the same apparent Km as does ATP with the submitochondrial particle preparation (Table I and Fig. 2) appears to argue that the synthetic analog is, in fact, site-directed to the same catalytic locus on submitochondrial particles as is ATP. However, inhibition by ADP yielded K_i values which differed according to the substrate hydrolyzed (Table I), a result that may be interpreted in several ways, but one that is clearly inconsistent with the concept of competitive inhibition. To resolve these questions, it was necessary to examine the manner in which the less complex soluble F1 preparation utilized the BzATP substrate analog.

BzATP as Substrate Analog for the Soluble F1-ATPase—BzATP also served as a substrate for soluble F1-ATPase hydrolytic activity in the absence of illumination. Table I shows that the apparent Km values for BzATP and ATP as substrates were nearly identical, while the Vmax for BzATP was reduced to 12% that for ATP. Again, it was observed that ADP inhibited the hydrolysis of both BzATP and ATP but exhibited different apparent K_I values depending on the substrate employed for hydrolysis.

**Photoinactivation of Isolated F1 with BzATP**—Fig. 3 shows that prior photoirradiation of purified F1 with BzATP inhibited approximately 70% of normal ATPase activity by removing that proportion of the enzyme from the reaction due to irreversible photoliganding. A series of assays over a range of ATP concentrations indicated that the enzyme, prephotolabeled with BzATP, had an unaltered apparent Km value relative to controls. The unilluminated control (with BzATP) and the control illuminated in the absence of BzATP displayed insignificant losses of ATPase activity (1.9 and 4.4%, respectively).

It is critical to note here that, as with the submitochondrial particle preparation, photolysis of purified F1 in the presence of 4-benzoylbenzoic acid alone caused virtually no loss (2.4%) of hydrolytic activity.

The addition of ATP to a photolysis incubation containing BzATP effectively protected against photoactivation by the BzATP analog. Under hydrolysis conditions (+Mg++) where equimolar (4 μmol/mg of F1) analog and ATP were co-incubated with F1, during 10 min of UV illumination, protection against photoinduced BzATP inhibition was better than 90%. These photoprotection data appear to support the belief that both substrates seek out a uniquely identifiable catalytic locus on F1. One may infer from these results that because of identical apparent Km values for both substrates with this preparation (see Table I), ATP is bound more effectively (k_on is smaller) and is hydrolyzed with greater efficiency (k_cat is larger) than is BzATP. Assuming for the moment that these factors hold, then the reason for the identical apparent Km values for F1 observed with both BzATP and ATP may be attributed to an equal and opposite alteration in both of the microscopic rate constants (k_on and k_cat), depending on the substrate employed. By this argument, the excellent protection against BzATP photoliganding by equimolar ATP could be understood and would support the proposal that both substrates are sequestered at a unique topological locus on F1 in preparation for catalysis at that site.

It may therefore be concluded from the above data that the photoactivated analog BzATP probably binds covalently to the catalytic locus of the isolated F1-ATPase, causing a substantial loss in activity of the incubation. The proportion of enzyme in the incubation which is not irreversibly bound with BzATP retains the ability to catalyze added ATP and displays an unaltered apparent Km.

**Covalent Photolabeling of Purified F1 by [3H]BzATP and [γ-32P]BzATP**—Fig. 4 shows the electrophoretic pattern ob-

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**Fig. 3. Effect of photolabeling of soluble F1 with BzATP.** Photolabeling conditions: soluble F1-ATPase (0.15 mg) was incubated for 10 min at 25 °C in 1.0-cm fluorescence cuvettes. The reaction contained 10 mM Tris-maleate (pH 7.2), 10 mM MgCl2. UV irradiation was performed as in Fig. 2. BzATP concentration was 4.0 μmol/mg of soluble F1 protein. Incubation volume was 1.0 ml. ATP concentration range for ATPase assay (29) was 0.3 to 2.0 mM.
tained via SDS-PAGE electrophoresis of soluble F₁, prephotolyzed with [³H]BzATP in the presence (Fig. 4A) or absence (Fig. 4B) of Mg²⁺. These experiments were performed such that the concentration of F₁ employed during photolysis, as well as that loaded onto the gel, was identical whether or not Mg²⁺ was present. As a result, it is seen that the total level of incorporation of label in either case also was nearly identical, but the distribution of label was clearly different. When F₁-ATPase was photolyzed with [³H]BzATP in the presence of Mg²⁺, the ratio of radioactive incorporation into the β subunit versus the α subunit was approximately 2:1. On the other hand, when the enzyme was photolyzed in the absence of Mg²⁺, the radioactive label was distributed with a β-to-α incorporation ratio of more than 7:1.

Fig. 4 shows incorporation of [γ³²P]BzATP in the presence (Fig. 5A) or absence (Fig. 5B) of Mg²⁺ in the photolysis incubation. In either case, radioactive label appeared exclusively in the β subunit, implying that the β subunit possesses a binding site for ATP, whereas the θ subunit does not.

DISCUSSION

The studies reported here are assessed on two levels: on aspects of photoaffinity labeling with benzophenone substrate analogs and on the mechanism of mitochondrial F₁ catalysis based on the use of 3'-O-(4-benzoyl)benzoyl adenosine 5'-triphosphate as substrate for this enzyme.

With regard to the usefulness of BzATP as a photoaffinity probe, the photoreactive benzophenone moiety of this analog possesses several advantageous properties compared with azido derivatives of adenine nucleotides that have more com-
**ATP and ADP Binding and the Catalytic Mechanism of F**

**PROBABLE MECHANISM**

![Diagram](https://via.placeholder.com/150)

**FIG. 6.** Probable reaction mechanism for covalent binding of BzATP to the ATPase.

Commonly been employed in photoaffinity enzyme studies to date. It is important to highlight a few of these advantages.

When benzophenone is excited by actinic illumination \(\lambda_{exc} \geq 340\text{ nm}\), a triplet diradical intermediate is generated at nearly 100% efficiency (35). This diradical triplet reacts by preferentially abstracting hydrogen from neighboring organic residues, rather than from the bulk aqueous environment (24). Such hydrogen abstraction followed by covalent insertion into a target molecule appears to be the principal route for dissipating the excited state of the benzophenone in the absence of physical quenching. This reaction route differs from that followed by the nitrene intermediate of azido derivatives, which can readily insert into water (24). Therefore, it would appear that productive (and efficient) photolabeling of an enzyme with azido analogs sometimes requires repetitive addition of the analog or a high initial analog concentration to the aqueous system (18, 19, 21-23) in order to compensate for the lower effective concentration of the active nitrene intermediate.

Fig. 6 illustrates a probable mechanism for the photochemical reaction of a benzophenone derivative of adenosine with F. The usefulness of BzATP as a photoactive ligand is enhanced by its relative stability at room temperature for more than 30 min to a broad range of pH, from 4 to 10, a situation that does not hold for several other types of adenine nucleotide chemical and photoaffinity probes commonly employed (15, 16, 19).

Our use of BzATP with membrane-affiliated and soluble F provides evidence that covalent modification of the enzyme via photoliganding is site-specific, rapid, and clean (see Figs. 4 and 5 and data on the ability of ATP to protect F from photolytic inactivation with BzATP).

In the absence of photoactivation, BzATP clearly substitutes for ATP as a substrate for mitochondrial ATPase. The apparent \(K_{m}\) values for BzATP with submitochondrial particles as well as with soluble F were, in each case, identical with those for ATP. This suggests that although the \(k_{cat}\) for BzATP hydrolysis or the \(k_{cat}\) of the enzyme-BzATP complex, or both, may differ because of the added steric bulk of the benzophenone functional group, both BzATP and ATP most probably interact with the enzyme at the catalytically functional site.

It is also worth focusing on the apparent \(K_{m}\) values for inhibition of ATPase that were obtained with either submitochondrial particles or soluble F. A number of earlier reports characterized ADP as a competitive inhibitor of ATPase activity, signifying a unique site on the enzyme that corresponds to the catalytic locus, where both ATP and ADP are capable of binding in a mutually exclusive fashion (27, 36). Our kinetic data, with both natural ATP and the BzATP analog as substrates in the presence of ADP as inhibitor, are not consistent with a competitive inhibition interpretation (see Table I). We observed that the ratio \(K_{m}\) of ATP to BzATP (BzATP) agreed rather closely with the ratio \(V_{max}\) of ATP to BzATP (i.e. between 6 and 9), despite the fact that each type of enzyme preparation, respectively, yielded identical \(K_{m}\) values with either ATP or BzATP as substrate (see Table I). A classical competitive inhibitor (37) cannot alter its \(K_{m}\) for an enzyme when various structural analogs are successful substrates for a unique catalytic site, especially when each one of the substrates yields the same apparent \(K_{m}\) for that enzyme. This suggests that the inhibition of the hydrolysis reaction by ADP could be due to its binding at an enzyme site other than that at which substrate is bound and hydrolyzed, yet one which can affect the functioning of the catalytic site.

Upon illumination, BzATP behaved as a site-directing photoligand with mitochondrial ATPase. Under hydrolyzing conditions (\(+\text{Mg}^{2+}\)), photolysis with low light fluence reduced the activity of the ATPase more than 70% in less than 10 min. In contrast, the photoreactive 4-benzoylbenzoic acid had almost
no effect on ATPase activity, whether or not the incubation was illuminated. Once covalently bound to the enzyme, the probe-enzyme complex was stable to centrifugal washing (sub-mitochondrial particle) or to prolonged dialysis (soluble F1).

The most intriguing results of these studies indicated differential, site-specific photoliganding of BzATP (and of resulting BzADP during Mg\textsuperscript{2+}-requiring, F\textsubscript{1}-mediated hydrolysis) to select subunit polypeptides of the F\textsubscript{1} holoenzyme. Fig. 4 illustrates that when BzATP is made radioactive in the adenine ring (as [\textsuperscript{3}H]BzATP), enzyme reaction conditions that support hydrolysis then yielded covalent labeling to both \(\alpha\) and \(\beta\) subunits (Fig. 4A). The absence of Mg\textsuperscript{2+}, and thus the absence of hydrolysis of [\textsuperscript{3}H]BzATP to yield [\textsuperscript{3}H]BzADP + Pi, allowed for only \(\beta\) subunit labeling (Fig. 4B). Furthermore, our experiments were conducted (see “Results”) such that the total amount of protein on each gel was conserved regardless of the enzyme incubation’s ability to support hydrolysis. Consequently, it may be observed that, upon electrophoretic resolution, the total polypeptide radioactive labeling by [\textsuperscript{3}H]BzATP (and [\textsuperscript{3}H]BzADP) under hydrolysis conditions (+Mg\textsuperscript{2+}) was equivalent to that in the absence of hydrolysis (−Mg\textsuperscript{2+}); that is, radioactivity in \(\alpha + \beta\) radioactivity in \(\beta\) is constant.

The simplest interpretation of these data would have a nucleoside triphosphate (ATP), as well as the Mg\textsuperscript{2+} binding site reside on the \(\beta\) subunit, where catalytic hydrolysis occurs, and a nucleoside diphosphate (ADP) binding locus on the \(\alpha\) subunit.

To support this proposal, we synthesized [\(\gamma\textsuperscript{-32P}\)]BzATP. Upon hydrolysis of [\(\gamma\textsuperscript{-32P}\)]BzATP in the presence of Mg\textsuperscript{2+}, BzADP + \(\text{32P}\) were generated, and any covalent photoliganding of the resulting nonradioactive BzATP to the \(\alpha\) subunit would not be observable with PAGE. We did, however, observe only unhydrolyzed [\(\gamma\textsuperscript{-32P}\)]BzATP binding via PAGE, and such binding was restricted to the \(\beta\) subunit (Fig. 5A). In the absence of hydrolysis (−Mg\textsuperscript{2+}), little BzADP arises, and again, exclusive \(\beta\) subunit labeling was observed (Fig. 5B).

Thus, it would appear that BzATP does not bind to the \(\alpha\) subunit, whereas BzADP does.

Together with our enzyme kinetics data, results from these site-directed photolabeling studies have provided the impetus for a proposal on the mechanism of ATP hydrolysis and, by extension, on that of ATP synthesis as well, catalyzed by F\textsubscript{1}.

Current concepts on the subunit stoichiometry and topology of F\textsubscript{1}, (16, 38) permit the oligomeric enzyme to be portrayed with 3 pairs of stacked \(\alpha/\beta\) subunits. With such a conception, each \(\alpha/\beta\) subunit pair possesses an interfacial domain or cleft at the spatial junction between the \(\alpha\) and \(\beta\) protomers. We propose that these clefts are accessible to ATP-Mg\textsuperscript{2+}, \(\text{H}_2\text{O}\), and to ADP, and that within each cleft reside the catalytic as well as the principal regulatory adenine nucleotide binding sites on the F\textsubscript{1}.

A mechanism of enzyme function that is consistent with our data and with observations from other laboratories is illustrated schematically in Fig. 7. Here we show an enlarged section of one \(\alpha/\beta\) stacked subunit pair, with its adenine nucleotide-accessible cleft comprising both catalytic and regulatory loci. We visualize the following sequential events comprising F\textsubscript{1}-mediated ATP hydrolysis (or, proceeding in the exact reverse, of ATP synthesis under appropriate energy-transducing conditions).

Starting with a totally “empty” cleft, a molecule of ATP-Mg\textsuperscript{2+} binds to the roof of the cleft, which is, synonymously, the \(\beta\) subunit. Cleavage of the \(\gamma\)-phosphoryl bond occurs, together with insertion of \(\text{H}_2\text{O}\), which induces the release of Pi, and ADP from the \(\beta\) liganding site. At the same time that a second ATP-Mg\textsuperscript{2+} binds to the recently emptied \(\beta\) site, the ADP that was generated from the first ATP-Mg\textsuperscript{2+} hydrolysis is sequestered tightly to the floor of the cleft, which is, synonymously, the \(\alpha\) subunit. To complete the catalytic sequence, the hydrolysis of the second ATP-Mg\textsuperscript{2+} at \(\beta\) is accompanied by the release of ADP from \(\alpha\).

Although no emphasis is given in Fig. 7 to conformational adjustments within this cleft upon the binding of ATP-Mg\textsuperscript{2+} to \(\beta\) and ADP to \(\alpha\), such topological alterations are probably intrinsic to the proposed mechanism and encourage speculation on differential equilibrium binding affinities displayed by ADP and ATP under varying experimental conditions (39). It might be argued that the proposed multiple adenine nucleotide binding sites on F\textsubscript{1} (10) are, in fact, manifestations of conformational alterations that obtain when ATP and/or ADP bind to their respective loci within the interfacial \(\alpha/\beta\) cleft.

This mechanistic sequence, albeit overly simplistic diagrammatically, explains how ADP is not a \textit{bona fide} competitive inhibitor of ATPase catalysis, why [\textsuperscript{3}H]BzATP hydrolysis results in both \(\alpha\) and \(\beta\) site binding, and why [\(\gamma\textsuperscript{-32P}\)]BzATP hydrolysis permits only \(\beta\) site binding to be observed in our PAGE system.

It is noteworthy that our model can be made compatible with various proposals that suggest conformational cooperativity between 2 or more adenine nucleotide binding sites on F\textsubscript{1}. This would require the additional assumption that such cooperativity involve both intracleft as well as cleft-cleft conformational alterations. For example, the sequence of steps (Fig. 7) involving the \(\beta\) (triphosphate) and \(\alpha\) (diphosphate) neighboring binding loci could be interpreted as a somewhat modified alternating site mechanism (40, 41). Our data on ADP inhibition of ATP hydrolysis (Table I), which suggest neighboring but nonidentical di- and triphosphate binding sites, appear more compatible with an intracleft cooperativity mechanism; but our results could easily accommodate the possibility of higher order cooperativity between pairs or triads of \(\alpha/\beta\) domains, as supported by the catalytic promotion studies of Grubmeyer and Penefsky (42). Given our present state of understanding, it is clear that alternative mechanistic interpretations are possible.

We wish to conclude with the suggestion that 3'-O-(4-ben-
zoyl)benzoyl adenosine 5'-triphosphate (as well as a host of other benzophenone-containing potential substrate analogs) is an effective photoaffinity probe whose use should be encouraged.

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Exploring the adenine nucleotide binding sites on mitochondrial F1-ATPase with a new photoaffinity probe, 3’-O-(4-benzoyl)benzoyl adenosine 5’-triphosphate.
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