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# PHOTOAFFINITY LABELING OF SOLUBLE CHLOROPLAST ADENOSINE 5′-TRIPHOSPHATASE WITH 3′-*O*-(4-BENZOYL)BENZOYL ADP

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**3′-*O*-(4-benzoyl)benzoyl ADP (BzADP) acts as a reversible inhibitor of the chloroplast coupling factor 1 ATPase ($CF_1$) when incubated with the enzyme in the dark. The $V_{max}$ of ATP hydrolysis is decreased and the kinetics of the reaction are altered from noncooperative to cooperative with respect to ATP. Photoactivation of the benzophenone group in BzADP by irradiation with ultraviolet light (366 nm) results in the covalent binding of BzADP to the enzyme and inactivation of its enzymic activity. Polyacrylamide gel electrophoresis of $CF_1$-ATPase in the presence of sodium dodecyl sulfate shows that the analog is bound primarily to the enzyme's $\beta$ subunit. Complete inactivation of the activated $CF_1$-ATPase occurs upon covalent binding of 2.45 mol BzADP/mol $CF_1$. Binding of BzADP and inactivation of the ATPase are prevented if ADP, but not ATP, is present during the photoactivation step. The presence of $Ca^{2+}$ during irradiation enhances the rate of BzADP covalent binding as well as the rate of inactivation of the enzyme.**

## Introduction

The chloroplast ATP synthetase is a protein complex that consists of two main portions: $CF_1$, a hydrophylic protein complex and $CF_0$, an intrinsic membrane protein complex. $CF_1$ contains the catalytic site(s) of the enzyme as well as the tight nucleotide binding site(s) (for review see Ref. 1). The tight binding site(s) were suggested to play part in the modulation of the enzyme activities [2–5]. The soluble $CF_1$ is a latent ATPase that can be activated by heat [6,7], incubation with high concentrations of thiol reagents [8], protease digestion [6,9] or incubation with the detergent octyl glucoside [10].

Affinity labeling is an important tool in the study of mechanism of enzyme action. DeBeneditti and Jagendorf described the inhibition of spinach $CF_1$ activity upon covalent binding of 5′-*p*-fluorosulfonylbenzoyl adenosine [11]. Another approach to affinity labeling involves the use of analogs that are reversible inhibitors of the enzyme, and can be activated by irradiation with ultraviolet light to produce a radical that will interact with the polypeptide amino acid side chains. Photoaffinity labeling of $F_1$-ATPases from mitochondrial, bacterial and chloroplast membranes has been recently reviewed [12]. Inactivation of the $CF_1$-ATPase upon covalent binding of 3′-O-[3-[*N*-(4-azido-2-nitrophenyl)-amino]-propionyl]-ADP (Arylazido ADP) to the $\alpha$ and $\beta$ subunits of $CF_1$ was reported [13,14]. The pattern of binding of $CF_1$ subunits varies with the different nucleotides used as photoaffinity labels, although their covalent binding generally results in the inactivation of the enzymic activity. Thus, arylazido ATP, which is hydrolyzed by $CF_1$, binds to the $\beta$ subunit only [13,14]. 8-Azido derivatives of ADP and ATP bind

Abbreviations: $CF_1$, soluble chloroplast ATPase; BzADP, 3′-O-(4-benzoyl)benzoyl ADP; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

to both the $\alpha$ and $\beta$ subunits of the enzyme [15]. 2-Azido ADP competes with ADP for the binding to the tight binding site(s) of the membrane-bound $CF_1$ and inhibits its hydrolytic activity as does ADP [16]. The analog binds covalently only to the $\beta$ subunit of the enzyme. 2-Azido-4-nitrophenyl phosphate, a photoaffinity analog of $P_i$ is covalently bound to the $\beta$ subunit of $CF_1$, and inhibits the ATPase activity [17].

Recently, 3′-O-(4-benzoyl)benzoyl ATP (Bz-ATP), a different type of photoreactive nucleotide analog was introduced [18]. This analog contains a benzophenone photosensitive group instead of the azido group in the compounds previously described [13–17]. BzADP binds tightly to the membrane-bound $CF_1$ [19], but, unlike 2-azido ADP, its binding does not result in the inactivation of the ATP utilizing reactions. Moreover, tight binding of BzADP protects against the inhibition caused by tight binding of ADP. The analog is also a competitive inhibitor of ADP and ATP in photophosphorylation or ATP hydrolysis and $P_i$-ATP exchange, respectively [19]. Upon irradiation, BzADP binds covalently to both the $\alpha$ and $\beta$ subunits of the membrane-bound $CF_1$ [19].

In this communication we describe the inactivation of the $CF_1$-ATPase upon interaction with BzADP. When present during the ATPase assay, where only noncovalent interactions occur, BzADP decreases the rate of ATP hydrolysis and changes the kinetics of the reaction from Michaelian to cooperative. Covalent binding of the analog to the enzyme, results in a concomitant decrease in the maximal velocity of the reaction. Complete inactivation occurs upon binding of 2.45 mol BzADP/mol $CF_1$. The label incorporated is found only in the $\beta$ subunit of $CF_1$.

## Materials and Methods

BzADP was synthesized by the procedure of Williams and Coleman [18] as modified by Bar-Zvi et al. [19]. Its concentration was determined assuming a molar absorption coefficient at 260 nm of 32000, based on the $P_i$ content and the molar absorbtion coefficients of ADP and 4-benzoylbenzoic acid. [$\gamma$-$^{32}$P]ATP was synthesized and purified as previously described [5]. $^{32}P_i$ was obtained from the Nuclear Research Center – Negev, Israel and [2-$^3$H]ADP from Amersham, U.K. All unlabeled nucleotides were purchased from Sigma Chemical Co.

*Enzyme preparation and activity assay*

$CF_1$, isolated from lettuce chloroplasts by chloroform extraction [20,21], was purified and stored at 4°C as a suspension in 50% $(NH_4)_2SO_4$ [22]. Just before use, the enzyme was precipitated by centrifugation at $12000 \times g$ for 5 min, the pellet was resuspended in 0.1–0.2 ml of a buffer solution containing 40 mM Tricine-NaOH (pH 8.0)/1 mM EDTA, and desalted by column centrifugation [23] with Sephadex G-25 preequilibrated with the suspension buffer.

$CF_1$ (0.2–0.5 mg/ml) was preincubated in small glass test tubes for 30 min in the dark with 40 mM Tricine-NaOH (pH 8.0), 2 mM EDTA and other components as indicated (total volume, 0.1–0.2 ml). After preincubation, the tubes were irradiated with a 366 nm wavelength light source from a Blak Ray lamp (model XX-15) at a distance of about 5 cm. Heating was minimized by interposing a glass plate between the lamp and the tubes. After irradiation for the indicated time, samples (10–20 $\mu$l) were taken out and kept in the dark until the irradiation procedure was completed. Finally, an activation buffer was added to give, in a final volume of 40 $\mu$l, the following concentrations: 40 mM Tricine-NaOH (pH 8.0); 2 mM EDTA; 25 mM ATP and 5 mM dithiothreitol. The samples were then heated at 60°C for 4 min to activate $CF_1$ [6,7]. ATPase assay was initiated by the addition of 0.96 ml reaction mixture (final concentrations: 40 mM Tricine-NaOH (pH 8.0), 10 mM $CaCl_2$ 10 mM and 5 mM [$\gamma$-$^{32}$P]ATP (with a specific activity of about $2 \cdot 10^4$ cpm/$\mu$mol) and quenched after 10 min incubation at 37°C by the addition of 0.4 ml of a 5% ammonium molybdate solution in 4 M HCl. 50 $\mu$l of a 20 mM $P_i$ solution followed by 2 ml of isobutanol-xylene (1 : 1, v/v) were added and phases were thoroughly mixed. Radioactivity was measured in a 1 ml aliquot of the upper organic phase [24]. Protein was determined according to Lowry et al. [25], using bovine serum albumin as a standard.

*Covalent binding of BzADP*

An ice-cold perchloric acid solution was added

(final concentration, 5%) to samples irradiated in the presence of Bz[$^3$H]ADP (0.5–20000 cpm/nmol). Samples were centrifuged for 2 min at 12000 × *g*, and the pellets were washed once with 0.2 ml of 5% perchloric acid. Washed pellets were resuspended in 5% sodium dodecyl sulfate and radioactivity was determined.

*Subunit distribution of the covalently bound Bz[$^3$H]ADP*

$CF_1$ was reacted with tritiated BzADP, irradiated and washed with perchloric acid as described above. The washed pellets were resuspended in sample buffer containing 5% sodium dodecyl sulfate/1% β-mercaptoethanol/10% glycerol/1% bromophenol blue. The pH value of the solution was adjusted to 7 with 1 M Tricine-NaOH (pH 8.0), and samples were incubated for 30 min at 37 °C. Electrophoresis in the presence of sodium dodecyl sulfate was performed according to Weber and Osborn [26], using 10% acrylamide gels. It is important to keep the temperature at 13–15 °C during electrophoresis, to reduce the release by hydrolytic cleavage of the esterified bound nucleotide moiety. After the run, slices (1 or 2 mm width) of one gel were incubated in polyethylene vials with 4 ml of a scintillation cocktail (toluene-triton X-100 base, containing 10% (v/v) Soluene-350) overnight, at room temperature in the dark, before counting. A second gel was stained with Coomassie Brilliant blue R [26] and scanned at 600 nm in a Cary-219 spectrophotometer.

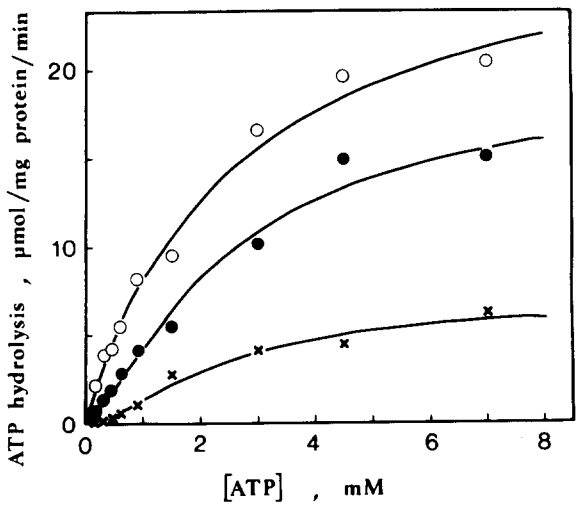


Fig. 1. Inhibition of ATP hydrolysis by BzADP. $CF_1$ was activated as described in Materials and Methods and separated from the activation medium by column centrifugation. ATP hydrolysis was assayed in the dark in the absence of BzADP (○) and in the presence of 100 µM (●) and in the presence of 500 µM (×) BzADP, respectively. The lines were calculated by nonlinear fitting [27] to the Hill equation.

## Results

*Reversible interaction of BzADP with $CF_1$*

Without photoactivation, BzADP present during the reaction in the dark inhibits the $Ca^{2+}$-ATPase activity of soluble $CF_1$ (Fig. 1). This decrease in the rate of hydrolysis, is accompanied by a change in the kinetics of the reaction, from the simple Michaelis-Menten type to cooperative kinetics with a change in the Hill constant from $1.00 \pm 0.07$ without BzADP to $1.39 \pm 0.14$ and $1.52 \pm 0.17$ with 100 and 500 µM BzADP, respectively. 4-Benzoyl-benzoate up to 3 mM had only a minor effect on the hydrolytic activity of the enzyme.

*Inhibition of the ATPase activity by covalent binding of BzADP*

Covalent binding of BzADP to $CF_1$ by irradiation with ultraviolet light leads to the inactivation of the enzyme (Fig. 2). The degree of inactivation depends both on the concentration of BzADP and the irradiation period. Inactivation of $CF_1$ by irradiation of BzADP seems to occur by a pseudo-first-order type of kinetics. The $t_{0.5}$ at BzADP concentrations of 37–525 µM vary between 47 and 11 min, respectively. Covalent binding of the analog to $CF_1$ affects mainly the $V_{max}$ of the ATPase reaction, while the Michaelis constant for ATP remains almost unchanged (Fig. 3). These findings suggest that inactive enzyme molecules result from the covalent binding of BzADP. The purity of the BzADP preparation used was checked carefully, since irradiation of 4-benzoylbenzoate with $CF_1$ also caused inactivation of the enzyme probably by nonspecific interaction of 4-benzoylbenzoate with the enzyme. In effect, the rate of inactivation of $CF_1$ by photoactivated 4-benzoylbenzoate is reduced when benzoic acid is present in the irradiation mixture. In addition, ADP did not prevent the photoinactivation by 4-benzo-

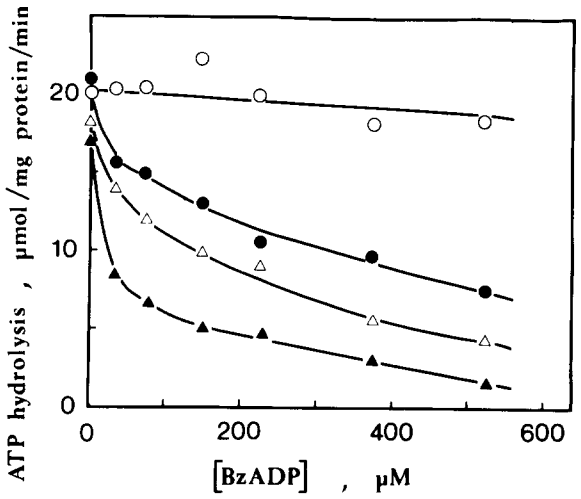


Fig. 2. Inhibition of $CF_1$ ATPase by covalent binding of BzADP: Effect of BzADP concentration and irradiation time. $CF_1$ was irradiated in the presence of the indicated concentrations of BzADP for 0 (○); 15 (●); 30 (△) or 60 (▲) min, and then activated and assayed as described in Materials and Methods.

ylbenzoate (not shown).

The photoinactivation of latent $CF_1$ by covalent binding of BzADP might affect the capacity of the latent enzyme to undergo activation and/or inhibit the catalytic activity of the activated enzyme. Therefore, we compared the enzymic activity of both the latent and activated enzyme, after modification by covalent binding of BzADP to the latent enzyme. As shown in Fig. 4, covalent binding of BzADP inhibits the hydrolytic activity of both the latent and heat-activated enzyme, suggesting that the catalytic process itself and not the activation of latent $CF_1$ is affected by interaction with BzADP. The hydrolysis rate of activated $CF_1$ is somewhat less inhibited than that of the latent enzyme (Fig. 4B). This might be due to partial hydrolysis of the bound probe during the heat activation step, and a lower degree of inactivation by the moiety that remains covalently bound.

The inactivation by BzADP is greatly prevented if ADP is present during the irradiation period (Fig. 5). On the other hand, even in the presence of a 130-fold excess of ATP to that of BzADP, no protective effect was observed. Irradiation of $CF_1$ with Bz[$^3$H]ADP results in the covalent binding of

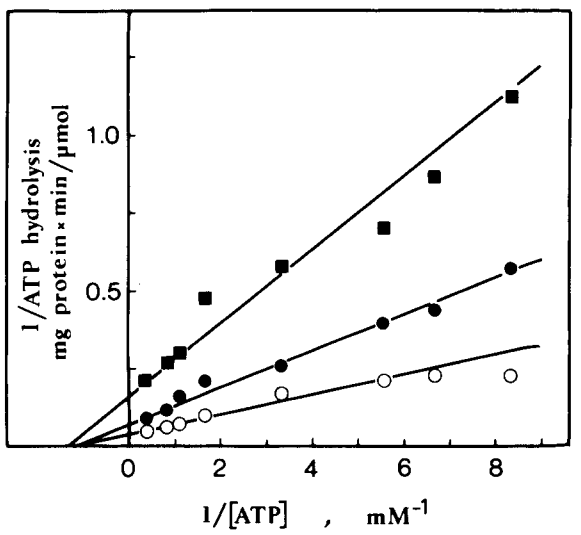


Fig. 3. Effect of photoinactivation of $CF_1$ by BzADP on the kinetic parameters of ATP hydrolysis. $CF_1$ was irradiated in the presence of 240 μM BzADP for 0 (○); 10 (●) and 30 (■) min. The activity was assayed as described in Fig. 1, after activation and separation from the activation medium.

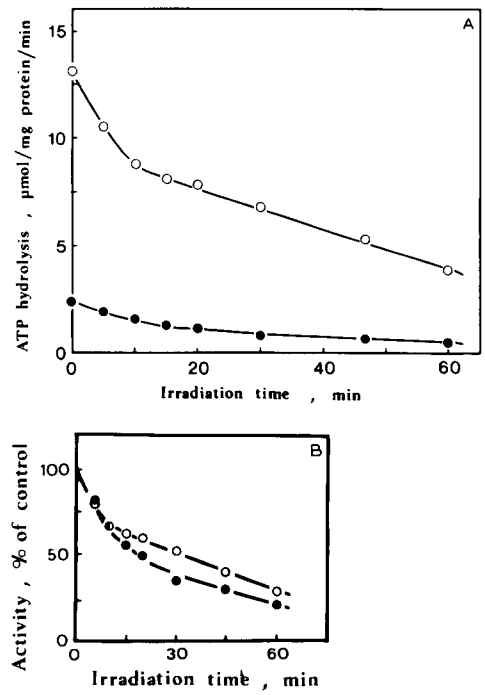


Fig. 4. Photoinactivation of the ATPase activity of activated and latent $CF_1$ by BzADP (A). $CF_1$ was irradiated in the presence of 200 μM BzADP. ATP hydrolysis was then assayed without (●) or after heat activation (○). Normalized curves are also presented (B).

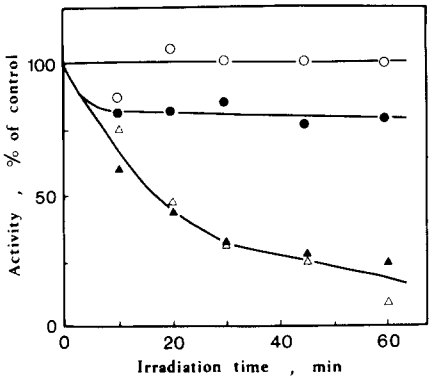


Fig. 5. Effect of adenine nucleotides on the photoinactivation of $CF_1$ by BzADP. $CF_1$ was irradiated for the indicated time as described in Materials and Methods. Nucleotides present during irradiation were: ○, none; △, 193 μM BzADP; ●, BzADP +25 mM ADP and ▲, BzADP+25 mM ATP.

the label to the enzyme (Fig. 6A). Irradiation in the presence of ADP prevents labeling of the enzyme by Bz[$^3$H]ADP, in agreement with the lack of inactivation of the enzyme under these conditions (Fig. 5). The rate of inactivation is correlated with the incorporation of the label into the enzyme. Both rates are increased in the presence of 10 mM $Ca^{2+}$ (Fig 6B). Assuming a molecular weight of 390000 for $CF_1$ [28], complete inactivation is obtained upon covalent binding of 2.45 mol

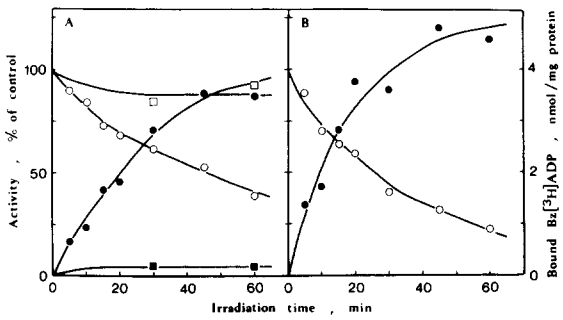


Fig. 6. Covalent binding of Bz[$^3$H]ADP to $CF_1$ by photoinactivation. 125 μM Bz[$^3$H]ADP and $CF_1$ were irradiated in a buffer solution containing 40 mM Tricine-NaOH (pH 8.0) and (A) 2 mM EDTA or (B) 10 mM $CaCl_2$ for the time indicated. After irradiation, samples were diluted 6-fold with 1 mM EDTA and 40 mM Tricine-NaOH (pH 8.0) and assayed for covalent binding (●, ■) or for ATPase activity (○, □) as described in Materials and Methods. (The concentrations of $Ca^{2+}$ and EDTA during the activation stage were 0.8 and 2.5 mM, respectively). □ and ■, irradiation in presence of BzADP and 25 mM ADP.

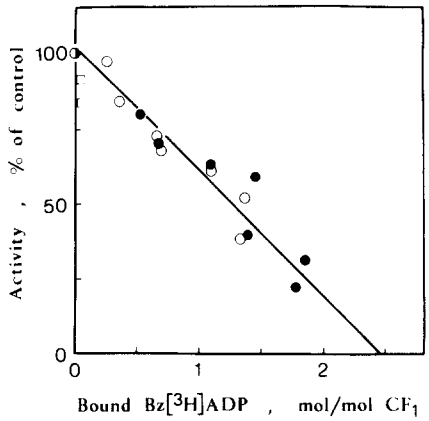


Fig. 7. Correlation between covalent binding of BzADP and inactivation of the ATPase. The data of Fig. 6 were replotted, assuming a molecular weight of 390000 for $CF_1$. Irradiation conditions: ○, +2 mM EDTA; ●, +10 mM $Ca^{2+}$ and □, +2 mM EDTA and 25 mM ADP.

Bz[$^3$H]ADP per mol $CF_1$ (Fig. 7). Inactivation of $CF_1$ by BzADP, in the absence or presence of 10 mM $Ca^{2+}$, gave a similar correlation of remaining activity and label incorporated. This suggests that although $Ca^{2+}$ increases the rate of modification of the enzyme (Fig. 6), it does not alter the inactivation pattern or the number of sites on the enzyme that interact with BzADP. Fig. 8 shows that irradiation of Bz[$^3$H]ADP with $CF_1$ results in the incorporation of the label only into the β subunit of the enzyme. Irradiation in the presence of $Ca^{2+}$ does not change this labeling pattern (not shown).

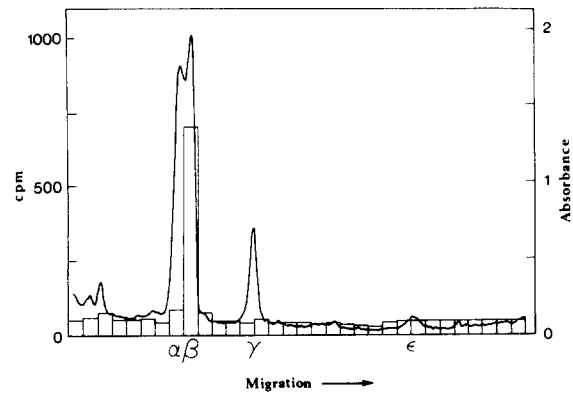


Fig. 8. Sodium dodecyl sulfate gel electrophoresis of $CF_1$ labeled with Bz[$^3$H]ADP. Photoaffinity labeling of $CF_1$, sample preparation, electrophoresis and analysis of the gels were done as described in Materials and Methods.

## Discussion

BzADP is a reversible inhibitor of ATP hydrolysis catalyzed by soluble $CF_1$, under conditions which do not lead to photoactivation of the benzophenone group (Fig. 1), and an irreversible inhibitor after it becomes covalently bound to the enzyme (Fig. 2). When BzADP acts as a reversible inhibitor, it decreases the $V_{max}$ of ATP hydrolysis and changes the kinetics of the reaction from noncooperative to cooperative (Fig. 1). This behavior is similar to that of ADP [29], and indicates that BzADP acts as an analog of ADP. The changes in the kinetic parameters might result from the binding of the nucleoside diphosphate analog to a regulatory site(s) that affects the properties of the catalytic site(s). Although this is the explanation we prefer, the possibility of BzADP binding to one catalytic site that consequently affects the reaction catalyzed by another catalytic site is not excluded.

BzADP binds covalently to sites that are probably specific for ADP, since ADP and not ATP prevent inactivation (Fig. 5) and binding (Fig. 6) of BzADP to $CF_1$. Shoshan et al. [21] showed that $CF_1$ binds 3 mol ADP, while ATP competes with the binding to only one ADP binding site. Bruist and Hammes [14], who identified several nucleotide binding sites on isolated $CF_1$, suggested that the catalytic site is able to bind both ADP and ATP. The inability of ATP in protecting $CF_1$ against photoinactivation by BzADP (Fig. 5) indicates that the analog probably reacts with a noncatalytic site. ATP was less effective than ADP in the protection against photoinactivation of $CF_1$ by 8-azido ATP and 8-azido ADP [15].

$Ca^{2+}$ increases the rate of both the photoinactivation of $CF_1$ by BzADP and the covalent binding of the analog to the enzyme (Fig. 6). However, it does not change the amount of bound BzADP needed to cause complete inactivation (Fig. 7). These results differ from the effect of $Ca^{2+}$ on the photoinactivation of $CF_1$-ATPase by 8-azido ADP [15], where $Ca^{2+}$ doubled the amount of bound nucleotide, but did not change the kinetics of inactivation. 2.45 Mol covalently bound BzADP per mol $CF_1$ were necessary for the complete inactivation of the enzyme (Fig. 7) in agreement with the values of 2 mol nucleotide per mol $CF_1$ reported for the inactivation of $CF_1$ with 8-azido derivatives of ADP and ATP in the absence of $Ca^{2+}$, when the molecular weight of $CF_1$ is taken as 325 000 [15]. However, the location of the label we found is rather different. While BzADP is incorporated into only the $\beta$ subunit of $CF_1$, the 8-azido derivatives of ADP and ATP and the aryl azido ADP analog are incorporated into both the $\alpha$ and $\beta$ subunits [15]. Furthermore, Bruist and Hammes [14] suggested that arylazido ADP binds to two types of binding sites, one that can also bind MgATP and results in the incorporation of the label, predominantly into the $\beta$ subunit. A second site is located near the interface between the $\alpha$ and $\beta$ subunits. On the other hand, arylazido ATP that was suggested to interact with the catalytic site of $CF_1$ is bound only to the $\beta$ subunit of the enzyme [14]. The difference in the location of the nucleotide binding sites based on the incorporation of the different analogs may be due to differences in both the nature of the reactive groups, and their place in the nucleotide molecule. Moreover, the pattern of labeling might also be influenced by the experimental conditions of the labeling procedure [30]. Binding of 2.45 mol BzADP/mol $CF_1$ (Fig. 7) only into the $\beta$ subunit of the enzyme (Fig. 8) may indicate that there may be three copies of the $\beta$ subunit per $CF_1$, in agreement with the $\alpha_3\beta_3\gamma\delta\epsilon_{1-2}$ subunits' stoichiometry recently suggested [28,31].

Bz[$\gamma$-$^{32}$P]ATP binds covalently only to the $\beta$ subunit of mitochondrial $F_1$ [18]. Since BzATP is also hydrolyzed by $F_1$, its binding to the catalytic site of the enzyme was suggested. On the other hand, Bz[$^3$H]ATP binds to both the $\alpha$ and $\beta$ subunits of $F_1$ [18]. Labeling of the tight nucleotide binding site(s) on the membrane-bound $CF_1$ results in the incorporation of Bz[$^3$H]ADP into both the $\alpha$ and $\beta$ subunits of the enzyme [19]. This difference in the labeling pattern between the membrane-bound and soluble $CF_1$ may indicate that different binding site(s) on the membrane-bound and the soluble enzyme participate, or that the BzADP binds to the same site(s) on both types of enzyme, and the difference in the labeling pattern results from differences in the conformations of the binding site(s) in these forms of $CF_1$. Further studies are needed to distinguish between these two possibilities.

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