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Photoaffinity labeling of the TF_1-ATPase from the thermophilic bacterium PS3 with 3′-*O*-(4-benzoyl)benzoyl ADP

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(1) 3′-*O*-(4-Benzoyl)benzoyl ADP (BzADP) was used as a photoaffinity label for covalent binding of adenine nucleotide analogs to the nucleotide binding site(s) of the thermophilic bacterium PS3 ATPase (TF_1). (2) As with the CF_1-ATPase (Bar-Zvi, D. and Shavit, N. (1984) Biochim. Biophys. Acta 765, 340–356) noncovalently bound BzADP is a reversible inhibitor of the TF_1-ATPase. BzADP changes the kinetics of ATP hydrolysis from noncooperative to cooperative in the same way as ADP does, but, in contrast to the effect on the CF_1-ATPase, it has no effect on the V_{max}. In the absence of Mg^{2+} 1 mol BzADP binds noncovalently to TF_1, while with Mg^{2+} 3 mol are bound. (3) Photoactivation of BzADP results in the covalent binding of the analog to the nucleotide binding site(s) on TF_1 and correlates with the inactivation of the ATPase. Complete inactivation of the TF_1-ATPase occurs after covalent binding of 2 mol BzADP/mol TF_1. Photoinactivation of TF_1 by BzADP is prevented if excess of either ADP or ATP is present during irradiation. (4) Analysis by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of the Bz[^{3}H]ADP-labeled TF_1-ATPase shows that all the radioactivity is incorporated into the β subunit.

Introduction

Chemical modification of functional groups of F_1-ATPases from different sources has provided useful information on the participation of these groups in the catalytic process. However, it is rather difficult to limit the covalent modification of different groups on a protein and to confine the modification only to the functional groups at the catalytic site. Derivatization of groups other than those at the catalytic site may result in the inactivation of the enzyme by interference with changes in the enzyme conformation necessary for catalysis. Affinity labeling may be more suitable for the specific modification of the enzyme's substrate binding sites, because such groups are expected to interact with the reactive analog more readily than other amino acid groups that do not take part in the catalytic process. Indeed, photoaffinity labeling of bacterial, mitochondrial and chloroplast F_1-ATPases with different photoreactive nucleotide analogs was described [1,2]. The

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Abbreviations: CF_1 and TF_1, coupling factors one ATPase from chloroplast and the thermophilic bacterium PS3, respectively; BzADP, 3′-*O*-(4-benzoyl)benzoyl ADP; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; P_i, inorganic phosphate.

nucleotide analogs are incorporated either into both the α and β subunits or only into one of these subunits [1,2]. No label is incorporated into the three smaller subunits of the F_1-ATPases. The adenine nucleotide analogs investigated so far appear to bind both to the catalytic and noncatalytic, regulating sites. The catalytic site(s) appear to reside in the β subunit and the regulating noncatalytic site(s) in the α subunit [3,4].

Recently, we have reported that 3′-*O*-(4-benzoyl)benzoyl ADP (BzADP), a photoreactive nucleotide analog, interacts with both the membrane-bound and the soluble CF_1-ATPase. Noncovalent binding of BzADP to soluble CF_1 inhibits the rate of ATP hydrolysis and changes the kinetics of the reaction from noncooperative to cooperative with respect to ATP [5]. Inactivation of the enzyme correlates well with the covalent binding of the analog to the β subunit of CF_1. The analog competes with ADP, for the tight nucleotide binding site(s) of the membrane-bound enzyme, but, in contrast to ADP tight binding of BzADP does not inactivate the enzyme [6]. Moreover, binding of BzADP protects the enzyme against the inactivation that occurs upon binding of ADP. Photoaffinity labeling of the tight nucleotide binding site of the membrane-bound CF_1 with BzADP results in the incorporation of the analog into both the α and β subunits of the enzyme [6].

Yoshida et al. [7] purified the TF_1-ATPase protein from the thermophilic bacterium PS3 and succeeded in obtaining stable isolated subunits that could be reconstituted into the active protein complex. Both the isolated α and β subunits contain nucleotide binding sites [8]. More recently, it was shown that the purified soluble TF_1, which does not contain endogenous adenine nucleotides, forms a 1 : 1 complex with ADP [9]. Binding of one ADP to TF_1 was sufficient to promote full acceleration of inactivation of the ATPase by DCCD modification of glutamyl residues on the β subunits. Binding of Mg^{2+} to form the ternary complex 1 : 1 : 1 $TF_1 \cdot ADP \cdot Mg^{2+}$ protects the enzyme against inactivation by DCCD [9].

In this communication we report on the interaction of BzADP with TF_1. Covalent binding of the analog to the enzyme results in the inhibition of the rate of ATP hydrolysis. Complete inactivation occurs upon binding of 2 mol BzADP/mol TF_1. As in the case of soluble CF_1, the label incorporated is found only in the β subunit of TF_1.

Materials and Methods

BzADP and Bz[^{3}H]ADP were synthesized according to Williams and Coleman [10] as modified by Bar-Zvi et al. [6] and Bar-Zvi and Shavit [5]. [γ-^{32}P]ATP was prepared by phosphorylation of ADP with $^{32}P_i$ by illuminated lettuce chloroplasts and purified as previously described [11]. $^{32}P_i$ was obtained from the Nuclear Research Center-Negev, Israel, and [2-^{3}H]ADP from Amersham, U.K. Unlabeled nucleotides were obtained from Sigma Chemical Co.

Enzyme preparation and assay

TF_1 was isolated and purified from the thermophilic bacterium PS3 as described [7] then lyophilized and stored at −20°C. This preparation contains 0.1 mol ADP and 0.2 mol Mg^{2+} per mol TF_1. The enzyme was dissolved just before use in 50 mM Hepes buffer (pH 7.3) at room temperature. ATPase activity was assayed at 23°C in a 1 ml mix containing 50 mM glycylglycine buffer (pH 8.6)/5 mM ATP (containing $(2–5) \cdot 10^7$ cpm/mmol [γ-^{32}P]ATP)/5 mM $MgCl_2$/3–5 μg TF_1. The reaction was quenched after 10 min by adding 0.4 ml of a 5% ammonium molybdate and 1 mM P_i solution in 4 M HCl, followed by 1.5 ml isobutanol/xylene (1 : 1, v/v). The tubes were vigorously mixed and the phases allowed to separate. Radioactivity was measured in a 1 m aliquot of the upper organic phase [12]. Protein was determined according to Bradford [13] using the lyophylized TF_1 as a standard.

Binding of nucleotides to TF_1

TF_1 (2–3 mg/ml) was incubated at 23°C in the dark in a medium containing 50 mM Hepes (pH 7.3), labeled nucleotide as indicated, and 1 mM EDTA or 5 mM $MgCl_2$. After 1 h or as indicated, the TF_1-nucleotide complex was separated from medium nucleotides and other low molecular-weight solutes by centrifugation of a sample of 50–100 μl through a Sephadex G-25 column, preequilibrated with 50 mM Hepes buffer (pH 7.3) in a 1 ml plastic syringe [14]. The radioactivity and

the protein content of each eluent fraction were determined.

Photoactivation of BzADP and covalent binding to TF_1

TF_1 (0.6–2 mg/ml) was preincubated for 1 h at room temperature in the dark with the indicated concentrations of BzADP in 50 mM Hepes buffer (pH 7.3) and 1 mM EDTA or 5 mM $MgCl_2$ in a final volume of 0.1–0.2 ml. After incubation, samples were irradiated with a 366 nm wavelength light source as described [5]. Samples (20 μl) were removed at the indicated time intervals and assayed for ATPase activity as described above and for incorporation of Bz[^{3}H]ADP as previously described [5].

Subunit distribution of the Bz[^{3}H]ADP incorporated

After covalent binding of Bz[^{3}H]ADP to TF_1 the acid-washed enzyme was resuspended in the sample buffer containing 5% sodium dodecyl sulfate/10 mM Tris-HCl (pH 8.0)/0.01% bromophenol blue/5% glycerol. The pH was adjusted to 7 with 0.5 M Tris-HCl (pH 8.0), and the samples were incubated for 30 min at room temperature. Electrophoresis in the presence of sodium dodecyl sulfate was done at 5–8°C according to Laemmli [15], using 11% polyacrylamide gels. Radioactivity and the protein content of the gels were determined as described [5].

Results

Inhibition of TF_1-ATPase by noncovalent binding of BzADP

The kinetics of ATP hydrolysis by TF_1 is affected by BzADP or ADP. In their presence the kinetics of the reaction is changed from noncooperative to cooperative with respect to ATP (Fig. 1). The Hill constant of 1.1 ± 0.1 without added adenosine diphosphates becomes 2.9 ± 0.3 with 0.1 mM BzADP, and 2.5 ± 0.2 with 1 mM ADP. BzADP is more effective than ADP – about a 10-fold higher concentration of ADP is necessary to give the same effect. ADP and BzADdP increase the $K_{0.5}$ for ATP, but have only a small effect on the V_{max}. The values for the Hill constant are increased in the presence of added adenosine

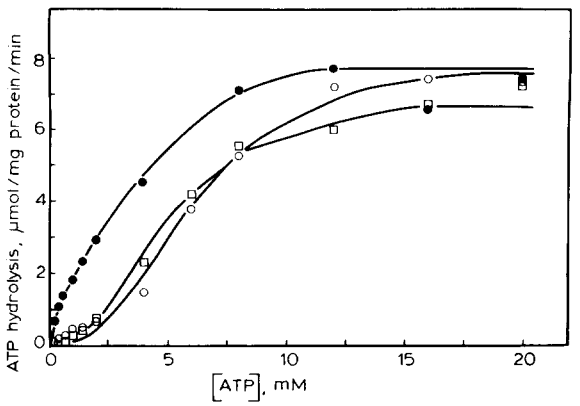

Fig. 1. Reversible inhibition of ATP hydrolysis by ADP and BzADP. ATP hydrolysis was assayed in the dark without added nucleoside diphosphate (●), with 1 mM ADP (□) or with 0.1 mM BzADP (○). The curves were obtained by nonlinear fitting to the Hill equation [16].

diphosphates, but are reduced to their original value upon increasing the Mg^{2+} concentration. 4-Benzoyl benzoate, up to 3.5 mM, does not inhibit ATP hydrolysis.

Noncovalent binding of Bz[^{3}H]ADP to TF_1 is enhanced by Mg^{2+} (Table I). At these nucleotide and Mg^{2+} concentrations about 2 mol of [^{3}H]ADP and 3 mol of Bz[^{3}H]ADP are bound per mol TF_1. Without Mg^{2+} and with EDTA present in the

TABLE I

TIGHT NONCOVALENT BINDING OF NUCLEOTIDES TO TF_1

TF_1 was incubated at 23°C for 1 or 2 h with 100 μM of the labeled nucleotides. The nonspecific binding is represented by the low level of labeled nucleotide remaining bound to TF_1 when 10 mM ADP and 100 μM of the labeled nucleotide were added together. Nucleotide binding was as described in Materials and Methods.

Additions	Nucleotide bound (mol/mol TF_1)			
	[^{3}H]ADP		Bz[^{3}H]ADP	
	EDTA	Mg^{2+}	EDTA	Mg^{2+}
None	1.10 ± 0.05	1.92 ± 0.17	0.93 ± 0.14	2.68 ± 0.45
+10 mM ADP [a]	0.30 ± 0.06	0.55 ± 0.06	0.43 ± 0.02	0.47 ± 0.02

[a] 10 mM unlabeled ADP was added after the first hour of incubation and the amount of labeled bound nucleotide was determined after an additional hour of incubation.

binding reaction mix, the levels of both of the bound nucleotides, [^{3}H]ADP and Bz[^{3}H]ADP, are similar and decreased to about 1 mol/mol TF_1. The relatively slow process of nucleotide binding reaches equilibrium within 1 h of incubation. The values reported here for [^{3}H]ADP bound to TF_1 in the absence of Mg^{2+} concur with those reported earlier [8,9]. However, in the presence of Mg^{2+}, we observe a maximal value of 2 mol [^{3}H]ADP bound/mol TF_1. The complex containing 3 mol ADP/mol TF_1 [8,9,17] is rather unstable. The displacement of the previously bound Bz[^{3}H]ADP upon addition of excess of ADP (Table I) indicates that the noncovalent binding of Bz[^{3}H]ADP may involve the same site to which ADP binds.

Covalent binding of Bz[^{3}H]ADP

Photoactivation of BzADP in the presence of TF_1 inactivates the enzyme. The time-course of inactivation with Mg^{2+} present in the irradiation mix shows that a $t_{0.5}$ of 10 min is attained at BzADP concentrations of 12–120 μM (Fig. 2B). However, without added Mg^{2+} and in the presence of EDTA, the degree of inactivation depends on the concentration of BzADP. The rate of inactivation appears to depend on the effectiveness of the interaction between the photoactivated benzophenone group and the amino acid side chains of the protein at or in the vicinity of the nucleotide binding site. In fact, the inactivation rate of TF_1 at saturating concentrations of BzADP (more than 100 μM) with EDTA is similar to that obtained in the presence of Mg^{2+}.

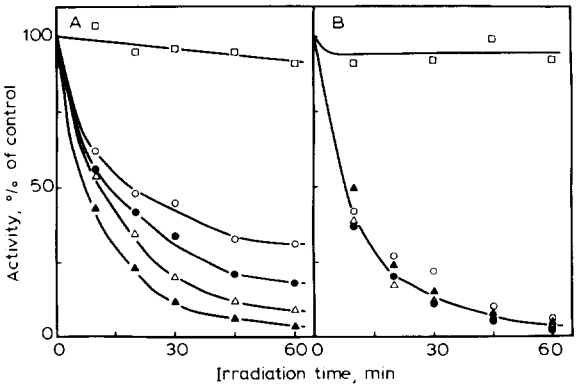

Fig. 2. Effect of Mg^{2+} and BzADP concentrations on the photoinactivation of the TF_1-ATPase. Reaction mixes of TF_1 and BzADP were irradiated for the time specified with 1 mM EDTA (A) or with 5 mM $MgCl_2$ (B). The BzADP concentration (μM) was: 0, (□); 12, (○); 24, (●); 84, (△); 120, (▲).

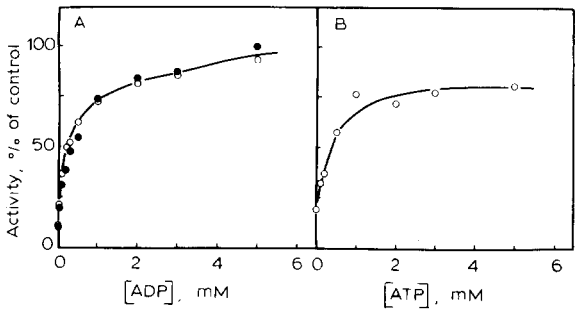

Fig. 3. Effect of ADP and ATP on the photoinactivation of TF_1 by BzADP. Mixes of TF_1, containing 50 mM Hepes buffer (pH 7.3), 60 μM BzADP, 1 mM EDTA (○) or 5 mM $MgCl_2$ (●), and either ADP (A) or ATP (B), were irradiated for 60 min. The corresponding nonirradiated samples were used as controls.

Inactivation of TF_1 by irradiation in the presence of BzADP is prevented by an excess of ADP or ATP (Fig. 3). ADP, with or without Mg^{2+}, was equally effective. In the absence of Mg^{2+}, ATP was as effective as ADP. ADP or ATP (0.5 mM) protected by about 50% against inactivation by irradiation in the presence of 60 μM BzADP. The protective effect is indeed exerted by ATP because only a small fraction of the ATP added (1–4%) underwent hydrolysis during the preincubation and irradiation intervals. The amount of ADP formed by this hydrolytic activity could not provide the degree of protection observed.

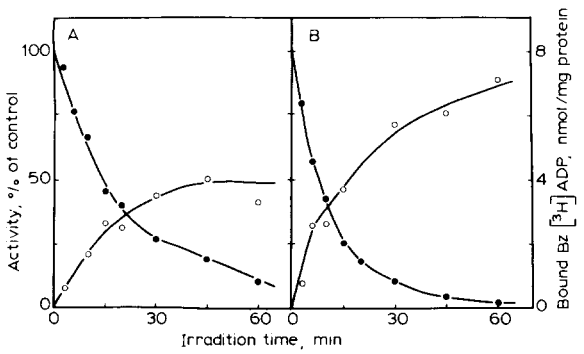

Fig. 4. Covalent binding of Bz[^{3}H]ADP to TF_1. Reaction mixes of TF_1 with 100 μM Bz[^{3}H)ADP and 1 mM EDTA (A) or 5 mM $MgCl_2$ (B) were irradiated as indicated. Assays of ATP hydrolysis (●) and covalently bound Bz[^{3}H]ADP (○) were as described in Materials and Methods.

Photoactivation of Bz[^{3}H]ADP in the presence of TF_1 results in the covalent binding of the analog to the protein (Fig. 4). As for the inactivation, the rate of covalent incorporation of Bz[^{3}H]ADP is enhanced when the irradiation is done in the presence of Mg^{2+}. As shown in Figs. 4 and 5 the covalent binding of Bz[^{3}H]ADP to TF_1 correlates well with the inactivation of the enzyme. Complete inactivation is attained after incorporation of 1.9 mol Bz[^{3}H]ADP/mol TF_1, independent of the Mg^{2+} concentration during irradiation. However, up to 2.7 mol Bz[^{3}H]ADP/mol TF_1 can bind covalently to TF_1 by irradiation of the analog in the presence of Mg^{2+}.

These high ratios of covalently bound BzADP obtained by irradiation of a mix without Mg^{2+} are rather puzzling, since without Mg^{2+} a 1 : 1 complex of TF_1 · BzADP is formed (Table I). To clarify this point we modified our usual labeling procedure by adding one more step, designed to remove excess free BzADP (together with other solutes) and leave in the mix to be irradiated only the noncovalently bound BzADP. As shown in Fig. 6, irradiation of the 1 : 1 TF_1 · BzADP complex obtained by binding of BzADP in the absence of Mg^{2+} (Table I) inactivates the TF_1-ATPase by about 50%. The degree of inactivation does not depend on the presence of Mg^{2+} during irradiation and is in agreement with the extrapolated value of 2 mol BzADP/mol TF_1 that is needed for complete inactivation. However, the degree of inactivation obtained by irradiation of the 1 : 3 : 3 TF_1-BzADP-Mg^{2+} complex (Table I, see also Ref. 9) formed by the binding of BzADP in the presence of Mg^{2+} is variable and depends on the irradiation conditions. When covalent binding is done with Mg^{2+} present, the inactivation of the ATPase is maximal (90%). On the other hand,

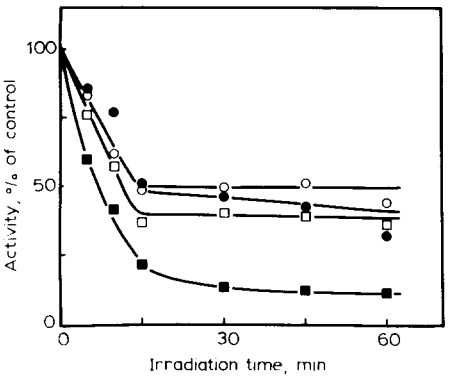

Fig. 6. Photoinactivation of the ATPase by irradiation of the TF_1-BzADP complex. TF_1 was incubated in the dark in a solution containing 50 mM Hepes buffer (pH 7.3), 80 μM BzADP and 1 mM EDTA (○, ●) or 5 mM $MgCl_2$ (□, ■). After 1 h the excess-free nucleotide and other solutes was removed by column centrifugation as described in Materials and Methods. To the protein-nucleotide complex 1 mM EDTA (○, □) or 5 mM $MgCl_2$ (●, ■) as added and the mixes were irradiated as indicated.

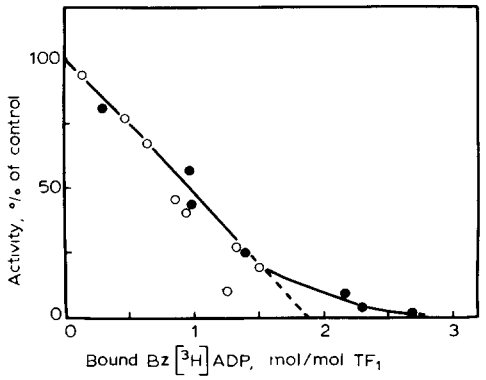

Fig. 5. Correlation between inactivation of the TF_1·ATPase and covalent binding of Bz[^{3}H]ADP. The data of Fig. 4 were replotted assuming a molecular weight of 380000 for TF_1 [18]. Irradiation was done in the presence of either 1 mM EDTA (○) or 5 mM $MgCl_2$ (●).

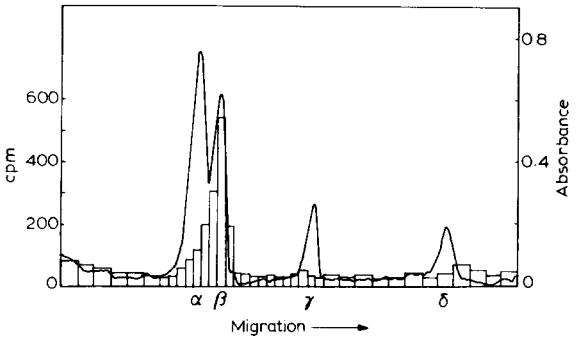

Fig. 7. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of labeled TF_1·Bz[^{3}H]ADP. Labeling of TF_1, sample preparation and analysis of the gels were as described in Materials and Methods. The ε subunit was electrophoresed out of the gel to improve separation between the α and the β subunits. This subunit did not contain Bz[^{3}H]ADP (not shown).

covalent binding without Mg^{2+} (in the presence of EDTA) results in 60% inactivation, probably because of the release of both the Mg^{2+} and the BzADP from the tight binding site, in agreement with the value of 1.2 mol BzADP per mol TF_1 calculated for a 60% degree of inactivation (Fig. 5).

Analysis of the subunit location of the covalently bound Bz[^{3}H]ADP shows that the labeled nucleotide is incorporated mainly into the β subunit of the protein (Fig. 7). This pattern is not affected by the presence or absence of Mg^{2+} during the irradiation procedure.

Discussion

The studies reported here show that photoaffinity labeling of TF_1-ATPase with an adenine nucleotide analog containing a photoactivatable benzophenone group inactivates the protein in a manner similar to that of CF_1-ATPase [5]. Noncovalent binding of BzADP reversibly inhibits the TF_1-ATPase and changes the kinetic parameters as with CF_1-ATPase except for the lack of inhibition of V_{max} (Fig. 1). This change in the kinetic behavior of the enzyme may result from the binding of the nucleoside diphosphate analog to a noncatalytic site with a subsequent effect on the properties of the catalytic site. Although we prefer this interpretation on the basis of several lines of evidence [3,5], surely our results do not rule out a direct effect by the binding of the ligand to the catalytic site itself. The effect of the Mg^{2+} concentration on the Hill constant indicates that the binding of Mg^{2+} and the formation of the ternary complex ($TF_1 \cdot ADP \cdot Mg^{2+}$) counteract the effect obtained by the binding of ADP. A decrease in the sensitivity of the TF_1-ADP complex to inactivation by DCCD, upon binding of Mg^{2+} and formation of the ternary complex, has also been reported [9].

Equimolar complexes are formed upon noncovalent binding of ADP and BzADP to TF_1 in the absence of Mg^{2+} (Table I). However, with Mg^{2+} present, 2:1 and 3:1 complexes of ADP and BzADP, respectively, are formed with TF_1. Our findings on the noncovalent binding of ADP and BzADP in the absence of Mg^{2+} concur with those reported by other workers for ADP [8,9]. With Mg^{2+} present, an intermediate 1:3:3 TF_1-ADP-Mg^{2+} complex is formed [9]. This ternary complex is unstable and dissociates when subjected to gel filtration, with the formation of the more stable 1:1:1 TF_1-ADP-Mg^{2+} complex. This complex is also obtained by the release of both the BzADP and Mg^{2+}, upon removal of the excess of free ligand prior to the covalent binding step. The fact that more BzADP than ADP appears to bind to TF_1 may be due either to a higher stability of the complex or to a higher affinity for the analog. The levels of nucleotide analogs, such as BzADP [6] and 2-azido ADP [19], bound to membrane-bound CF_1 were also higher than those of ADP. A plausible explanation is that the complex between the protein and the analog is more stable than that with ADP, and that more of the bound ADP is lost during isolation than that of the analog.

Both ADP and ATP effectively protect against photoinactivation of TF_1 by BzADP (Fig. 3), as expected from the rather similar dissociation constants of both ADP and ATP, with the isolated subunit of TF_1, with and without Mg^{2+} [8]. In contrast, inactivation of CF_1 by photoinactivation of BzADP was prevented only by ADP and not ATP [5]. Both the rate of photoinactivation of TF_1 and the covalent binding of Bz[^{3}H]ADP are enhanced if irradiation is done in the presence of Mg^{2+} (Fig. 4). However, the amount of bound Bz[^{3}H]ADP needed for complete inactivation does not depend on the presence of Mg^{2+} (Fig. 5). The Mg^{2+}-enhanced rate of photoinactivation may be due to a more efficient covalent binding of the labeled nucleotide analog. In the presence of Mg^{2+}, a higher level of the nucleotide analog is noncovalently bound to TF_1 (Table I). Retention of high levels of ligand per binding site increases the chances of fruitful interactions with the protein amino acid side chains upon excitation of the bound ligand molecules. Similar results were also obtained for the photoinactivation of CF_1 by covalent binding of BzADP in the presence of Ca^{2+} [5]. However, irradiation in the absence of Mg^{2+} also results in the incorporation of more than 1 mol BzADP/mol TF_1, suggesting that the covalent binding of one molecule of the analog does not prevent the binding of another one.

The incorporation of BzADP into the β subunit of TF_1 and the lack of effect of Mg^{2+} on the pattern of incorporation is very similar to that

reported for CF_1 ($\pm Ca^{2+}$) [5]. Labeling of the β subunit of the mitochondrial F_1 with Bz[γ-^{32}P]ATP was also reported [10]. However, with Bz[^{3}H]ATP the label is distributed between both the α and β subunits of F_1 [10]. Prolonged photoactivation results in the incorporation of 2.7 mol BzADP/mol TF_1 (Fig. 5) in accordance with the proposed stoichiometry of three copies of the β subunit per TF_1 [18]. Nevertheless, blocking of 2 of the β subunits is necessary for the complete inactivation of the enzyme. Modification with DCCD of two out of the three subunits of TF_1 was also reported to cause complete inactivation of the enzyme [9]. Since the stoichiometry of the reaction between the carboxylate anion of a glutamate residue on the β subunit and the reagent has not been clearly established because of a possible rearrangement of the *O*-acylisourea formed initially, these findings should not be interpreted in terms of a 2/3-of-the-sites reactivity [17]. We have also shown that 2.5 mol of BzADP are needed per mol of CF_1 for complete inactivation of the CF_1-ATPase [5]. In the latter case, the difference appears to be due to the standard used for protein determination. When the lyophylized TF_1 is replaced by bovine serum albumin as protein standard, as in the CF_1 assay, we indeed obtain a value of about 2.3 mol BzADP bound per TF_1.

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