Interaction of the chloroplast ATP synthetase with the photoreactive nucleotide 3′-*O*-(4-benzoyl)benzoyl adenosine 5′-diphosphate

Dudy Bar-Zvi, Marjorie A. Tiefert* and Noun Shavit

Department of Biology, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

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The photoreactive nucleotide 3′-*O*-(4-benzoyl)benzoyl ADP (BzADP) is not a substrate for photophosphorylation but is a strong competitive inhibitor (K_i 2–25 μM) with respect to ADP and ATP in photophosphorylation or ATP hydrolysis and P_i–ATP exchange reactions, respectively. The analog binds tightly to the membrane-bound CF_1, competes with the tight binding of ADP, and prevents the inactivation of the enzyme by tight binding of ADP. Upon irradiation with long wavelength ultraviolet light, the tightly bound BzADP becomes covalently attached to both the α- and β-subunits of the enzyme.

Photoaffinity labeling *ATPase* *Analog* *Tightly-bound nucleotide* *Chloroplast*

1. INTRODUCTION

Upon energization, the membrane-bound ATP synthetase of chloroplasts catalyzes the synthesis of ATP. It retains bound nucleotides tightly under non-energized conditions (review [1]). The tight binding sites apparently have regulatory rather than catalytic functions [2–5], although this question is not yet completely settled [6]. One approach to studying the role of the various nucleotide binding sites has been to label them covalently with nucleotide analogs that can be photoactivated by irradiation with ultraviolet light. When analog binding and photolabeling of isolated CF_1 are done simultaneously, 8-azido-ATP, 8-azido-ADP, or 3′-*O*-(3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl)-ADP (arylazido-ADP) binds to both the α- and β-subunits of the enzyme [7–9]. Interaction arylazido-ATP with isolated CF_1, or 2-azido-ADP with the tight nucleotide binding site(s) of membrane-bound CF_1, followed by photoactivation of the analogs, results in the labeling of the β-subunit only [8,10]. The ATPase activity of the soluble enzyme is inhibited by all 4 of the tested analogs, and when the arylazido-ATP is not covalently bound it serves as a substrate for the enzyme's hydrolytic activity [7,8]. Under non-covalent binding conditions, 2-azido-ADP competes with ADP for tight binding, but its effect on ATP synthesis of hydrolysis was not tested [10]. Arylazido-ADP is a strong inhibitor of photophosphorylation and also competes with ADP for tight binding. However, photolabeling was not reported [11].

An adenine nucleotide analog, 3′-*O*-(4-benzoyl)-benzoyl ATP (BzATP), which contains a benzophenone rather than an azido as the photoactivatible group, was shown to react with mitochondrial

Abbreviations: CF_1, coupling factor 1, the hydrophilic portion of the ATP synthetase/hydrolase of chloroplast membranes containing the active site(s); BzADP, 3′-*O*-(4-benzoyl)benzoyl adenosine 5′-diphosphate; chl, chlorophyll; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; tricine, *N*-tris(hydroxymethyl)methylglycine; $K_{i(s)}$ and $K_{i(i)}$, K_i values corresponding to effects on the slope and intercept of the double reciprocal plot, respectively

* Present address: The Institutes for Applied Research, Ben-Gurion University of the Negev, Beer Sheva 84110, Israel

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F_1-ATPase [12]. Unlike the active nitrene derived from the azido group, the diradical triplet generated from benzophenone does not react with water. Thus, photolabeling with BzATP or BzADP ought to be more efficient than labeling with azido analogs. In addition, modification of the 2'- or 3'-positions of adenosine nucleotides usually does not have much effect on the ability of the analogs to interact with the tight binding or catalytic sites of CF_1 on chloroplast membranes [13].

Here, we describe the labeling of the tight nucleotide binding site(s) on CF_1 by the interaction of BzADP with chloroplast thylakoids. Without photoactivation, this analog is competitive with the appropriate unmodified adenine nucleotide during photophosphorylation, ATP hydrolysis, and P_i–ATP exchange. The analog binds tightly to membrane-bound CF_1 and competes with the tight binding of ADP. Upon photoactivation, the tightly bound Bz[^{3}H]ADP becomes covalently bound to both the α- and β-subunits of CF_1.

2. MATERIALS AND METHODS

Chloroplast thylakoid membranes were prepared from lettuce leaves by conventional procedures [3]. $^{32}P_i$ was purchased from the Nuclear Research Center (Negev), [2-^{3}H]ADP from Amersham (Bucks) and Soluene-350 from Packard. Unlabeled nucleotides were obtained from Sigma Chemical Ca (St Louis MO). Photophosphorylation, ATP hydrolysis, P_i–ATP exchange, and tight binding of nucleotides were assayed as in [3,14]. Analysis of the kinetic data was done using the non-linear fitting procedure in [15]. Chlorophyll was determined as in [16] and protein according to [17] using bovine serum albumin as a standard. Electrophoresis on 10% polyacrylamide gels in the presence of sodium dodecyl sulfate was done as in [18], at 15°C to reduce hydrolytic cleavage of the label during the run.

BzADP was prepared and purified essentially as in [12]. Lowering the ratio of 1,1'-carbonyldiimidazole and 4-benzoylbenzoic acid to ADP by a factor of 5 yielded a transparent solution of the first two reagents in dimethylformamide, and resulted in a final yield similar to that reported. The lyophilized ammonium salt of BzADP obtained after purification on a Sephadex LH-20 column (2.4 × 38 cm for 2 mmol ADP) was dissolved in a minimal volume of water, applied to a Dowex-W50 column (H^+ form), and eluted with water. Fractions containing BzADP were combined, brought to pH 8.0 with concentrated Tris base and lyophilized. Bz[2-^{3}H]ADP (0.3 mCi/μmol) was prepared as above from [2-^{3}H]ADP, but was kept as an ammonium salt, since only very small amounts were added to the assay mixtures.

3. RESULTS

3.1. *Kinetic effects of BzADP on photophosphorylation and its partial reactions*

A survey of the effects of BzADP on the activities of chloroplast membranes was done under conditions where covalent binding of the analog

Table 1

Apparent kinetic parameters for the inhibition of chloroplast ATP synthetase activities by reversible interaction with BzADP

Activity	Parameter	Value (μM)	Type of inhibition
1. Photophosphorylation [ADP] varied	$K_{i(s)}$	1.2 ± 0.1	Competitive
[P_i] varied	$K_{i(s)}$	24.6 ± 2.8	Mixed
	$K_{i(i)}$	51.6 ± 4.0	
2. P_i–ATP exchange [ATP] varied	$K_{i(s)}$	20.4 ± 6.3	Probably competitive
3. ATP hydrolysis	$K_{i(s)}$	21.3 ± 7.8	Competitive
4. Postillumination tight [^{3}H]ADP binding[a]	$K_{i(s)}$	1.7 ± 0.3	Competitive

[a] Binding of [^{3}H]ADP was quenched by the addition of 5 μM FCCP and 5 mM unlabeled ADP

Activities were measured at several BzADP concentration (0–200 μM) and the data analysed by non-linear regression as in [14,15]

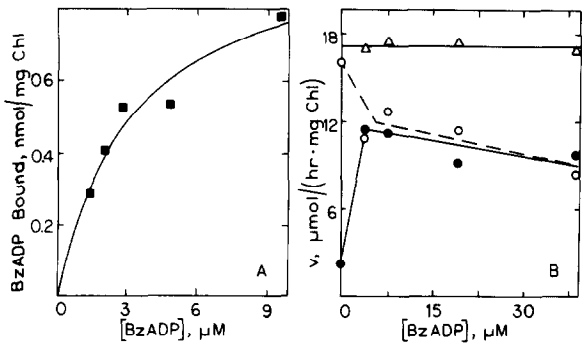

Fig.1. Non-covalent binding of BzADP to membrane-bound CF_1. (A) Tight binding of Bz[^{3}H]ADP. Reaction conditions were as in section 2. Binding was quenched by the addition of 5 μM FCCP and 5 mM ADP. Tightly bound Bz[^{3}H]ADP was determined after release of the labeled analog by adding perchloric acid to the washed chloroplast thylakoids. The line was fit by non-linear regression [15]. (B) Protection, by BzADP, of P_i–ATP exchange against inhibition due to ADP. BzADP at the indicated concentrations was added alone (○) or together with 5 μM ADP (●) in the dark 5 s after the activation step, and ATP + $^{32}P_i$ (5 mM each) were added after an additional 60 s after the activation step.

was found not to occur. The analog was added in darkness to preilluminated membranes, or it was incubated with membranes during only short periods of illumination with white light ($\leqslant$30 s, incandescent lamps). BzADP itself is not phosphorylated by lettuce chloroplasts. However, it strongly inhibits phosphorylation, competitive with ADP and mixed-type with respect to P_i (table 1), as expected for a reversible, non-phosphorylatable ADP analog [19]. Benzoylbenzoic acid alone did not inhibit ATP synthesis. When added together with the substrates in a dark postactivation step, BzADP also reversibly inhibits the P_i–ATP exchange and ATP hydrolysis reactions, apparently in a competitive manner with respect to ATP, with $K_{i(s)}$ values of about 20 μM (table 1).

3.2. *Interaction of BzADP with the tight nucleotide binding site(s)*

BzADP also interacts with the tight nucleotide binding site(s) on CF_1. As shown in table 1, BzADP inhibits the postillumination tight binding of [^{3}H]ADP in a competitive manner. Indeed, upon addition to preilluminated thylakoids, the analog binds tightly but non-covalently (fig.1A). The app. K_d for binding is 3.5 ± 1.1 μM and the maximal binding is 1.10 ± 0.15 nmol/mg chl.

When ADP is added in a post-illumination step to activated membranes before ATP is added, the enzyme is deactivated, resulting in the inhibition of the P_i–ATP exchange and ATPase activities. This inhibition can be prevented by the addition of other nucleoside diphosphates together with ADP [2,3]. Fig.1B shows that BzADP also protects against the inhibitory effect of ADP. The protection is maximal at [BzADP] $\leqslant$ 5 μM when it is added together with ADP before addition of ATP and P_i. BzADP added by itself partly inhibits the P_i–ATP exchange when it is allowed to bind for 1 min before adding ATP plus P_i. However, when the analog is added simultaneously with the substrates, it does not inhibit the reaction under these conditions (high [ATP]).

Table 2

Release of tightly bound nucleotides upon irradiation with long wavelength ultraviolet light

Addition	Irradiation time (min)	[^{3}H]ADP released	
		nmol/mg chl	% bound [^{3}H]ADP
None	1	0.10	7.4
	2	0.15	11.2
	10	0.43	31.2
Methyl viologen, 0.5 mM	10	1.01	72.9
NH_4Cl, 10 mM	10	0.03	2.3

After tight binding of [^{3}H]ADP (light + dark), chloroplast membranes were washed 3 times with 25 mM tricine–NaOH (pH 8.0), 50 mM NaCl, 1 mM $MgCl_2$ and then resuspended in the same solution. Samples (0.5 ml final vol.) were placed in 10 ml beakers on ice and irradiated with the above additions with long wavelength ultraviolet light (366 nm) from a Mineralight lamp (model UVSL-25) at a distance of about 3.5 cm. Release was quenched as described under table 1. After centrifugation, a sample of the supernatant was counted to determine the amount of nucleotide released. The total amount present in the membranes was determined by extraction with 3% perchloric acid, or by illumination with white light for 1 min at about 20°C in the presence of methyl viologen and 0.1 mM unlabeled ADP, and was 1.38 nmol/mg chl

Table 3

Photolabeling of chloroplast thylakoids with Bz[^{3}H]ADP

Irradiation time (min)	Bound label (nmol/mg chl)	
	Non-covalent	Covalent
0	0.53	0.03
30	0.35	0.13
60	0.30	0.14

Chloroplast thylakoid membranes were allowed to bind Bz[^{3}H]ADP (8.7 μM) tightly in a dark postillumination step as in section 2. Binding was terminated by adding 10 μM FCCP. The membranes were washed twice with a cold solution containing 25 mM tricine–NaOH (pH 8.0), 50 mM NaCl, 1 mM $MgCl_2$ and 10 μM FCCP and resuspended in the same solution to 50–100 μg chl/ml. Samples of 1 ml in 5 cm diameter Petri dishes were irradiated with Blak-Ray lamp (model XX-15) (366 nm) at about 5 cm for the times indicated. After quantitative transfer to centrifuge tubes, perchloric acid (5% final) was added. The samples were centrifuged and the precipitates washed once with perchloric acid. The two supernatants were used for determination of non-covalently bound nucleotide. The pellets were decolorized by extraction with acetone, then solubilized with 5% sodium dodecylsulfate for determination of covalently bound label

3.3. *Photoaffinity labeling of the ATP synthetase*

Preliminary experiments, where chloroplast membranes containing bound BzADP were irradiated with ultraviolet light under conditions that did not interfere with the chloroplasts' catalytic ability, gave no functional evidence for irreversible incorporation of BzADP (not shown). We observed that irradiation of the thylakoid membranes with long wavelength ultraviolet light (366 nm), which does not damage their activity [20] and is optimal for photoactivation of the benzophenone group, also induces release of ADP previously tightly bound to CF_1. This release occurs even without an added electron carrier and is prevented by addition of an uncoupler (table 2). Therefore, to obtain covalent binding of Bz[^{3}H]ADP, we irradiated membranes containing tightly bound Bz[^{3}H]ADP with a higher intensity ultraviolet light source for relatively long periods of time in the presence of an uncoupler (table 3). The treatment partly bleached the pigmented membranes, thus precluding later study of their enzymatic activities. However, up to one-third of the tightly bound BzADP became covalently bound under these conditions. Extraction of CF_1 from membranes labeled by this method, followed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate, showed that both the α- and β-subunits of CF_1 were labeled (fig.2).

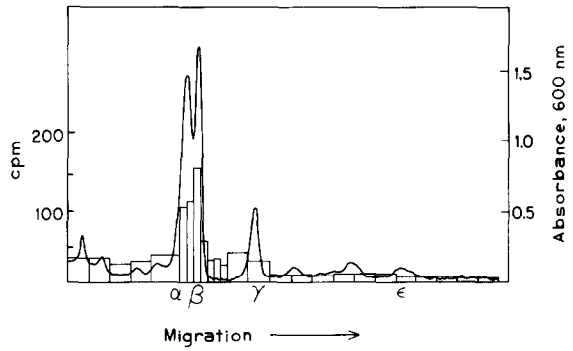

Fig.2. Photoaffinity labeling of CF_1 subunits with Bz[^{3}H]ADP. Covalent binding of Bz[^{3}H]ADP to the membrane-bound enzyme was done as described under table 3. After irradiation, samples were centrifuged and the pellets resuspended in a solution containing 1 mM EDTA and 1 mM tricine (pH 8.0). $CHCl_3$, 1/2 the volume of the thylakoid suspension, was added and the mix was vigorously stirred for 30 s followed by centrifugation at 12 000 × g for 5 min. The upper phase was transferred to another centrifuge tube, an aliquot was taken for protein determination, and ice cold perchloric acid was added to the remaining sample to a final concentration of 5%. The sample was then centrifuged for 2 min at 12 000 × g and the precipitate washed with cold 5% perchloric acid and centrifuged again. The washed pellet was resuspended in buffer containing 5% sodium dodecylsulfate, 10 mM P_i, 1% β-mercaptoethanol, 10% glycerol and 1% bromophenol blue. The solution was adjusted to pH 7 by the addition of 1 M tricine–NaOH (pH 8.0). Samples were incubated 30 min at 37°C and electrophoresis was done in duplicate as in section 2. Slices (1–2 mm) of one gel were incubated overnight at room temperature in the dark with 4 ml scintillation mixture (toluene–Triton X-100 based containing 10% Soluene-350) before counting. The duplicate gel was stained with Coomassie brilliant blue R as in section 2 and absorption at 600 nm was determined in a Cary-219 spectrophotometer.

4. DISCUSSION

It is clear that BzADP interacts non-covalently with the membrane-bound CF_1 and is a potent inhibitor of all the enzyme activities. The mode of inhibition of the various reactions (table 1) suggests that the analog can bind reversibly to the catalytic site. Thus, BzADP acts as a competitive inhibitor with respect to ADP in photophosphorylation, and to ATP in the ATP hydrolysis and P_i–ATP exchange reactions. In addition, the analog inhibits phosphorylation in a non-competitive manner with respect to P_i, as expected for a non-phosphorylatable analog of ADP [19]. The higher K_i values obtained for the competitive inhibition of the ATP-utilizing reactions do not necessarily suggest different binding sites, they may result from the different modes of energization established during photophosphorylation and in ATP hydrolysis and P_i–ATP exchange. It has already been shown that the K_m values for ADP in photophosphorylation depends on the energization levels [21]. In addition, differences in the K_i values for the inhibition of ATP synthesis and hydrolysis by naphtoyl derivatives of ADP and ATP were suggested to result from differences in the energization level existing during the assays of these reactions [22]. However, nucleotide diphosphate analogs of ADP may bind to both catalytic and non-catalytic (regulatory) sites, if indeed these sites are completely separate. Even though our results fit the equation for competitive inhibition well (table 1), they also fit well a velocity equation developed by assuming interaction of the analog with both catalytic and non-catalytic sites [14].

BzADP also is capable of binding to the tight site on CF_1 (fig.1). This is probably also the site where ADP binds tightly, since both nucleotides compete for binding to the enzyme (table 1), and prior binding of BzADP prevents tight binding of ADP and consequent inhibition of the enzyme (fig. 1B). This behavior is similar to that reported for GDP [3] and suggests that the site to which these analogs bind is regulatory rather than catalytic. The higher amounts of tightly bound Bz[^{3}H]ADP in comparison to those of [^{3}H]ADP, observed under identical experimental conditions [3,23], may not represent binding of the analog to an additional site, but may be mainly due to a greater retention of the tightly bound nucleotide analog on CF_1 during the washing procedure [10,23].

Photoaffinity labelling of both the α- and β-subunits of membrane-bound CF_1 (fig.2) may occur even if only one binding site is involved, if groups on one of the subunits are close enough to the site on the other subunit where the nucleotide analog binds. In addition, the type of the photoreactive group and analog used can contribute to differences in labeling patterns. Thus, labeling of both the α- and β-subunits of soluble CF_1 occurred when 8-azido derivatives of ADP and ATP [7] or arylazido-ADP [8,9] were used. Moreover, crosslinking between the α- and β-subunits of bacterial [24] and mitochondrial oligomycin-sensitive ATPase [25] occurred when photoaffinity labeling was done with 3′-arylazido-8-azido ATP. On the other hand, photoaffinity labeling of membrane-bound CF_1 with 2-azido-[^{32}P]ADP results in the incorporation of the analog only by the β-subunit [10]. Preferential labeling of the β-subunit was also obtained when photolabeling of isolated CF_1 was done with BzADP, as will be reported elsewhere. Variation in the degree of labeling of subunits can also depend on the experimental conditions [26]. The pattern of subunit labeling and its relation to the functionally defined nucleotide binding sites is being investigated further.

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