

Investigation of nucleotide binding sites on chloroplast coupling factor 1 with 3'-O-(4-benzoyl)benzoyl adenosine 5'-triphosphate

(photoaffinity labeling/bioenergetics/ATP synthesis)

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ABSTRACT The subunit locations of each of the three nucleotide binding sites of soluble chloroplast coupling factor 1 have been studied with the photoaffinity label 3'-O-(4-benzoyl)benzoyl-ATP. This derivative is an effective inhibitor of ATPase activity. Photolysis of the radioactive label when bound to each of the three nucleotide sites on the coupling factor has been examined. For the nucleotide site that normally binds ADP very tightly, NaDodSO₄/polyacrylamide gel electrophoresis after photolysis indicates that primarily the β polypeptide chain is appreciably labeled (86%), although some labeling of the α polypeptide chain is found (14%). For the site that binds MgATP tightly, 97% of the radioactivity is found on the β polypeptide chain. The α and β polypeptide chains are labeled in approximately equal amounts when photolysis is carried out with the nucleotide analog bound to the third site.

The dicyclohexylcarbodiimide-sensitive ATPase of chloroplast thylakoid membranes is the final enzyme in oxidative phosphorylation and is responsible for the synthesis of ATP (cf. ref. 1). Chloroplast coupling factor 1 (CF₁) is the water-soluble portion of this complex and possesses a latent ATPase activity specific for Ca²⁺ (2). CF₁ has five different types of polypeptide chains (α , β , γ , δ , and ϵ); recent reports suggest a subunit stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ (3).

Solubilized CF₁ possesses nucleotide binding sites that function in both catalysis and its regulation (4, 5). Thus far, three specific sites have been identified, each exhibiting distinct characteristics. A single site, whose function is controversial, binds ADP very tightly (site 1; see refs. 6–8). ADP or ATP can exchange into this site, but only ADP is found bound to the enzyme; when trinitrophenyl-ADP binds to this site, hydrolytic activity is abolished. A second site binds MgATP very tightly (site 2). Whether or not this site is occupied does not affect the catalytic activity of the enzyme, although its role may be regulatory on membrane-bound CF₁ (8). The third site binds either ADP or ATP with a dissociation constant in the micromolar range (site 3), and it is a likely candidate for a catalytic site (8).

Photoactivatable nucleotide analogs have been used extensively to determine the polypeptide chain location of each of these sites. By inserting covalently into selected regions on the enzyme, these compounds have been invaluable as site-specific labels (9). Experiments with 3'-O-[3-N-(4-azido-2-nitrophenyl)aminopropionyl]-ADP and -ATP have shown that primarily the β polypeptide chain is labeled after photolysis of this analog when bound to the site to which MgATP binds tightly and that the α and β polypeptides are labeled in approximately equal amounts when the label is bound to site 3 (7). Thus far, photoaffinity labeling of the site normally occupied by ADP has not been possible. Recently, however, photoreactive labeling with 2-azidoadenosine diphosphate bound to membrane-bound CF₁ at a site normally containing

ADP has resulted in labeling of the β polypeptide (10). A similar experiment with 3'-O-(4-benzoyl)benzoyl-ADP resulted in the labeling of both the α and β polypeptide chains (11).

In this investigation, the interactions of the photoaffinity label 3'-O-(4-benzoyl)benzoyl-ATP (BzATP) with soluble CF₁ were studied. This substrate analog is an effective inhibitor of ATPase activity, as well as a derivative capable of binding to each nucleotide site. Studies using radioactive BzATP as a site-specific photoaffinity label on soluble CF₁ have permitted the isolation of labeled polypeptides associated with binding at each of the three nucleotide sites. For the site that binds both ADP and ATP reversibly, the α and β polypeptides again were labeled in approximately equal amounts; for the other two sites, predominantly the β polypeptide was labeled.

MATERIALS AND METHODS

Chemicals. ATP, 1,1'-carbonyldiimidazole, and Tris were from Sigma; 4-benzoylbenzoic acid was from Aldrich; [2,3-³H]ATP (ammonium salt) with a specific activity of 32 Ci/mmol (1 Ci = 37 GBq) was purchased from Amersham. All other reagents used were high quality commercial grades, and all solutions were prepared with distilled deionized water.

Enzyme. CF₁ was prepared from fresh market spinach following procedures slightly modified from Lien and Racker (12) and Binder *et al.* (13). Purified enzyme was stored as an ammonium sulfate precipitate in saturated ammonium sulfate, 20 mM Tris·SO₄/2 mM EDTA, pH 7.1, and kept at 4°C for no longer than 1 month. Assays for enzymatic activity were performed periodically by heat-activating a solution (1–2 mg/ml) of latent solubilized CF₁ for 5.0 min at 64°C in the presence of 40 mM ATP/10 mM dithiothreitol/2 mM EDTA/40 mM Tris·HCl, pH 8.0 (3). The activity was typically 15–20 μ mol/(mg·min). For the purified latent enzyme, both free and dissociable nucleotides were removed prior to all experiments by passage through two consecutive 3-ml centrifuge columns containing Sephadex G-50 pre-equilibrated with appropriate buffer (14). An extinction coefficient of 0.483 cm²·mg⁻¹ (277 nm) was used to determine all enzyme concentrations (7).

Synthesis of BzATP. BzATP was prepared following the procedure of Williams and Coleman (15) with only minor modifications. Under an atmosphere of dry nitrogen, 4-benzoyl benzoic acid (final concentration, 0.14 M) and 1,1'-carbonyldiimidazole (final concentration, 0.43 M) were dissolved in 5.0 ml of distilled anhydrous dimethylformamide and stirred for 15 min at room temperature. After the initial reaction, 25 ml of an aqueous ATP solution (final concentration, 0.03 M) was added to the medium, and the synthetic reaction was allowed to proceed for 19 hr in the dark. Sol-

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Abbreviations: CF₁, chloroplast coupling factor 1; BzATP, 3'-O-(4-benzoyl)benzoyl-ATP.

vent was removed by lyophilization, and excess reagents were extracted with acetone. The residual precipitate was dissolved in 12.5 mM sodium acetate (pH 5.0) and purified by high-performance liquid chromatography on an Altex C₁₈ reversed-phase column (10 mm × 25 cm) using a Beckman Model 342 Gradient Liquid Chromatograph and a 12.5 mM sodium acetate, pH 5.0/methanol gradient. The product, which eluted as a sharp band at 40%–50% methanol, was characterized by proton-NMR and absorbance spectroscopy ($\lambda_{\max} = 260$ nm) and constituted a 15%–20% yield relative to ATP. [2,8-³H]BzATP was prepared identically, using [2,8-³H]ATP diluted with nonradioactive ATP. Specific activities obtained for several preparative batches ranged between 1 and 12 mCi/mmol. An extinction coefficient of 3.25×10^4 cm⁻¹·M⁻¹ at 260 nm was used to determine the concentration of analog (unpublished results).

Photoaffinity Labeling. Covalent labeling of each of the nucleotide binding sites on CF₁ using [2,8-³H]BzATP was performed as follows. Enzyme was solubilized in 2 mM EDTA/50 mM Tris·HCl, pH 8.0/6.0 mM MgCl₂, and freed from dissociable nucleotides using G-50 Sephadex columns pre-equilibrated with appropriate buffer. Photolysis was carried out in a 1-cm water-cooled quartz cuvette for 8 min at a distance of 10 cm from a Hanovia 200 xenon-mercury arc lamp fitted with a Corning filter to remove unnecessary emission <300 nm and >410 nm. Radioactive nucleotide incorporation stoichiometries were determined by measuring the radioactivity of 10- μ l aliquots of labeled enzyme solution in 10 ml of ACS scintillation fluid (Amersham) in a Beckman SP-100C liquid scintillation counter. Unless otherwise specified, all reactions were carried out at room temperature (19°C ± 2°C) either in the dark or under a 25 W red safety lamp prior to photolysis.

To label the site binding ADP tightly (site 1), the site binding MgATP tightly was first blocked by incubating 15 μ M CF₁ with 240 μ M ATP in 6.0 mM MgCl₂/2 mM EDTA/50 mM Tris·HCl, pH 8.0, for 10 min. Passage through two Sephadex G-50 centrifuge columns pre-equilibrated with 50 mM Tris·HCl, pH 8.0/2 mM EDTA removed dissociable nucleotides as well as free Mg²⁺, and the enzyme was incubated for 10 hr in a 20-fold molar excess of [2,8-³H]BzATP. After exchange into site 1 had occurred, dissociable nucleotide was similarly removed and the labeled CF₁ was photolyzed.

The site binding MgATP tightly (site 2) was labeled by incubating 10 μ M CF₁ with 200 μ M [2,8-³H]BzATP in 6.0 mM MgCl₂/2 mM EDTA/50 mM Tris·HCl, pH 8.0, for 1 min. After incorporation of analog, dissociable nucleotides were removed as described above, and labeled enzyme was incubated for 8 hr in 200 μ M ATP to remove any label from the nondissociable ADP site. Photolysis of the sample followed removal of excess ATP.

To label the third site, the MgATP site was blocked by incubating 15 μ M enzyme for 10 min with 200 μ M ATP and 6 mM MgCl₂ in 50 mM Tris·HCl (pH 8.0) and 2 mM EDTA. After unbound nucleotide was removed, the enzyme solution was placed in the photolysis cuvette and irradiated immediately upon the addition of \approx 1.5-fold excess [2,8-³H]BzATP. Again, unbound nucleotide was removed, and stoichiometries were determined.

Gel Electrophoresis. In preparation for polyacrylamide gel electrophoresis, 100- μ g quantities of enzyme were denatured at 100°C for 3 min in 60 mM Na₂CO₃/0.60% (wt/vol) NaDodSO₄/30 mM dithiothreitol. Approximately 90- μ g samples of CF₁ with bromothymol blue and glycerol added were applied to a 13% (wt/vol) acrylamide/0.35% (wt/vol) N,N'-methylene bisacrylamide gel, and electrophoresed for 1 hr at 70 V and then for 5 hr at 100 V. Protein bands were fixed with Coomassie blue stain, destained, and sliced into 3 × 10 mm (diameter) sections. After dissolution of gel bands in 500 μ l of H₂O₂ for 2 hr at 70°C, excess peroxide was de-

stroyed by adding catalase, and the radioactivity of the sample was determined in 10 ml of ACS scintillation fluid.

RESULTS

BzATP as a Substrate Analog for Purified CF₁. The effectiveness of BzATP as a substrate analog for purified solubilized CF₁ was determined by using the standard phosphate assay on the activated form of the enzyme. To prevent undesirable photoactivation of this nucleotide derivative, each of these experiments was performed in the dark until the reaction had been quenched. With ATP (2.5 mM) as the substrate, normal ATPase activities of 19 μ mol/(mg·min) were obtained, whereas in assays performed with either BzATP as the substrate or an equimolar mixture (2.5 mM) of both BzATP and ATP, no significant hydrolytic activity was observed. These results indicate that while BzATP itself is not appreciably hydrolyzed by activated CF₁, it does function as an effective inhibitor of ATP hydrolysis. A complete characterization of the inhibition was not carried out, but the inhibition was primarily noncompetitive.

Site-Specific Photoaffinity Labeling of Nucleotide Binding Sites on CF₁. With BzATP as a photoactivatable nucleotide analog, each of the three nucleotide binding sites present on latent CF₁ was covalently labeled with [2,8-³H]BzATP. Prior to this, BzATP was shown to displace [2,8-³H]ATP from site 1, and MgBzATP was found to bind tightly to site 2, analogously to MgATP (7). Subsequent denaturation of the enzyme followed by NaDodSO₄/polyacrylamide gel electrophoresis resulted in the separation of all five polypeptide chains. Each chain was then analyzed for incorporation of radioactivity to determine the specific location of each nucleotide binding site. The α and β chains were completely resolved: an unstained area of gel separated the Coomassie blue-stained bands associated with the α and β polypeptide chains.

Earlier work using photoactivatable aryl-azido derivatives of ADP and ATP proved unsuccessful in identifying the subunit location of site 1 on latent purified CF₁ (7). With BzATP as a new photoactivatable ligand, however, specific labeling of the β polypeptide occurred (Table 1; Fig. 1): 86% of the covalently bound nucleotide detected after electrophoresis was on the β chain. By preblocking site 2 with MgATP and then incubating the enzyme in excess [2,8-³H]BzATP, a stoichiometry of 1.18 per CF₁ was observed after removal of both free and dissociable nucleotide from the enzyme. In addition, the time course for incorporation of [2,8-³H]BzATP onto CF₁ approaches stoichiometric equivalency (unpublished results). Since analog bound at sites 2 and 3 is readily removed by gel chromatography when separating enzyme from free nucleotide, these data provide strong evidence for the site 1-specific incorporation of analog onto the enzyme and labeling of predominantly the β polypeptide. However, a small amount of labeling of the α polypeptide has been found in several different experiments.

Table 1. Radioactive incorporation after site-specific labeling with BzATP

Sites labeled	Stoichiometry, mol of substrate per mol of CF ₁	% radioactivity	
		α	β
1	1.18	14	86
2	0.80	3	97
3	0.25	47	53
1 and 2	2.08	5	95

Percentage of total radioactivity for α and β polypeptides was obtained from 3 × 10 mm (diameter) sections of polyacrylamide gels (Fig. 1).

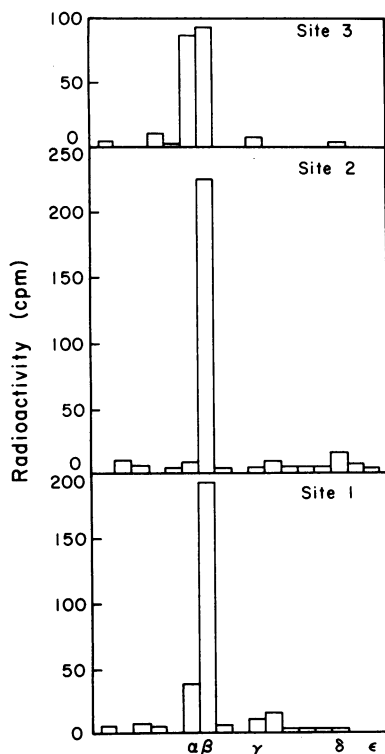


FIG. 1. ^3H radioactivity (cpm) of polyacrylamide gel sections (3×10 mm diameter) after electrophoretic separation of polypeptide chains in CF_1 previously labeled with $[2,8\text{-}^3\text{H}]\text{BzATP}$ (12.0 mCi/mmol) at sites 1, 2, and 3, respectively. Gels were analyzed in 17 sections from the top left to the bottom right. Locations of polypeptide chains are indicated on the abscissa.

Incubation of $[2,8\text{-}^3\text{H}]\text{BzATP}$ with latent CF_1 in the presence of Mg^{2+} for 1 min followed by gel filtration to remove excess nucleotide, photoactivation, and electrophoresis revealed that $>95\%$ of all radioactivity incorporated into the gel was located on the β polypeptide (Table 1; Fig. 1). To ensure that label was not present in site 1 at the time of photolysis, a second experiment was performed prior to photolysis, in which labeled enzyme was freed from excess nucleotide and Mg^{2+} and then incubated an additional 8 hr in excess ATP. The same results were obtained in both cases and correlate well with earlier studies that identified the β polypeptide as being labeled when site 2 contains the photoaffinity label (7).

Because of the relatively high dissociation constant associated with nucleotides binding at site 3, separation of free nucleotide before photolysis was not possible, and photoactivation of the BzATP analog had to be carried out *in situ*. After blocking site 2 with MgATP , an equivalent concentration of $[2,8\text{-}^3\text{H}]\text{BzATP}$ was added to a solution of CF_1 that was photolyzed immediately. Removal of free nucleotide label yielded a stoichiometry of 0.25 mol of label per mol of CF_1 , indicating the inherent difficulty in attempting to label site 3 efficiently. However, specificity of labeling was attained by using a concentration of $[2,8\text{-}^3\text{H}]\text{BzATP}$ that was equivalent to or only in slight excess of the enzyme concentration. Table 1 and Fig. 1 give the results obtained after electrophoretic analysis and indicate that α and β polypeptides are labeled nearly equivalently when BzATP is targeted for site 3.

Further confirmation of the previous results indicating that both sites 1 and 2 reside primarily on the β polypeptide of latent CF_1 was obtained by labeling both sites 1 and 2 together. Table 1 shows that when CF_1 is incubated with $[2,8\text{-}^3\text{H}]\text{BzATP}$ in the presence of Mg^{2+} and then photoacti-

vated after removal of free nucleotide from the medium, the label/enzyme stoichiometry increases to 2.08, indicating that both sites have been labeled with analog. When the labeled CF_1 subunits are then separated on polyacrylamide gels, 95% of all bound label is found to comigrate with the β polypeptide.

DISCUSSION

The use of BzATP as a photoactivatable nucleotide analog has proven to be successful in the site-specific labeling of each of three nucleotide binding sites present on purified solubilized CF_1 . Although covalent incorporation of label is not an efficient process, its utility as a covalently inserting analog should not be underestimated. (The stoichiometries for sites 1 and 2 in Table 1 are not a measure of covalent incorporation because the nucleotides are tightly bound prior to photolysis.) In particular, BzATP possesses some unique advantages over many of the azido nucleotides that have been used in previous studies with CF_1 . Apart from its stability over the pH 4–10 range, BzATP is virtually nonreactive with water and appears to exhibit a very selective covalent insertion pattern (15). Williams and Coleman (15) have postulated a probable mechanism of reaction consistent with their own results using BzATP on mitochondrial coupling factor 1. According to their interpretation, photolysis stimulates excitation of the carbonyl group in the benzophenone moiety to form a highly reactive diradical triplet. Radical oxygen then abstracts a hydrogen atom from nearby methylene residues present in the enzyme, resulting in a transient caged radical pair, which quickly degenerates to form a carbon-carbon covalent bond between substrate and enzyme. The formation of a carbon-carbon single bond is a major advantage over less stable bonds with nitrogen formed from the more commonly used nitrene intermediates.

In the results presented here, BzATP has been shown to function first as an effective inhibitor of CF_1 ATPase activity, and second as a ligand that can be induced to covalently insert into any one or all of the desired binding sites on the enzyme. The inhibition observed appears to be similar to that found with the trinitrophenyl derivatives of ADP and ATP (8).

Prior to the investigation reported here, attempts to identify the subunit location of the site on soluble CF_1 containing tightly bound ADP had failed. Earlier studies using arylazido derivatives of ADP and ATP resulted in the location of the MgATP at a site on or near the β polypeptide, and site 3 near an interface between both α and β subunits (7). Recently, the location of a site on membrane-bound CF_1 containing tightly bound ADP has been identified as the β polypeptide with 2-azidoadenosine diphosphate as the label (10) and as both the α and β polypeptides with BzATP as the label (11). In our experiments, however, use of the BzATP substrate analog has resulted in the labeling of all three well-characterized nucleotide binding sites on soluble purified CF_1 . Analysis of the data obtained after electrophoresis of the labeled denatured enzyme confirms previous results, with a different nucleotide analog, that binding of the analog at site 2 results predominantly in labeling of the β polypeptide (97%) and binding at site 3 results in equal labeling of α and β polypeptides. In addition, binding at site 1 results predominantly in labeling of the β polypeptide (86%) on soluble CF_1 , although some labeled α polypeptide is consistently labeled.

The simplest interpretation of these results is that all three nucleotide sites are located in a similar region of the enzyme. However, conformational differences result in different labeling patterns. The extreme case is site 3, where one-half of the radioactivity is found on the α polypeptide. Thus, all three nucleotide sites are probably near the interface of the α and β polypeptides, although predominantly on the β poly-

peptide. Whether the conformational differences are due to preexisting asymmetry in the structure of the enzyme or to ligand-induced asymmetry is not known. A similar dilemma exists in considering whether the mechanism of the enzyme involves alternating activity of equivalent nucleotide binding sites (cf. refs. 16 and 17) or a structural asymmetry in the function of the nucleotide binding sites that is retained during the catalytic cycle (cf. refs. 8 and 18).

The next step in defining the location of the nucleotide binding sites is to purify the individual radioactive peptides and to carry out amino acid analyses and protein sequencing.

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