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# Photoaffinity labeling with 2-azidoadenosine diphosphate of a tight nucleotide binding site on chloroplast coupling factor 1

(ATPase/adenine nucleotide analog)

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**ABSTRACT** **An analog of ADP containing an azido group at the C-2 position of the purine ring has been synthesized and used as an affinity probe of the membrane-bound coupling factor 1 of spinach chloroplast thylakoid membranes. The 2-azido-ADP inhibited light-induced dark binding of ADP at the tight nucleotide binding site on the thylakoid membranes. The 2-azido-ADP itself bound tightly to the thylakoid membranes, with 1 $\mu$M as the concentration giving 50% maximum binding. Tight binding of the analog required the thylakoid membranes to be energized, and the nucleotide remained bound after repeated washings of the membranes. The maximum extent of tight binding of the analog (1.2–1.3 nmol/mg of chlorophyll) was stoichiometric with the known coupling factor 1 content of thylakoid membranes but somewhat higher than that observed for ADP (0.5–0.9 nmol per mg of chlorophyll). Tight binding of 2-azido-ADP was decreased by the simultaneous addition of ADP. UV photolysis of washed thylakoid membranes containing tightly-bound 2-azido-[$\beta$-$^{32}$P]ADP resulted in the covalent incorporation of label into the membranes. Isolation of the chloroplast coupling factor 1 from these membranes followed by NaDodSO$_4$ gel electrophoresis demonstrated that the analog was covalently bound to the $\beta$ subunit of the coupling factor complex.**

Energy-transducing membranes from mitochondria, chloroplasts, and bacteria contain a proton-translocating coupling factor or ATPase. These coupling factors are comprised of an intrinsic membrane portion, $F_0$, which conducts protons, and an extrinsic complex, $F_1$, which catalyzes the ATP synthesis reaction (1–3). The $F_1$ coupling factors ($MF_1$, $CF_1$, and $BF_1$ from mitochondria, chloroplasts, and bacteria, respectively) are readily released from the membrane, and the soluble enzymes have been studied extensively. The $F_1$ ATPase consists of five types of subunits designated $\alpha$ through $\varepsilon$ in order of decreasing molecular weight. Although the stoichiometry of the subunits is still controversial, the two that are most frequently proposed are $\alpha_3\beta_3\gamma\delta\varepsilon$ and $\alpha_2\beta_2\gamma_{1-2}\delta_{1-2}\varepsilon_{1-2}$ (see ref. 4).

The soluble coupling factors have been found to contain tightly bound adenine nucleotides ($K_d < 10^{-10}$ M) that are not removed by charcoal treatment and do not readily exchange with medium nucleotides. Although there is variability among the many observations, one or two ADP and one or two ATP are usually found associated per $F_1$ (5). Although the function of these sites remains obscure, a variety of regulatory and catalytic roles have been proposed (6–9). The tightly bound nucleotides of membrane-bound coupling factors have been shown to exchange with medium nucleotides upon energizing the membrane (5). Illumination of chloroplast thylakoids induces the binding of up to 1 mol of medium ADP per mol of $CF_1$ (10). It has been proposed that ADP binding to this tight nucleotide site inhibits the ATPase activity of the membrane-bound $CF_1$ complex (11–13).

Several lines of evidence suggest that the nucleotide binding sites are located on the $\alpha$ and $\beta$ subunits of the $F_1$ complexes. Magnusson and McCarty (14) observed that extensive trypsin digestion of $CF_1$, which leaves only the $\alpha$ and $\beta$ subunits intact, resulted in the retention of bound nucleotides. In addition, by using purified individual subunits from bacterial coupling factors, nucleotides have been observed to bind only to the $\alpha$ and $\beta$ subunits (15, 16). The use of a variety of alkylating (17–19) and photoaffinity (20–27) analogs of ADP and ATP has resulted in the labeling of the $\alpha$ and $\beta$ subunits exclusively.

Although the location of the tight nucleotide binding sites remains to be determined, the catalytic site is generally considered to be located on the $\beta$ subunit (1–3). Using 3′-arylazido-ATP, Hammes and co-workers (23, 24) recently identified the location of a MgATP tight binding site on soluble spinach $CF_1$ as primarily the $\beta$ subunit. Their attempts to identify the nondissociable ADP site were unsuccessful.

The difficulty in the localization of the tight binding site stems largely from the strict specificity observed for these sites (28, 29) and the subsequent failure of modified nucleotides to bind to them. The 3′-aryl derivatives may cause difficulties in the interpretation of results in multisubunit enzymes because of the distance (≃10 Å) between the nucleotide and the photolabile moiety (20–25). The failure of 8-azido-ADP and -ATP derivatives to displace tightly bound nucleotides (22, 26, 27) is probably the result of the *syn* conformation induced by the 8-substitution (30) because circular dichroism studies on nucleotides bound to isolated subunits indicate that the bound nucleotides have an *anti* conformation (15). However, nucleotides substituted in the 2-position would be expected to retain an *anti* conformation because steric interactions would tend to disfavor the *syn* arrangement. Harris *et al.* (28) have found that isoguanosine (2-hydroxyadenosine) nucleotides are capable of binding to the tight sites of coupling factors. Similarly, 2-amino-ADP has been shown to be an excellent substrate for photophosphorylation (29). The ability of 2-substituted derivatives to obtain an *anti* conformation and participate in coupling factor activities suggests that 2-azido-adenosine derivatives should make excellent photoaffinity probes of the adenosine nucleotide binding sites of the coupling factors. In other systems, adenosine deaminase has been shown to be irreversibly inhibited by 2-azido-adenosine (31), and platelet membranes have been nonspecifically labeled with 2-azido-ADP (32). The specific, stoichiometric incorporation of 2-azido-AMP into the allosteric sites of fructose-1,6-bisphosphatase has been reported (33).

In this communication, it is shown that 2-azido-ADP was an effective inhibitor of ADP tight binding to chloroplast thylakoid

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Abbreviations: $CF_1$, chloroplast coupling factor 1; $K_{0.5}$, concentration giving 50% maximum binding.

membranes. In addition, 2-azido-ADP itself was bound tightly and was incorporated covalently into the membranes upon UV irradiation. Labeled 2-azido-ADP comigrated with the $\beta$ subunit of $CF_1$ isolated from irradiated membranes.

## MATERIALS AND METHODS

**Synthesis of 2-Azido-[$\beta$-$^{32}$P]ADP.** The 2-azido-ADP (Fig. 1) was prepared from 2-chloroadenosine (Sigma) by the methods of Schaefer and Thomas (34), Yoshikawa *et al.* (35), and Michelson (36). Purification was obtained with a DEAE-cellulose ($HCO_3^-$) column and a linear gradient of 0–0.4 M triethylammonium bicarbonate (pH 7.8). Analysis of the product by high-performance liquid chromatography showed that the product was at least 90% pure and contained no contaminating adenine nucleotides. The nucleotide was radioactively labeled by using the ADP–$P_i$ exchange reaction catalyzed by polynucleotide phosphorylase (37). A preliminary experiment indicated that 2-azido-ADP was a substrate for the exchange reaction at a rate ≈10% of that observed for ADP under identical conditions. A 0.5-ml solution containing 100 mM Tris (pH 8.7), 5 mM $MgCl_2$, 0.3 mM EDTA, 3.8 mM 2-azido-ADP, 2.5 mM $KH_2PO_4$, 5 mCi (1 Ci = 3.7 × $10^{10}$ becquerels) of $H_3{}^{32}PO_4$ (New England Nuclear), and 7.5 units of polynucleotide phosphorylase (P-L Biochemicals) was incubated at 25°C for 9 hr. The reaction was stopped by the addition of 0.1 ml of 10% (wt/vol) trichloroacetic acid. After centrifugation, the supernatant was extracted with Freon/tri-*n*-octylamine as described by Khym (38), diluted, and applied to a 0.5 × 4 cm column of DEAE-Sephadex A-25. After the column was washed with 3 column volumes of water, inorganic phosphate was eluted with 10 ml of 0.27 M triethylammonium bicarbonate (pH 7.8), and 2-azido-ADP was eluted with 15 ml of 0.4 M buffer. The peak fractions were pooled and repeatedly evaporated from methanol to remove excess volatile salts. The product had a specific activity of 250 cpm/pmol, contained <1% inorganic phosphate, and migrated as a single spot on thin-layer chromatography. In all experiments, the 2-azido-ADP was added as a methanolic solution of its triethylammonium salt. Final methanol concentrations were kept below 2.5% and had no detectable effect on control samples. An extinction coefficient of 15,500 $M^{-1}$ $cm^{-1}$ at 270 nm was used for the determination of all analog concentrations.

**Preparation of Chloroplasts.** Spinach chloroplast thylakoid membranes were prepared by using a procedure adapted from Strotmann *et al.* (39). Freshly harvested leaves were homogenized in 0.3 M sucrose/50 mM NaCl/1 mM $MgCl_2$/10 mM tricine·NaOH, pH 7.8. The homogenate was strained through two layers of nylon cloth. The thylakoid membranes were isolated by centrifugation at 3,000 × *g* for 4 min, resuspended in 10 mM NaCl/2 mM tricine·NaOH, pH 7.8, and collected by centrifugation at 12,000 × *g* for 2 min. The membranes were then washed in 50 mM NaCl/1 mM $MgCl_2$/2 mM tricine·NaOH, pH 7.8, collected by centrifugation at 3,000 × *g* for 2 min, and resuspended in a small amount of the same medium. All operations were performed at 0–4°C.

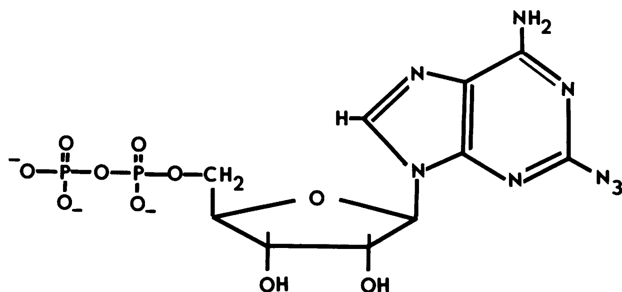


FIG. 1. The structure of 2-azido-ADP.

**Assay of Tightly Bound Nucleotides.** Thylakoid membranes (100 $\mu$g of chlorophyll) were suspended in 0.5 ml of 50 mM KCl/2 mM $MgCl_2$/25 mM tricine·KOH, pH 8.0/100 $\mu$M phenazine methosulfate/2 mM sodium ascorbate. The sample was stirred at 20°C during a 60-sec illumination period. After 10 sec in the dark, labeled adenine nucleotide was added. [$^3$H]ADP (200–400 cpm/pmol) was prepared by using [2,8-$^3$H]ADP (25–40 Ci/mmol, New England Nuclear); 2-azido-[$\beta$-$^{32}$P]ADP was prepared as described above. The reaction was quenched 60 sec later by the addition of unlabeled ADP and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to final concentrations of 10 mM and 5 $\mu$M, respectively. The thylakoid membranes were washed three times in 50 mM KCl/25 mM tricine·KOH, pH 8.0. A portion of the resuspended membranes was removed for a chlorophyll assay (40). Another aliquot was diluted 1:1 in 30% trichloroacetic acid to release the tightly-bound nucleotides. The precipitate was removed by centrifugation at 11,000 × *g*, and a sample of the supernatant was assayed for radioactivity in Aquasol (New England Nuclear) in a Packard 460C liquid scintillation counter. Dark controls, prepared in the same way, contained <5% of the labeled nucleotide found in the light-treated sample. These control values were subtracted from all sample values.

**Photolabeling of Membrane-Bound $CF_1$.** Thylakoid membranes (3 mg of chlorophyll) were suspended in 15 ml of the tight binding reaction medium. The sample temperature was maintained at 20°C during a 90-sec illumination period. The 2-azido-[$\beta$-$^{32}$P]ADP (11.2 $\mu$M) was added to the reaction after a 10-sec dark incubation, and the reaction was quenched 90 sec later with unlabeled ADP and CCCP as before. The membranes were washed and resuspended as described above and assayed for chlorophyll content and for tightly bound 2-azido-ADP. The chlorophyll concentration was then adjusted to 0.2 mg per ml with the wash medium, and the solution was placed in a flat-bottomed container to a depth of 2–3 mm. The sample was stirred on ice during a 10-min irradiation with a Mineralight model SL UV lamp (254 nm) equipped with a visible wavelength filter. After photolysis, covalent incorporation of 2-azido-ADP was measured as acid precipitable radioactivity. Samples were spotted on Whatman GF/C glass fiber filters and washed extensively with 10% trichloroacetic acid and with 95% ethanol. The filters were dried and counted in Aquasol.

**Isolation of Labeled $CF_1$.** The photolyzed membranes were washed five times with sodium pyrophosphate (pH 7.8) to remove ribulose-1,5-bisphosphate carboxylase, resuspended in 0.3 M sucrose/2 mM Tris/tricine, pH 7.8, and the solution was stirred at room temperature for 30 min to release $CF_1$ (41). The membranes were removed by centrifugation at 48,000 × *g* for 30 min at room temperature. The supernatant was concentrated by pressure dialysis on an Amicon PM-10 membrane to ≈1 ml. The resulting crude $CF_1$ was stored at 4°C as a precipitate in 50% saturated $(NH_4)_2SO_4$/0.5 mM EDTA/10 mM Tris sulfate, pH 7. After resuspending the precipitate in 50 mM tricine·NaOH (pH 8.0), the protein concentration of the crude $CF_1$ was determined by using the Lowry assay with bovine serum albumin as the standard.

**$NaDodSO_4$ Gel Electrophoresis.** A 7.5–15% polyacrylamide gradient slab gel with a 4% acrylamide stacking gel was prepared (42). Protein samples were dissolved in 3% $NaDodSO_4$/10% glycerol/62.5 mM Tris tricine, pH 6.8/0.005% bromphenol blue/5% mercaptoethanol and heated at 100°C for 2 min. Both low molecular weight standards from Bio-Rad and purified spinach $CF_1$ were used as molecular weight markers. After electrophoresis, the Coomassie brilliant blue (R250)-stained gel was

partitioned, and a portion was dried and subjected to autoradiography with X-Omat AR film (Kodak). To determine the quantitative distribution of label, the stained bands corresponding to the five coupling factor subunits were cut from the undried lanes and dissolved by incubation in 0.5 ml of $HClO_4$ (69–72%) and 0.3 ml of 30% $H_2O_2$ at 65°C overnight. The resulting samples were assayed for radioactivity in Aquasol.

## RESULTS

**Inhibition of Tight Binding of ADP by 2-Azido-ADP.** In order to measure tight binding of adenine nucleotides, labeled nucleotide was added in the dark to chloroplast thylakoid membranes that previously had been illuminated in the absence of nucleotides. After quenching with uncoupler and excess ADP, the membranes were washed repeatedly and assayed for tightly bound nucleotide. ADP was found to bind to a maximum extent of 0.56–0.86 nmol/mg of chlorophyll. The concentration required to yield 50% maximum binding ($K_{0.5}$) was 2 $\mu$M. These values are in good agreement with the extent and apparent $K_d$ commonly observed (10–13). The simultaneous addition of 2-azido-ADP and ADP lowered the observed extent of tight binding of ADP (Fig. 2).

**Light-Induced Tight Binding of 2-Azido-ADP.** 2-Azido-ADP was bound tightly to thylakoid membranes (Fig. 3). This binding was dependent on prior energizing of the membranes and was reversed upon reenergizing (results not shown). The $K_{0.5}$ for 2-azido-ADP binding was 1 $\mu$M, which is similar to the value observed for ADP binding. The maximum extent of analog binding was 1.2–1.3 nmol/mg of chlorophyll. The extent of analog tight binding was severely inhibited by simultaneous addition with ADP (Fig. 3).

The maximum extent of tight binding of 2-azido-ADP was consistently higher than that of ADP with a given thylakoid membrane preparation. Under the conditions of the experiment, the 2-azido-ADP analog was unreactive, and it is clear that tight binding did not involve a covalent chemical reaction between the analog and the membranes because the assay required the release of the label upon acid precipitation. A simple explanation for the apparent difference in binding is that the analog is able to bind to an additional site to which ADP cannot. However, in the experiment shown in Fig. 3, ADP (22 $\mu$M), which bound to a maximum level of 0.56 nmol/mg of chlorophyll (results not shown), displaced up to 0.79 nmol of 2-azido-ADP per mg of chlorophyll. This result suggests that ADP and 2-azido-ADP both interact with the same site(s) on the thylakoid membrane.

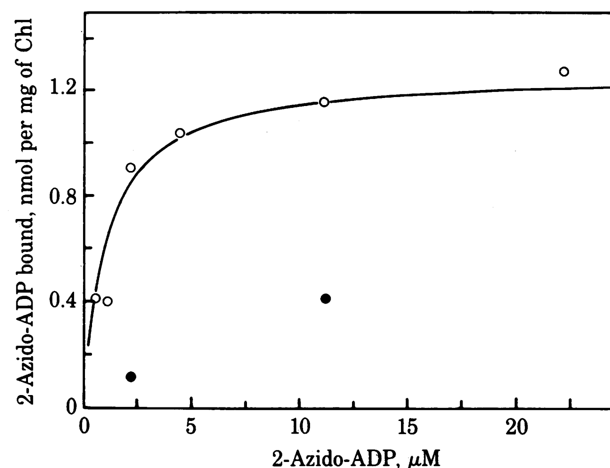


FIG. 3. The effect of ADP on light-induced tight binding of 2-azido-ADP to thylakoid membranes. Tight binding of 2-azido-[$\beta$-$^{32}$P]ADP to chlorophyll thylakoid membranes was carried out in the absence (○) or presence (●) of ADP (22 $\mu$M). Chl, chlorophyll.

Two other possibilities could account for the disparity in binding. (*i*) Tight binding of ADP induces strong negative cooperativity between two nucleotide binding sites on $CF_1$, whereas the analog does not induce cooperativity and is able to bind to both sites. (*ii*) Tightly bound ADP is more easily lost than tightly bound 2-azido-ADP during the assay procedure, resulting in unequal recoveries. The second explanation is favored by the observation that under these conditions, the level of 2-azido-ADP tight binding was approximately equal to the stoichiometry of the coupling factor on the thylakoids [1.3 nmol/mg of chlorophyll (41)]. The tight binding of ADP was always substoichiometric to the level of $CF_1$ and considerably more variable (ranging from 0.56 to 0.86 nmol/mg of chlorophyll) than that observed for 2-azido-ADP (1.2–1.3 nmol/mg of chlorophyll). These results are consistent with the loss of tightly bound ADP during the assay procedure.

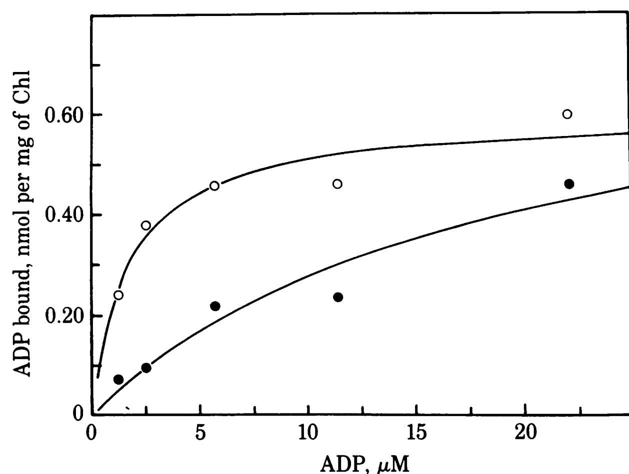


FIG. 2. The effect of 2-azido-ADP on light-induced tight binding of ADP to thylakoid membranes. Tight binding of [2,8-$^3$H]ADP to chloroplast thylakoid membranes was carried out in the absence (○) or presence (●) of 2-azido-ADP (4 $\mu$M). Chl, chlorophyll.

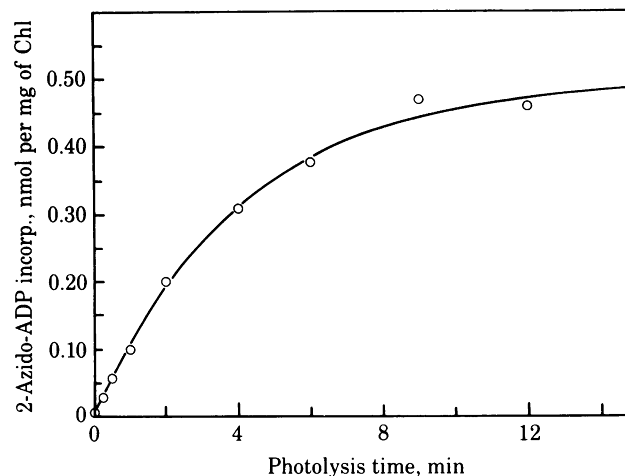


FIG. 4. Time course of covalent photoincorporation of 2-azido-ADP into thylakoid membranes. Thylakoid membranes containing tightly bound 2-azido-[$\beta$-$^{32}$P]ADP were prepared as for Fig. 3 and illuminated with UV light for various lengths of time. The covalent incorporation of 2-azido-[$\beta$-$^{32}$P]ADP was measured as acid precipitable radioactivity. Chl, chlorophyll; incorp., incorporated.

**Photoaffinity Labeling of Membrane-Bound $CF_1$.** Once bound to the tight binding sites, adenine nucleotides remain bound in the absence of medium nucleotides (5). The binding of 2-azido-ADP to the tight sites of thylakoid membranes provides an ideal situation for photoaffinity labeling because the photolysis may be performed after the removal of unbound analog. Because the only nucleotides present are those bound to the tight binding sites, no nonspecific labeling should occur. Two other advantages in this system are evident. (*i*) An assay of the amount of tightly bound analog allows one to determine the nucleotide content of the membranes prior to photolysis and provides the means to calculate the efficiency of the photoincorporation. (*ii*) Protection studies are unnecessary because the specificity of binding is determined prior to the photolysis (Fig. 3).

Thylakoid membranes containing 1.23 nmol of 2-azido-[$\beta$-$^{32}$P]ADP tightly bound per mg of chlorophyll ($\approx$0.95 mol of analog per mol of $CF_1$) were prepared. The washed membranes were irradiated for various lengths of time with UV light. Fig. 4 shows the time course of photoincorporation of 2-azido-[$\beta$-$^{32}$P]ADP into thylakoid membranes. The covalent incorporation of the analog (as measured by acid precipitable radioactivity) required UV light and followed an exponential path that would be expected for a first-order process. The maximum extent of photoincorporation was 0.5–0.6 nmol/mg of chlorophyll, which represents insertion of 40% of the bound analog into the membranes. Irradiation of thylakoid membranes containing tightly bound labeled ADP did not result in any detectable covalent incorporation of label (results not shown). Irradiation in the absence of 2-azido-ADP had no measurable effect on the extent of ADP binding measured subsequently (result not shown).

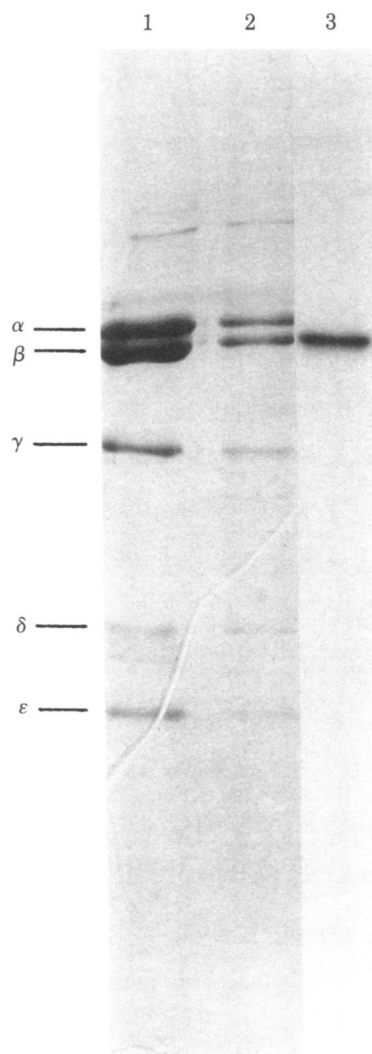


FIG. 5. $NaDodSO_4$/polyacrylamide gel electrophoresis of $CF_1$ photolabeled with 2-azido-[$\beta$-$^{32}$P]ADP. Washed thylakoid membranes containing tightly-bound 2-azido-[$\beta$-$^{32}$P]ADP were irradiated with UV light for 10 min. After isolation of the crude $CF_1$, gel electrophoresis and autoradiography were performed. Lanes: 1, purified spinach $CF_1$ (25 $\mu$g); 2, 2-azido-[$\beta$-$^{32}$P]ADP-labeled crude $CF_1$ (5 $\mu$g); 3, autoradiograph of lane 2.

Table 1. Distribution of 2-azido-[$\beta$-$^{32}$P]ADP covalently incorporated into subunits of isolated $CF_1$

| Lane | $CF_1$, $\mu$g | Radioactivity per subunit, cpm | | | | |
|---|---|---|---|---|---|---|
| | | $\alpha$ | $\beta$ | $\gamma$ | $\delta$ | $\varepsilon$ |
| 1 | 10 | 109 | 1,242 | 21 | 24 | 21 |
| 2 | 5 | 52 | 686 | 20 | 20 | 21 |

Crude $CF_1$ isolated from thylakoid membranes containing covalently incorporated azido-[$\beta$-$^{32}$P]ADP (145 cpm/pmol) was electrophoresed in a $NaDodSO_4$/polyacrylamide gel. The gel was sliced, and the bands corresponding to the $CF_1$ subunits were digested and assayed for radioactivity.

**Localization of the Covalently Incorporated 2-Azido-ADP.** The tight nucleotide binding sites on the thylakoid membranes are generally believed to be on the $CF_1$ complex (43). In order to identify which subunit of the $CF_1$ complex contained the photoincorporated 2-azido-ADP, thylakoid membranes containing 0.56 nmol of 2-azido-[$\beta$-$^{32}$P]ADP covalently incorporated per mg of chlorophyll were prepared as above. The labeled $CF_1$ was isolated and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis. In Fig. 5, lanes 1 and 2 show the Coomassie blue-stained protein bands from purified $CF_1$ and the crude $CF_1$ extract, respectively. The five bands associated with the $\alpha$, $\beta$, $\gamma$, $\delta$, and $\varepsilon$ subunits of $CF_1$ are clearly identifiable in the crude $CF_1$ of lane 2. Lane 3 shows the autoradiograph prepared from lane 2 and demonstrates that only the band corresonding to the $\beta$ subunit of $CF_1$ was labeled with $^{32}$P. These data were further corroborated by slicing and counting the bands from parallel lanes containing crude $CF_1$. These results, shown in Table 1, demonstrate that whereas less than 6% of the total radioactivity comigrated with the $\alpha$ subunit, more than 94% of the bound label comigrated with the $\beta$ subunit. This suggests that the tight ADP binding site is located on the $\beta$ subunit of the $CF_1$ complex.

## DISCUSSION

The existence of multiple adenine nucleotide binding sites has complicated the study of the $F_1$ ATPases. The tight nucleotide binding sites in particular have resisted study with analogs due to the strict structural requirements for binding (5, 28, 29). In this study we observed the ability of 2-azido-ADP to compete for and bind to the tight ADP binding site(s) on thylakoid membranes. This result is consistent with studies on the specificity of coupling factor functions (5, 28, 29). The tight binding of 2-azido-ADP to the thylakoid membranes was similar to the tight binding of ADP in the following ways. (*i*) No substantial tight binding occurred without energizing the membranes. (*ii*) Prior energizing of the membranes resulted in the subsequent dark binding of nucleoside diphosphate in amounts approaching a 1:1 stoichiometry with the $CF_1$ content of the membranes. (*iii*) The apparent $K_{0.5}$ for binding of the nucleoside diphosphates was 1–2 $\mu$M. (*iv*) Once tightly bound, the nucleotide did not dissociate upon repetitive washings nor did it exchange with medium nucleotides. In addition, 2-azido-ADP and ADP each

were found to inhibit the binding of the other at micromolar concentrations. Together these results suggest that ADP and 2-azido-ADP bind to the same tight binding site(s) on the thylakoid membranes.

The most interesting observation of this study is that UV irradiation of thylakoid membranes containing tightly bound 2-azido-[$\beta$-$^{32}$P]ADP caused covalent incorporation of label into the $\beta$ subunit of the $CF_1$ complex. This result implies that the tight ADP binding site(s) of the $CF_1$ is (are) located on the $\beta$ subunit. The appearance of a small amount of label in the $\alpha$ subunit may prove to be significant in view of suggestions of nucleotide binding sites on the $\alpha$ subunits (16–19, 25, 26) or at the interface between the $\alpha$ and $\beta$ subunits (20, 21, 25). Hammes and co-workers (23, 24) have localized a tight MgATP binding site on the $\beta$ subunit of the soluble $CF_1$ ATPase near its interface with the $\alpha$ subunit. Because the removal of unbound analog prior to the UV irradiation diminishes the possibility of nonspecific labeling, it is possible that tight nucleotide binding sites exist on the $\alpha$ subunit that are not effectively loaded or labeled under the conditions used in this study.

The identification of the $\beta$ subunit of the $CF_1$ as the primary binding site of the tightly bound ADP, the tightly bound MgATP (24), and the catalytic site (see refs. 1–3) suggests direct mechanistic interactions. Although the tightly bound nucleotides are generally considered not to be catalytically competent (2, 3, 44), their exact roles in the synthesis and hydrolysis of ATP remain to be firmly established. In view of suggestions of alternate site cooperativity in catalysis (see ref. 44) and considering that the observed levels of tightly bound ADP (10–13) and tightly bound MgATP (23, 24) are stoichiometric with the $CF_1$ complex rather than with the $\beta$ subunit content, tight nucleotide binding and catalysis may be mutually exclusive events of a $\beta$ subunit in the coupling factor complex.

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