Characterization of nucleotide-binding sites on the chloroplast coupling factor 1 using two photolabile analogs

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Nucleotide-binding sites on the chloroplast coupling factor 1 (CF_1) have been probed using two photoreactive ADP analogs: 2-azido-ADP (2-N_3-ADP) and 2′,3′-*O*-(4-benzoyl)benzoyl-ADP (Bz-ADP). Photolabeling of the isolated CF_1 with 2-N_3-ADP results in incorporation of the analog exclusively into the β-subunit of the enzyme. The location of the nucleotide-binding site(s) within the β-subunit of the CF_1 was investigated using peptide mapping. Within the discrimination limits of this technique, it is concluded that the azido- and benzoyl-modified analogs both bind to the same conformation of the nucleotide-binding site(s) of soluble CF_1. Bz-ADP, however, labels the binding site(s) on membrane-bound CF_1 in a slightly different manner.

Coupling factor *ATPase* *Photoaffinity analog* *Chloroplast* *Azido-ADP* *2′,3′-O-(4-Benzoyl)benzoyl-ADP*

1. INTRODUCTION

The CF_1 of chloroplast thylakoid membranes contains one or more tightly bound adenine nucleotides [1–3]. These nucleotides are predominantly in the form of ADP although small amounts of tightly bound ATP are also observed. In the de-energized state, the tightly bound nucleotides are essentially non-exchangeable (K_{app} < 1 nM). Upon energization of the thylakoid membrane, however, the nucleotides become loosely bound (K_{app} = 2–5 μM) and exchangeable with nucleotides from the medium. The soluble coupling factor binds a total of three adenine nucleotides (ADP + ATP) per molecule of CF_1. At least two species of bound ADP have been described, and in the presence of Mg^{2+} and ATP, a single molecule of Mg-ATP is bound very tightly. It is not yet clear how these sites may relate to or interact with the catalytic site for ATP synthesis and hydrolysis, or to the tight nucleotide-binding sites on the membrane-bound CF_1.

Experiments using photoreactive nucleotide analogs have identified which subunits of the coupling factor interact with ADP and ATP [3–8]. Using 2-N_3-ADP, it has been observed that both tightly bound ADP and newly synthesized, tightly bound ATP are located on the β-subunit of the thylakoid-bound enzyme [5,6]. Peptide mapping of the photolabeled β-subunits demonstrated that the same polypeptide regions of the β-subunit interact with the base portions of both the tightly

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Abbreviations: CF_1, chloroplast coupling factor 1; 2-N_3-ADP or 2-N_3-ATP, 2-azidoadenosine 5′-diphosphate or -triphosphate; Bz-ADP or Bz-ATP, 2′,3′-*O*-(4-benzoyl)benzoyladenosine 5′-diphosphate or -triphosphate; octylglucoside, *n*-octyl β-D-glucopyranoside; LDS, lithium dodecyl sulfate; chl, chlorophyll; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DTT, dithiothreitol

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bound ADP and ATP [7]. In contrast, the α- and β-subunits of the thylakoid-bound CF_1 are both labeled by tightly bound Bz-ADP [8]. Isolated CF_1 also displays differences in labeling, depending upon which analog is used [3,4,8,9].

2. MATERIALS AND METHODS

2-N_3-ADP was prepared from 2-chloroadenosine as described by Czarnecki [10]. The compound was labeled with $^{32}P_i$ using the NDP-P_i exchange reaction catalyzed by polynucleotide phosphorylase. Bz-ADP was synthesized, using either [β-^{32}P]ADP (prepared from ADP and $^{32}P_i$ by NDP-P_i exchange as above) or [8-^{14}C]ADP [7].

The chloroplast coupling factor was released from spinach thylakoid membranes [11], and the enzyme purified by chromatography on DEAE-Sephadex in buffered solutions containing $(NH_4)_2SO_4$ (100–300 mM). The concentration of the purified, desalted protein was determined using the Lowry assay with BSA as a standard. Octylglucoside-dependent Mg^{2+}-ATPase assays were performed as described by Pick and Bassilian [12].

The soluble CF_1 was photolabeled in reaction mixtures containing 40 mM Tricine (pH 8.0), 2 mM EDTA, 0.5 mg CF_1/ml. The nucleotide analog was added and the mixture incubated in the dark for 30 min at ambient temperature. Samples were irradiated with ultraviolet light using either the Mineralight UVSL-25 (for 2-N_3-ADP) or the Blak-Ray (for Bz-ADP).

Thylakoid membranes were prepared from market spinach [11] and suspended (0.3 mg chl/ml) in a reaction mixture containing 20 mM Tricine (pH 8.0), 50 mM KCl, 8 mM $MgCl_2$, 0.1 mM phenazine methosulfate, 10 mM DTT. The samples were illuminated for 90 s and the photoaffinity analog added in the dark to a final concentration of 10 μM. The samples were stirred for 90 s and the binding reaction quenched by the addition of the uncoupler CCCP (11.6 μM final concentration). The membranes were washed, resuspended (0.2 mg chl/ml), and irradiated on ice with UV light.

Covalent incorporation of the analogs was quantified by liquid scintillation counting of acid-precipitated samples. Electrophoresis was performed according to Laemmli, except that SDS was replaced by LDS, and electrophoresis was carried out at 4°C. Partial proteolytic digestion and electrophoresis of the β-subunit was carried out as described by Cleveland et al. [13] with LDS substituted for SDS.

3. RESULTS

The results shown in fig.1 indicate that, as with Bz-ADP, only the β-subunit of the soluble CF_1 is photolabeled by 2-N_3-ADP. The extent of covalent incorporation in the experiment shown was 1.27 mol 2-N_3-ADP per mol CF_1. Even at higher ratios of incorporation, only the β-subunit was photolabeled (not shown). To determine the effect of 2-N_3-ADP modification on ATPase activity,

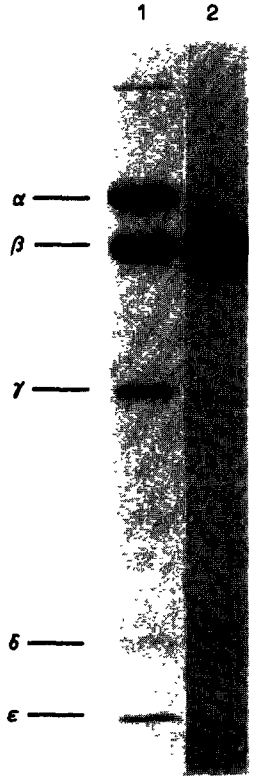

Fig.1. Photolabeling of soluble CF_1 by 2-N_3-ADP. Purified CF_1 was incubated with 2-N_3-[β-^{32}P]ADP (11.8 μM), then irradiated with UV light and analyzed by gel electrophoresis as described in section 2. Lanes: 1, Coomassie stained, photolabeled CF_1; 2, autoradiogram of lane 1.

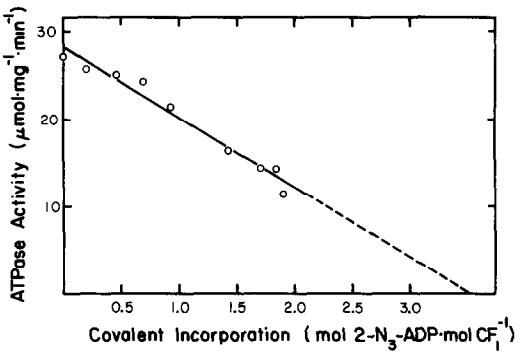

Fig.2. ATPase activity of CF_1 photolabeled with 2-N_3-ADP. Purified CF_1 was incubated with 14.3 μM 2-N_3-[β-^{32}P]ADP. The sample was irradiated with UV light and aliquots were removed during the irradiation to obtain a range of levels of photoincorporation. The extent of covalent incorporation of the analog and the Mg^{2+}-ATPase activity were assayed as described in section 2.

samples of CF_1 containing various amounts of covalently incorporated 2-N_3-[β-^{32}P]ADP were prepared by varying the time of irradiation. The extent of photoincorporation was determined and the ATPase activity measured for each sample. The results of such experiments (fig.2) indicate: (i) that covalent incorporation of 2-N_3-ADP into the β-subunit of the soluble CF_1 inhibits the catalytic activity of the enzyme; and (ii) that complete inactivation of ATPase activity extrapolates to slightly more than 3 mol 2-N_3-ADP per mol CF_1. These results are similar to those obtained with the benzoyl-modified analog [8], although the extent of incorporation required for complete inhibition is somewhat higher with the azido-modified analog.

To determine whether the two analogs bind to the same portion of the β-subunit, we have used the peptide mapping technique of Cleveland et al. [13]. The protease V8 from *S. aureus*, which hydrolyzes peptide bonds adjacent to aspartate and glutamate residues, produces a specific and reproducible digestion pattern from the CF_1 β-subunit. Panel A of fig.3 shows the protein stained electrophoretic pattern from digestions of isolated CF_1 β-subunits using increasing amounts of protease. Two major polypeptides (~32 and 27 kDa) are produced by digestion with 0.05 μg protease (lane 2). The 32 kDa polypeptide is relatively susceptible to proteolytic degradation while the 27 kDa species is only slowly digested by increasing amounts of protease (lanes 3,4). (Incorporation of photoaffinity analogs into the β-subunit does not affect the electrophoretic pattern of pro-

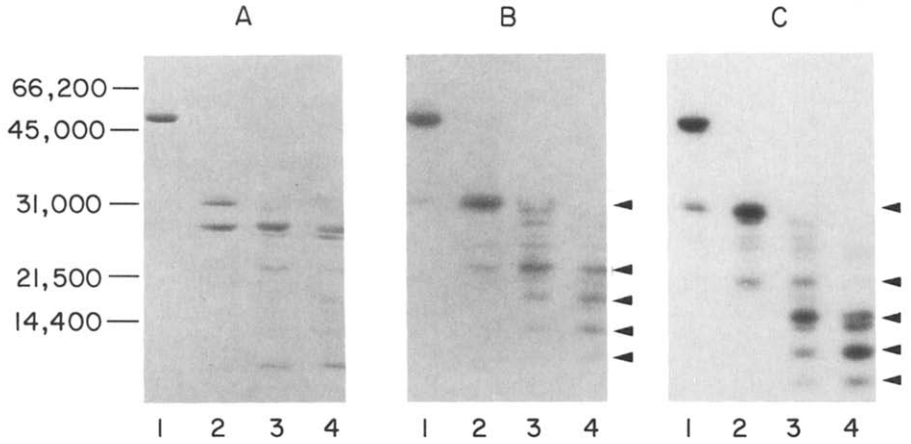

Fig.3. Partial proteolysis of the β-subunit of photolabeled, soluble coupling factors. Soluble CF_1 was photolabeled with either 2-N_3-ADP or Bz-ADP. The extents of covalent incorporation of the ADP analogs were 1.5 and 0.89 mol/mol CF_1, respectively. The subunits were then separated by LDS-PAGE. Slices containing the β-subunit placed in the stacking wells of a second gel and protease V8 (*S. aureus*) were added – 0, 0.05, 0.5 and 2.5 μg protease in lanes 1–4, respectively. Proteolysis and LDS-PAGE were performed as described [13]. (A) Coomassie-stained proteolysis pattern of unlabeled CF_1 β; (B) autoradiogram of proteolysis pattern of 2-N_3-[β-^{32}P]ADP-labeled CF_1 β; (C) autoradiogram of proteolysis pattern of Bz-[β-^{32}P]ADP-labeled CF_1 β. Wedges indicate the most heavily labeled bands.

teolytic fragments obtained; not shown.) The polypeptide distribution of the covalently incorporated photoaffinity analogs is shown in fig.3, panel B (2-N_3-[β-^{32}P]ADP) and fig.3, panel C (Bz-[β-^{32}P]ADP). A comparison of panels B and C indicates that the two patterns are essentially identical. In both cases, the label is found primarily in the protease-sensitive 32 kDa polypeptide (lanes B2,C2). With increasing amounts of protease the label is found in major polypeptides of apparent molecular mass ~22, 17–18 (doublet), 14.5 kDa and then smaller fragments which migrate behind the dye front (lanes B3, C3, B4, C4). This pattern is indistinguishable from that obtained with thylakoid-bound CF_1 photolabeled with either 2-N_3-ADP or 2-N_3-ATP [7].

Fig.4 shows a composite of the Coomassie-stained and photolabeled polypeptide patterns of the β-subunits from the thylakoid-bound CF_1 labeled with Bz-[β-^{32}P]ADP. The protein digestion pattern (lanes 1, 3, 5, 7) and the labeling pattern (lanes 2, 4, 6, 8) appear to be nearly identical to those observed with the isolated enzyme (fig.3). However, one important change in labeling between the membrane-bound enzyme and that in solution is seen. The fragment of ~14.5 kDa, though clearly produced (lane 7), is not labeled (lane 8, arrow).

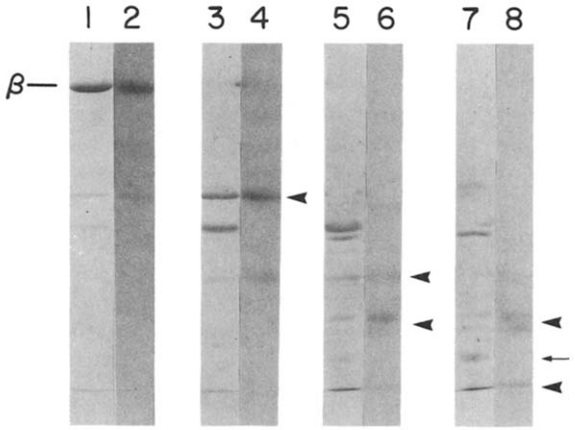

Fig.4. Partial proteolysis of the β-subunit of the membrane-bound CF_1 photolabeled with Bz-ADP. Thylakoid membranes were labeled with tightly bound Bz-[β-^{32}P]ADP as previously described. The extent of covalent incorporation was 0.36 nmol Bz-ADP/mg chl. Proteolytic digestion was carried out as described in fig.3. Lanes: 1, 3, 5, 7, Coomassie-stained proteolysis pattern; 2, 4, 6, 8, autoradiograms of preceding lanes, respectively; 1, 2, no protease added; 3, 4, 0.05 μg protease; 5, 6, 0.5 μg protease; 7, 8, 2.5 μg protease.

4. DISCUSSION

Despite differences in the chemical properties of the photoreactive moieties of 2-N_3-ADP and Bz-ADP, we have found that these analogs interact quite similarly with purified CF_1. Both compounds covalently label only the β-subunit and the complete inactivation of ATPase activity requires incorporation of approx. 3 mol analog per mol CF_1. Since there are three β-subunits per CF_1, these results suggest that each β-subunit contains a nucleotide-binding site which inactivates ATPase activity when modified in either its base-binding or ribose-binding portion. The identical photolabeling patterns of the proteolytic fragments from Bz-ADP-labeled and 2-N_3-ADP-labeled β-subunits indicate that both the base-binding and ribose-binding portions of the nucleotide-binding site on the soluble enzyme are located on a portion of the β-subunit with apparent molecular mass greater than 14.5 kDa.

The pattern of labeling of the thylakoid-bound CF_1 β-subunit by Bz-ADP is also quite similar to that observed for the isolated enzyme. However, the absence of label in one of the polypeptide fragments (~14.5 kDa), which in all other cases is heavily labeled, suggests that the ribose-binding portion of the β-subunit has a somewhat different conformation when the CF_1 is associated with the membrane.

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