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Photoaffinity labeling of the tight ADP binding site of the chloroplast coupling factor one (CF_1): the effect on the CF_1-ATPase activity

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Chloroplast thylakoid membranes contain tightly bound ADP which is intimately involved in the mechanism of photophosphorylation. The photoaffinity analog 2-azido-ADP binds tightly to spinach thylakoid membrane-bound coupling factor one (CF_1) and, in a manner similar to ADP, inhibits the light-triggered ATPase activity (Czarnecki, J.J., Abbott, M.S. and Selman, B.R. (1983) Eur. J. Biochem. 136, 19–24). Ultraviolet irradiation of thylakoid membranes containing noncovalently, tightly bound 2-azido[β-^{32}P]ADP results in the inactivation of both the methanol-stimulated MgATPase activity of the membrane-bound CF_1 and the octylglucoside-dependent MgATPase activity of the solubilized enzyme. There is a linear correlation between the loss of enzyme activity and the covalent incorporation of the photoaffinity analog. Full inactivation of catalytic activity is estimated to occur upon incorporation of 1.07 mol analog and 0.65 mol analog per mol enzyme for the methanol- and octylglucoside-stimulated activities, respectively. Since 2-azido-ADP modifies only the β subunit of the CF_1 and since there are probably three β subunits per CF_1, these results indicate strong cooperativity among β subunits and between the site of tightly bound nucleotides and the catalytic sites.

Introduction

It has been well established that the F_1 coupling factor ATPases contain tightly bound ADP and ATP, whose functions in the mechanism of ATP synthesis and hydrolysis remain to be established. Illumination of chloroplast thylakoid membranes results in the release of tightly bound nucleotides whereas rebinding occurs in the dark [1–5]. An inverse correlation has been observed between the levels of tightly bound nucleotides and the rates of ATP synthesis and hydrolysis [3–7]. Additionally, kinetic correlations have been made between the release of tightly bound ADP and the activation of catalytic activity [8,9]. Although tightly bound ADP appears not to be the initial phosphoryl acceptor [10], tightly bound nucleotides have been proposed to be kinetically competent and to act as intermediates in the mechanism of ATP synthesis [11,12]. Other workers, however, have proposed that the tightly bound nucleotides are kinetically incompetent and are bound at sites different from the catalytic site [1,5,13,14]. It is possible that more than one type of tight site exists [15].

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Abbreviations: CF_1, chloroplast coupling factor 1; Chl, chlorophyll; 2-azido-ADP or 2-N_3-ADP, 2-azido-adenosine 5′-diphosphate; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; 8-N_3-ADP, 8-azido-adenosine 5′-diphosphate; Bz-ADP, 3′-*O*-(4-benzoyl)benzoyl-adenosine 5′-diphosphate; 3′-arylazido-ADP, 3′-*O*-[3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl]adenosine 5′-diphosphate; enzymes: ATPase, CF_1, E.C. 3.6.1.3.

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Recently, we have localized the site of binding for tightly bound ADP on the β subunit of the CF_1 complex by the utilization of the photoaffinity analog 2-N_3-ADP [16,17]. The noncovalent binding of 2-N_3-ADP to the thylakoid membranes correlates with the inhibition of the light-activated ATPase activity [17]. In order to understand more fully the role of the tightly bound ADP, we have examined the effect of the covalent incorporation of the photoaffinity analog on the catalytic activity of the CF_1 complex.

Materials and Methods

The 2-azido-ADP was prepared and radioactively labeled as described previously [17]. Determinations of analog-specific activities and concentrations were based upon assay with pyruvate kinase coupled to NADH oxidation by lactate dehydrogenase [18]. Aqueous stock solutions of the analog were incubated with 0.1 M HCl for 45 min and then neutralized with NaOH just prior to use [18].

Thylakoid membranes were prepared as described previously from market spinach [3]. Chlorophyll concentrations were determined using the method of Arnon [19]. Photoaffinity labeling with 2-N_3-[β-^{32}P]ADP was performed as previously described [17]. It has previously been demonstrated that, under these conditions, the tightly bound 2-N_3-[β-^{32}P]ADP covalently labels only the β subunit of the CF_1 [16,17]. At various time points during the labeling, samples were removed and aliquots were assayed for the covalent incorporation of the analog. Samples were acid precipitated on Whatman GF/F glass fiber filters and washed extensively with 10% trichloroacetic acid, 5% trichloroacetic acid, and then ethanol. The filters were placed in Aquasol (New England Nuclear) and counted by liquid scintillation. The remaining photolyzed membranes were washed once and either assayed for methanol-activated ATPase activity [20] or resuspended in 0.3 M sucrose containing 2 mM Tris-Tricine (pH 7.8) in order to release the coupling factor from the membranes [21]. The solubilized coupling factor was assayed for octylglucoside-activated ATPase activity [22]. The concentration of CF_1 in the low salt extracts was determined using an immunoelectrophoretic assay [23]. The amount of solubilized CF_1 containing covalently incorporated analog was determined by acid precipitation on filters as described above with the addition of 20 μg carrier protein (bovine serum albumin).

The octylglucoside-dependent Mg^{2+}-ATPase of the solubilized CF_1 was assayed using the method of Pick and Bassilian [22]. In a total volume of 0.1 ml, samples containing 0.6 μg CF_1/40 mM octylglucoside/ 4 mM [γ-^{32}P]ATP ($1.7 \cdot 10^6$ cpm/μmol)/1.2 mM $MgCl_2$/0.1 mM EDTA/30 mM Tricine (pH 8.0) were incubated at 37° for 2 min. The reaction was stopped by the addition of 2 ml of a solution containing 1% ammonium molybdate and 0.8 M $HClO_4$. Following the addition of carrier K_2HPO_4 (4 μmol), the inorganic phosphate was extracted with 2 ml of a solution of isobutanol and benzene (1 : 1, v/v), and 1 ml was counted by liquid scintillation.

The methanol-activated Mg^{2+}-ATPase activity of the thylakoid membranes was assayed essentially as described by Anthon and Jagendorf [20]. The irradiated thylakoids (10 μg Chl) were incubated in 25 mM Bicine (pH 8.8)/25 mM NaCl/2.5 mM $MgCl_2$/34% (v/v) methanol for 30 s at 37°C. [γ-^{32}P]ATP [$(1-3) \cdot 10^6$ cpm/μmol] was added to a final concentration of 5.0 mM (final volume, 0.5 ml), and the reaction was allowed to proceed for 2 min. The reaction was terminated, and the samples treated as described above for the octylglucoside-dependent assay.

Results

2-Azido-adenine nucleotides require short wavelength (less than 300 nm) ultraviolet light in order to activate the reactive nitrene [18]. Irradiation of thylakoid membranes with low-intensity 254 nm ultraviolet light, however, results in a large decrease in their photophosphorylation activity (data not shown). This ultraviolet-induced damage appears to occur primarily to components involved in light harvesting and/or electron transfer rather than the coupling factor complex per se (as measured by the ATPase activity, see below) and precluded an accurate assessment on the impact of the specific covalent modification of the CF_1 complex on photophosphorylation or the light-induced ATPase activities. In order to circumvent this

problem, the effect of the covalent modification of CF_1 on the activity of the enzyme was studied by measuring the ATPase activity of the latent enzyme after activation. Activation of the Mg^{2+}-specific ATPase activity was achieved by methanol stimulation of the thylakoid-bound enzyme [20,24–26] or octylglucoside stimulation of the solubilized enzyme [22,27].

The effect of covalent modification on the membrane-bound Mg^{2+}-ATPase activity

Following energization of the thylakoid membranes, 2-N_3-[β-^{32}P]ADP or ADP was allowed to bind to the CF_1 in the dark. The washed thylakoids, containing either tightly bound 2-N_3-[β-^{32}P]ADP (1.42 nmol/mg Chl) or ADP, were irradiated with ultraviolet light for varying lengths of time to covalently incorporate different amounts of 2-N_3-[β-^{32}P]ADP into the CF_1. Under the conditions of the irradiation, covalent incorporation of ADP was not detectable and there was less than a 12% loss of tightly bound ADP. The specificity of the photolabeling is ensured by the high affinity of tight binding ($K_d < 10^{-10}$ M), the specificity of the tight nucleotide binding sites [28], the absence of medium nucleotides during the irradiation, and the mutual competition of ADP and 2-N_3-ADP for the tight nucleotide binding sites [16,17].

As shown in Fig. 1A and previously [16,17] the covalent incorporation of the photoaffinity analog into the thylakoid membranes increased with the time of photolysis, and the label was associated exclusively with the coupling factor complex [17]. Ultraviolet irradiation of control membranes, containing tightly bound ADP, resulted in only small decreases in their methanol-activated, Mg^{2+}-ATPase activity. In contrast, the ATPase activity of membranes containing tightly bound 2-N_3-[β-^{32}P]ADP was rapidly inactivated upon ultraviolet irradiation. The time course of the photoinactivation corresponded to the appearance of covalently bound analog, suggesting that the inactivation of the ATPase activity was the result of the covalent incorporation of the photoaffinity analog into its binding site.

As shown in Fig. 1B, there is a linear correlation between the extent of covalently bound analog and the loss of the methanol-activated Mg^{2+}-ATPase activity. Although a maximum of 40–55% of the tightly bound 2-N_3-ADP was observed to be covalently incorporated (Figs. 1 and 2; Refs. 16 and 17), an extrapolation reveals that the methanol-activated ATPase activity would have been fully inactivated upon the incorporation of 1.07 mol of analog per mol CF_1 complex.

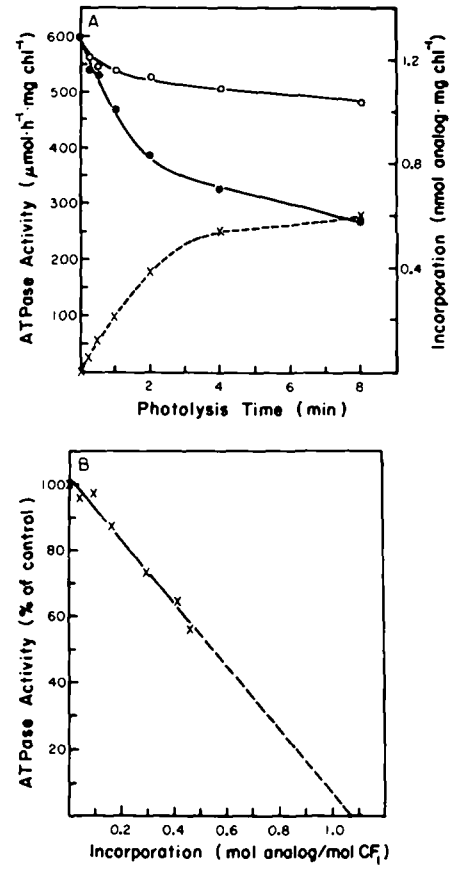

Fig. 1. Photoincorporation of tightly membrane-bound 2-azido-ADP: inactivation of the methanol-stimulated MgATPase activity of the membrane-bound CF_1. (A) Time-course of ultraviolet irradiation. Washed thylakoid membranes, containing tightly bound ADP (○) or 2-azido-[β-^{32}P]ADP (1.42 nmol/mg Chl) (●), were prepared and ultraviolet irradiated for the times indicated as described in Materials and Methods. The methanol-activated ATPase activities (○, ●) and covalent incorporation of the analog (×) were determined as described in Materials and Methods. (B) Stoichiometry of inactivation versus covalent incorporation. The data of (A) were replotted. The CF_1 content is based upon an estimation of 1.3 nmol CF_1/mg Chl [21]. The percent activity is expressed relative to the activity of the control membranes (containing tightly bound ADP) irradiated for an equivalent amount of time to account for nonspecific ultraviolet damage.

The effect of photoincorporation of 2-N_3-ADP on the octylglucoside-activated Mg^{2+}-ATPase activity of the soluble CF_1

In order to examine the effect of the covalent modification on the solubilized enzyme, chloroplast thylakoid membranes, containing either tightly bound 2-N_3-[β-^{32}P]ADP (1.48 nmol/mg Chl) or ADP were prepared and ultraviolet irradiated as above. The irradiated membranes were subsequently stirred in a low ionic strength medium to release the coupling factor complex, and the soluble extract was analyzed for CF_1 content, covalently incorporated analog, and octylglucoside-activated, Mg^{2+}-ATPase activity.

As shown in Fig. 2A, the proportion of the soluble CF_1-ATPase containing photoincorporated analog appeared to be 30–50% less than that of the membrane-bound CF_1 when based upon an estimate of 0.4 mg CF_1 per mg chlorophyll [21,23]. This suggests that either the modified CF_1 is retained preferentially during the low salt CF_1 extraction procedure or that the extent of photoincorporation into the soluble CF_1-ATPase was underestimated.

The effect of the ultraviolet treatment on the octylglucoside-dependent ATPase activity is shown in Fig. 2B. Only a small decrease of the ATPase activity was observed for CF_1 isolated from ultraviolet-irradiated control membranes containing tightly bound ADP. In contrast, soluble CF_1, isolated from irradiated thylakoid membranes that contained tightly bound 2-N_3-[β-^{32}P]ADP, had significantly decreased octylglucoside ATPase activity, in proportion to the extent of covalently incorporated analog. A replot of the data (Fig. 2C) indicates that there was a linear correlation between the covalent incorporation of the analog and the loss of the ATPase activity. In addition, an extrapolation of the data shows that total inactivation of the ATPase activity occurs upon the covalent incorporation of approx. 0.65 mol of analog per mol CF_1 complex. Since it is unlikely that the photoincorporation of the analog can affect more than one soluble CF_1 complex, these results suggest that covalent incorporation of 1 mol 2-N_3-ADP per mol CF_1 completely inactivates the ATPase activity of the solubilized coupling factor. Together with the data obtained from the methanol-activated, membrane-bound ATPase, these results indicate that covalent modification of

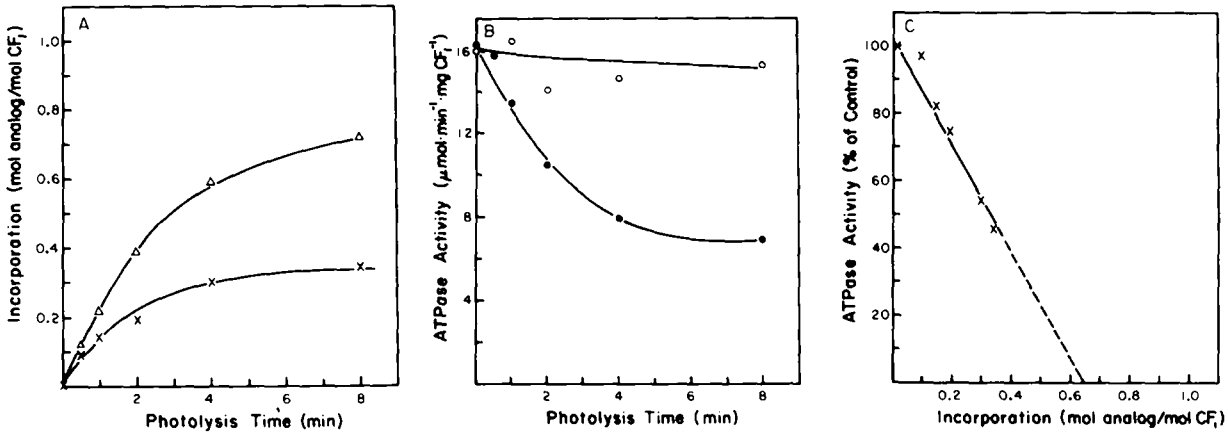

Fig. 2. Photoincorporation of tightly membrane-bound 2-azido-ADP: inactivation of the octylglucoside-dependent MgATPase activity of the soluble CF_1. Washed thylakoid membranes containing tightly bound ADP or 2-azido-[β-^{32}P]ADP (1.48 nmol/mg Chl) were prepared and ultraviolet irradiated as described in Materials and Methods for the times indicated. (A) Time-course of photoincorporation. The covalent incorporation of the analog was determined as described in Materials and Methods before ($\triangle$) and after ($\times$) the isolation of the CF_1 complex. (B) Time-course of photoinactivation. CF_1 was isolated from irradiated membranes which contained tightly bound ADP (O) or 2-azido-[β-^{32}P]ADP (●). The octylglucoside-activated MgATPase activities and CF_1 concentrations were determined as described in Materials and Methods. (C) Stoichiometry of inactivation vs. covalent incorporation. The data of (A) and (B) were replotted. The CF_1 content is based upon an estimated molecular weight of 400000 [34]. The activity is expressed relative to the activity of the control (CF_1 isolated from membranes containing tightly bound ADP which were irradiated for an equivalent amount of time) to account for nonspecific ultraviolet damage.

a single β subunit at the site of tightly bound ADP results in the complete inhibition of the CF_1 complex.

Discussion

A large number of studies have been published using nucleotide analogs as affinity labels to study the coupling factors (see Ref. 29). Most of these studies have focused on low affinity sites of the solubilized enzyme. Under these conditions, binding is freely reversible, and previous studies have been complicated by both non-specific and multi-subunit labeling and uncertainty in terms of the occupancy of nucleotide binding sites. For instance, Wagenvoord et al. [30] have observed that 8-N_3-ADP, which does not bind to the tight nucleotide binding sites, inactivated the soluble CF_1-ATPase upon photoincorporation of 2 or 4 mol (in the presence of Ca^{2+}) per mol enzyme. Similarly, Bar-Zvi and Shavit [31] recently observed inactivation of the soluble lettuce CF_1-ATPase upon photoincorporation of 2.45 mol 3′-*O*-(4-benzoyl)benzoyl-ADP (Bz-ADP) into the β subunit, but it is not known if the tight nucleotide binding sites on the soluble CF_1 were modified under the conditions used. Whereas the Bz-ADP was observed by Bar-Zvi et al. [32] to compete for the tight ADP binding sites on the membrane-bound coupling factor, it stabilized rather than inhibited the light-triggered ATPase activity, photolabeled both the α and β subunits, and the effects of the photoincorporation of tightly membrane-bound Bz-ADP on the CF_1-ATPase activity were not examined. Carlier et al. [33], using a 3′-arylazido-ADP derivative, observed 70% inactivation of the Ca^{2+}-ATPase upon the incorporation of 0.4 mol/mol enzyme into the α and β subunits. In a later work, Bruist and Hammes [15] employed 2′-arylazido-ATP to localize a MgATP tight binding site on the β subunit of the soluble CF_1 but no definitive effects on the activity of the enzyme were reported. Additionally, their attempts to label the site of a non-dissociable ADP on the soluble coupling factor were unsuccessful.

The work presented here is unique in that it represents observations of a single type of high affinity site which has been extensively studied in terms of its nucleotide binding characteristics and its interactions with events revolving around photophosphorylation. Photoaffinity labeling under conditions where the ligand does not dissociate from its binding site ensures the specificity of the site of covalent incorporation.

We have previously demonstrated that 2-N_3-ADP binds tightly to thylakoid membranes at the site of ADP tight binding and labels the β subunit of the CF_1 complex [16,17]. The CF_1 complex probably contains three β subunits which are thought to be the locations of the catalytic site(s) [29]. The inactivation of the octylglucoside-dependent and methanol-activated Mg^{2+}-ATPase activities of the soluble and membrane-bound coupling factors, respectively, upon modification of a single β subunit suggests that the binding of ADP to one tight site totally inactivates the CF_1 complex. This implies heterogeneity and a strong cooperativity among β subunits. This is consistent not only with the observed stoichiometries of ADP and 2-N_3-ADP tight binding to the thylakoid membranes and the accompanying inhibition of the light-triggered ATPase activity [17], but is also consistent with previous work which utilized a graphical analysis to indicate that one ADP inhibits 2.6 ATPase active sites [3].

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