Characterization of a Phosphate Binding Domain on the α-Subunit of Chloroplast ATP Synthase Using the Photoaffinity Phosphate Analogue 4-Azido-2-nitrophenyl Phosphate†

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ABSTRACT: The photoaffinity phosphate analogue 4-azido-2 nitrophenyl phosphate (ANPP) was shown previously (Pougeois, R., Lauquin, G. J.-M., and Vignais, P. V. (1983) *Biochemistry 22*, 1241−1245) to bind covalently and specifically to a single catalytic site on one of the three β-subunits of the isolated chloroplast coupling factor 1 (CF_1). Modification by ANPP strongly inhibited ATP hydrolysis activity. In this study, we examined labeling of membrane-bound CF_1 by ANPP by exposing thylakoid membranes to increasing concentrations of the reagent. ANPP exhibited saturable binding to two sites on CF_1, one on the β-subunit and one on the α-subunit. Labeling by ANPP resulted in the complete inhibition of both ATP synthesis and ATP hydrolysis by the membrane-bound enzyme. Labeling of both sites by ANPP was reduced by more than 80% in the presence of P_i ($\geq$10 mM) and ATP ($\geq$0.5 mM). ADP was less effective in competing with ANPP for binding, giving a maximum of ~35% inhibition at concentrations $\geq$2 mM. ANPP-labeled tryptic peptides of the α-subunit were isolated and sequenced. The majority of the probe was contained in three peptides corresponding to residues Gln^{173} to Arg^{216}, Gly^{217} to Arg^{253}, and His^{256} to Arg^{272} of the α-subunit. In the mitochondrial F_1 (Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) *Nature 370*, 621−628), all three analogous peptides are located within the nucleotide binding pocket and within close proximity to the γ-phosphate binding site. The data indicate, however, that the azidophenyl group of bound ANPP is oriented at approximately 180° in the opposite direction to the adenine binding site with reference to the phosphate binding site on the α-subunit. The study has confirmed that ANPP is a bona fide phosphate analogue and suggests that it specifically targets the γ-phosphate binding site within the nucleotide binding pockets on the α- and β-subunits of CF_1. The study also indicates that in the resting state of the chloroplast F_1-F_0 complex both the α- and β-subunits are structurally asymmetric.

The chloroplast ATP synthase, like all F-type ATPases, consists of two structurally and functionally distinguishable parts, a membrane-embedded portion, CF_0,[1] which acts as a proton-specific channel, and a peripheral membrane portion, CF_1, which contains the catalytic site(s). The hydrophilic CF_1 portion is composed of five different subunits having a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ (*1*, *2*).

The subunit composition and general subunit organization of the F_1 portion is very similar in all of the F-type ATPases. The α- and β-subunits alternate in a hexameric ring surrounding a central mass that is comprised of one or more of the smaller subunits (*3*). There are six nucleotide binding sites per molecule located at the six interfaces between α- and β-subunits in the hexameric ring (*4*, *5*). Three catalytic sites are considered to reside primarily on the three β-subunits with minor structural contributions from part of each adjacent α-subunit. Conversely, three putative regulatory sites are located primarily on the α-subunits with minor contributions from the adjacent β-subunits. The three catalytic sites are structurally asymmetric. This was observed most clearly in the crystal structure of the mitochondrial enzyme, which showed that one β catalytic site was occupied by ADP, another was occupied by AMP−PNP (an ATP analog), and the third was unoccupied. The observed asymmetry was consistent with the *alternating sites* hypothesis proposed by Boyer and colleagues (reviewed in ref *6*) in which each catalytic site alternates between three different conformational states during a single catalytic cycle. In contrast, each of the three noncatalytic sites appeared in the crystal structure to be more or less structurally equivalent, each being occupied by AMP−PNP.

Two P_i binding sites were identified on mitochondrial F_1 using a centrifugal column technique (*7*), one with a dissociation constant of 37 μM and a second nonsaturable

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[1] Abbreviations: CF_o, the hydrophobic proton channel portion of the chloroplast ATP synthase (EC 3.6.1.34); CF_1, the hydrophilic catalytic portion of the chloroplast ATP synthase; ANPP, 4-azido-2-nitrophenyl phosphate; BTP, 1,3-bis[tris(hydroxymethyl)methylamino]-propane; chl, chlorophyll; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethansulfonic acid); PMS, phenazine methosulfate. AMP−PNP, adenyl-β,γ-imidodiphosphate; TNP−ATP/ADP, 2′(3′)-(trinitrophenyl)-ATP or ADP.

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site. A single phosphate binding site with a dissociation constant of 170 μM has been identified on CF_1 using a similar technique (*8*). The photoaffinity phosphate analogue 4-azido-2-nitrophenyl phosphate (ANPP), also labeled just a single site on CF_1 (*9*). ANPP binding to this site was independent of magnesium ions and was strongly inhibited by P_i and ATP. Following photoactivation, most of the probe was attached to β-Tyr^{328} with some incorporation into the two adjacent residues Val^{329} and Pro^{330} on the β-subunit. There was no detectable amount of label incorporated into α-subunits. Complete inhibition of the calcium-dependent ATPase activity of CF_1 was extrapolated to binding of 1 mol of ANPP/mol of enzyme, strongly suggesting that ANPP binds to a catalytic site. The indication that binding of ANPP to a single catalytic site results in nearly complete inhibition is entirely consistent with the *alternating sites* hypothesis as a valid description of the catalytic mechanism of the isolated F_1 enzyme.

We have extended this approach by examining the effects of photoaffinity probes on the membrane-bound form of the enzyme with the expectation that we would observe similar results to those found with the isolated enzyme. Remarkably, under conditions essentially identical to those that lead to specific labeling of the β-subunit of isolated CF_1, ANPP labels two sites on the membrane-bound enzyme, one on β and one on α. Binding of ANPP at both sites is competitive with phosphate, ATP, and to a lesser extent, ADP. By comparing the results of sequence analysis of ANPP-labeled peptides with the recently published crystal structure of the mitochondrial F_1, we conclude that ANPP binds to the γ-phosphate site of the ATP binding pocket in the same orientation on both α- and β-subunits. The data argue strongly for the existence of structural asymmetry among the nucleotide binding sites on the α-subunits of membrane-bound CF_1 and point to differences between the conformational states of the isolated and membrane-bound forms of the enzyme.

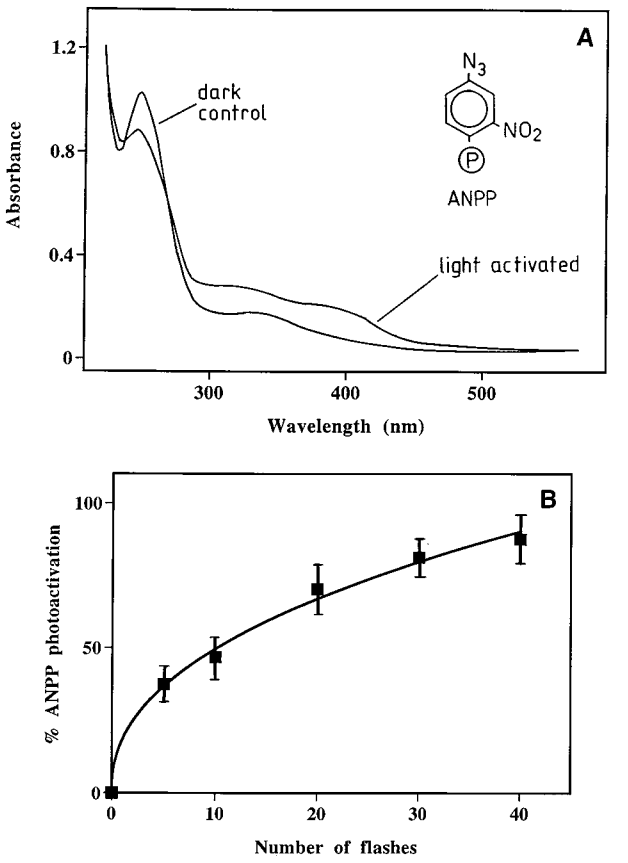

FIGURE 1: Spectrophotometric determination of bound ANPP. After 25 min of preincubation in an ice bath in the dark, thylakoid solutions were transferred to an ice-cooled Petri dish, and ANPP was covalently bound to the membrane-bound CF_1 by flash photoactivation. Treated thylakoids were washed 4 times using 10 mM NaCl. CF_1 was isolated (*13*) and desalted by passage successively through three centrifuge columns equilibrated with 10 mM BTP buffer, pH 7.5, containing 2 mM EDTA. (A) Photoactivated sample contained 0.7 μM CF_1−ANPP complex. For comparison, an unactivated sample containing 0.7 mM ANPP in the presence of 1.24 μM CF_1 is shown. (B) Yield of photoactivated, covalently bound ANPP is shown as a function of the number of light flashes (40 mW/flash).

MATERIALS AND METHODS

Materials. Sephadex G-25, PMS, DEAE-cellulose, dithiothreitol, HEPES, and bovine serum albumin were purchased from Sigma. ATP was obtained from Boehringer Mannheim, 2-nitro-4-aminophenyl phosphate was from Aldrich Chemical Co., and radiochemicals were from NEN, Dreieich. All other reagents used were from Merck and of highest analytical purity. ANPP was synthesized as described by Lauquin et al. (*10*). To avoid hydrolysis, it was crystallized and kept dry in the dark at −20 °C until needed.

Thylakoids were isolated from spinach (*Spinacia oleracea* L. cv. Butterfly) leaves as described by Strotmann et al. (*11*). The chlorophyll concentration was measured using the method of Arnon (*12*). CF_1 was isolated as described by Nelson (*13*), sedimented in 2 M $(NH_4)_2SO_4$, 4 mM ATP, and 10 mM HEPES buffer, pH 8.0, and collected by centrifugation. The protein was suspended in 300 μL of 50 mM BTP buffer, pH 7.5, containing 2 mM EDTA and passed three times successively through 3-mL Sephadex G-25 columns (*14*) equilibrated with the same buffer. For the separation and preparation of CF_1 subunits, the desalted enzyme was incubated in a medium containing 2% SDS, 2% β-mercaptoethanol, and 10% sucrose prior to electrophoresis (*15*). Gel electrophoresis was performed according to Laemmli (*16*), modified as described by Schägger et al. (*17*) in order to visualize protein bands during their separation. CF_1 subunits were separated on 10% acrylamide tube gels or isolated by preparative polyacrylamide gel electrophoresis using a Biometra Elucon E71 extractor.

Photolysis and Labeling. Upon illumination of ANPP, a reactive nitrene is formed that can bind covalently to proteins. Thylakoid suspensions equivalent to 100 μg of chl/mL in 10 mM HEPES−NaOH (pH 8), 10 mM NaCl, and 5 mM $MgCl_2$ were preincubated on ice for at least 25 min prior to illumination in ice-cold Petri dishes. In most experiments, photoirradiation was achieved using a flash lamp (Metz 40CT4) giving 40 mW of white light per flash. As shown in Figure 1A, the absorption maximum at 247 nm of the untreated probe was reduced in magnitude upon photoactivation whereas the absorption maxima at 335 and 397 nm were enhanced. The absorption spectra of the untreated and light-activated ANPP intersect at 273 nm. The point of intersection was used to calculate the sum of activated and nonactivated ANPP, and the percentage of photoactivated ANPP was calculated from the ratio of $E_{273\ nm}/E_{335\ nm}$. These

calculations were used to optimize the conditions for photoactivation and to estimate the amount of bound label per CF_1.

The relationship between photoactivation and the number of flashes is shown in Figure 1B. In all subsequent experiments involving this technique of flash photolysis, 40 flashes were used, which resulted in approximately 80% of photoactivation of ANPP. In a few experiments (indicated in the figure legends), a 347-nm laser (40 mW pulse intensity) or 2 min of continuous illumination with white light from a 500-W Hg lamp at an intensity of 1 kW/m^2 was used instead of the flash lamp. All three methods resulted in identical changes in the absorption spectrum of ANPP. Following photolysis, thylakoids were pelleted and washed three times with the above medium without added ANPP. Thylakoids were resuspended in medium containing 30 mM HEPES−NaOH (pH 8.0), 50 mM NaCl, 0.5 mM $MgCl_2$, and 50 μM PMS adjusted to the appropriate chlorophyll concentration (0.5−2 mg/mL).

Spectrohotometric determination of bound ANPP was performed using a double-beam spectrophotometer (Kontron Uvikon 930). After 25 min of preincubation in the dark to allow binding to come to equilibrium (*8*), ANPP was covalently attached to membrane-bound CF_1 by photoactivation. Treated thylakoids were washed four times with 10 mM NaCl. CF_1 was isolated and passed successively through three centrifuge columns equilibrated with 10 mM Bis−Tris−propane buffer, pH 7.5, containing 2 mM EDTA. The amount of bound analogue was calculated from its absorption at 247 nm using the molar absorption coefficient ϵ_{247} = 16 550 (*10*) and using as a blank a sample of untreated CF_1 adjusted to the same protein concentration.

Isolation and Sequencing of Tryptic Peptides. To localize the positions of the ANPP label within the α- and β-subunits, the isolated subunits were subjected to tryptic proteolysis using the method of Cross et al. (*18*). After prolonged tryptic digestion (>16 h at 35 °C), peptides were separated by reverse-phase HPLC as described elsewhere (*18*). Tryptic peptides containing covalently attached ANPP were identified by their strong absorbance at 247 nm and were sequenced by automated Edman degradation using the core sequencing facility at the School of Medicine, Hannover.

Activity Assays. For photophosphorylation measurements, isolated thylakoids (equivalent to 0.1 mg of chl/mL) were incubated in a medium containing 10 mM HEPES−NaOH (pH 8.0), 50 mM NaCl, and 5 mM $MgCl_2$. Thylakoids were incubated for 25 min in the dark in the presence of ANPP to obtain association/disassociation equilibrium of the analogue prior to photolysis and assay. ATP synthesis was measured by detecting incorporation of ^{32}P into ADP following sedimentation of inorganic phosphate as described by Sugino and Miyoshi (*19*). ATP hydrolysis by isolated CF_1 was measured by following release of $^{32}P_i$ from [γ-^{32}P]-ATP as described elsewhere (*8*). Protein concentrations were measured using the method of Bradford (*20*).

RESULTS

Thylakoid membranes were exposed to ANPP under conditions essentially identical to those which led to labeling of a single site on the β-subunit of isolated CF_1 (*9*). Following photolysis, CF_1 was isolated from the membranes, and the amount of covalently attached ANPP was measured photometrically by measuring the absorbance at 247 nm due to the presence of the probe (Figure 1A). Under the conditions of illumination used (40 flashes), ca. 80% of the ANPP present was routinely photolyzed (Figure 1B). The results shown in Figure 2 indicated saturable binding of ANPP at two sites on CF_1. Examination of the subunit distribution of the bound ANPP following separation of subunits by SDS polyacrylamide gel electrophoresis (Table 1) indicated that ANPP was incorporated primarily into the α- and β-subunits with little or no incorporation into the smaller subunits. The ratio of label was ~2:1 for β:α at 50 μM ANPP and approached 1:1 at 200 μM ANPP. The same result was obtained in several identical trials indicating that the α site has a slightly lower affinity for ANPP than the β site.

Pougeois et al. (*9*) showed earlier that inorganic phosphate competitively blocked ANPP binding to CF_1, the maximal effect requiring about 10 mM P_i. They also showed that the

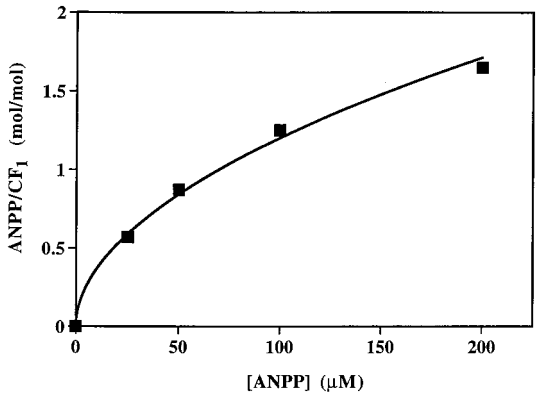

FIGURE 2: Correlation between ANPP binding and the concentration of free ANPP in the incubation medium. Following flash photoactivation (Figure 1), thylakoids were washed 4 times with 10 mM NaCl. CF_1 was isolated and passed successively through three subsequent centrifuge columns equilibrated with 10 mM BTP buffer, pH 7.5, containing 2 mM EDTA. The ANPP content was calculated using its molar absorption coefficient at 247 nm.

Table 1: Binding of ^{32}P-Labeled ANPP to the α- and β-Subunits of Membrane-Bound CF_1

	treatment[a]			
gel band (dpm)	50 μM ANPP	50 μM ANPP + 10 mM P_i	200 μM ANPP	200 μM ANPP + 10 mM P_i
CF_1 α	174.7	34.6	423.7	87.6
CF_1 β	309.4	18.9	553.9	104.0
CF_1 γ	2.1	0.3	2.8	1.1
CF_1 δ	1.8	0.3	2.2	0.8
CF_1 ϵ	1.2	0.2	1.9	0.6
unknown 1	1.4	0.2	2.5	0.7
unknown 2	1.2	0.2	1.8	0.6
blank slice	1.1	0.2	1.5	0.5

[a] Thylakoid membranes were photolabeled with ^{32}P-ANPP (prepared as described by Lauquin et al. (*10*) using ^{32}P-labeled orthophosphate, 5 mCi/μmol) in the absence and presence of 10 mM orthophosphate. CF_1 was isolated according to Nelson (*13*) and subjected to electrophoresis on 12% polyacrylamide gels. Gels were stained with Coomassie blue. 3-mm gel slices corresponding to protein bands were excised from the gel, and the radioactivity present was measured by liquid scintillation counting. Each gel lane contained a total of 30 μg of protein taken from samples with specific activities (dpm/μg of protein) of 57.3, 6.4, 19.4, and 103.1 for the 50 μM ANPP lane, the 50 μM ANPP + 10 mM P_i lane, the 200 μM ANPP lane, and the 200 μM ANPP + 10 mM P_i lane, respectively.

Table 2: Competitive Inhibition of ANPP Binding by P_i, ATP, and ADP

concn of inhibitor added (mM)[a]	inhibitor (% inhibition of ANPP binding)		
	P_i	ADP	ATP
0.05		15	60
0.1		21	68
0.2	39		
0.5	58	29	82
1.0	71	31	84
2.0	75	35	84
10.0	82		

[a] Thylakoid membranes were incubated for 25 min on ice in the dark in 10 mM HEPES buffer, pH 8.0, containing 10 mM NaCl, 5 mM $MgCl_2$, 50 μM ANPP, thylakoids corresponding to 100 μg/mL, and either P_i, ATP, or ADP at the concentrations indicated. Following incubation, mixtures were transferred to an ice-cooled Petri dish and exposed to photoactivating conditions using a 500-W Hanau lamp (light intensity: 1.3 kW/m^2). Treated thylakoids were washed 4 times with 10 mM HEPES buffer, pH 8.0, containing 10 mM NaCl and 5 mM $MgCl_2$. The CF_1 was isolated from the treated membranes and passed successively through three centrifuge columns equilibrated with the same buffer. The ANPP content was calculated using its molar absorption coefficient at 247 nm. In the control experiment, 1.08 mol of ANPP bound/mol of CF_1. This corresponds to 100% labeling.

same amount of protection was afforded by 1−2 mM ATP; however, millimolar concentrations of ADP were only partially effective in blocking ANPP binding. For example, at 10 mM ADP in the presence of Mg^{2+}, the maximum protection against ANPP inhibition was ~55% (*9*). We have observed a very similar response of ANPP labeling to the presence of phosphate in the medium by the membrane-bound form of the enzyme (Table 2). Ten millimolar P_i provided the maximal response, protecting against ANPP labeling by >80% and reducing labeling of both α and β sites to a similar extent as determined by electrophoresis of the radioactively labeled CF_1 (not shown). ATP was as effective as phosphate, but maximal inhibition was observed at a lower concentration (1−2 mM). In contrast, ADP blocked ANPP binding to a much lower extent (~35% maximum). The degree to which substrates could block ANPP labeling was found to be strongly dependent on the order of their addition. If the membranes were preincubated with ANPP for 10−20 min prior to addition of 10 mM P_i, the effectiveness of the P_i as a competitor was almost completely lost (data not shown). This is most likely to indicate that the on-rate for ANPP binding to the enzyme is significantly faster than its off-rate. ANPP is similar in this respect to the nitrophenyl nucleotide analogues trinitrophenyl ATP or ADP (*21*). It follows that the nitrophenyl group must contribute significantly to binding, although the binding specificity is clearly dictated by the phosphate or nucleoside groups.

To examine the effect of ANPP binding on CF_1F_0 activity, thylakoids were treated with ANPP, and the amount of ANPP incorporated into CF_1 was determined spectrophotometrically after isolating the enzyme from the treated membranes. Treatment of thylakoids with ANPP strongly inhibited ATP synthesis (Figure 3) in a manner that is consistent with a competition between binding of ANPP and binding of inorganic phosphate. ANPP treatment of thylakoids had no measurable effect on either the rate of uncoupled electron transport or on generation of the transmembrane pH gradient (data not shown), indicating that the inhibitory effect of

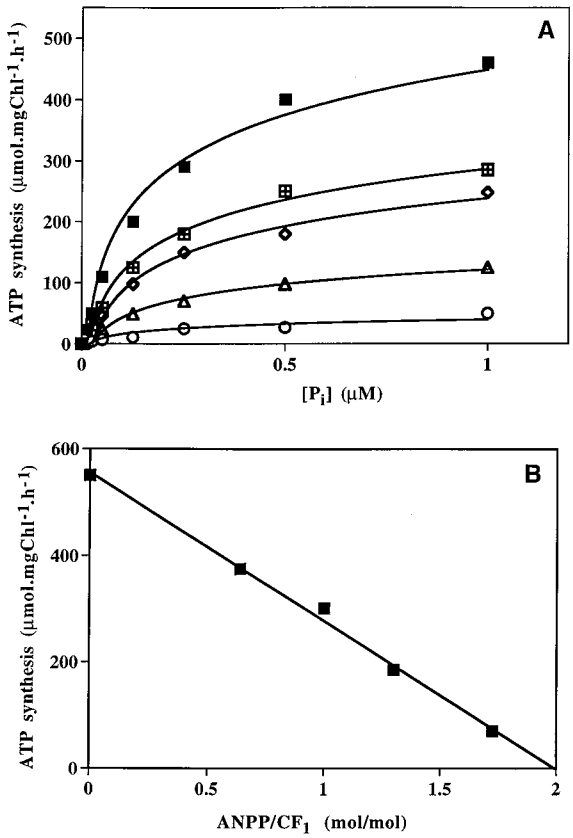

FIGURE 3: Correlation between the amount of covalently bound ANPP and inhibition of ATP synthesis. Photolabeling of the membrane bound CF_1 was performed as described in the legend to Figure 2. Treated thylakoid membranes were washed three times in a medium consisting of 10 mM HEPES, pH 8.0, 50 mM NaCl, and 5 mM $MgCl_2$. The final pellet was resuspended in 30 mM HEPES−HCl buffer, pH 8.0, containing 50 mM NaCl, 0.5 mM $MgCl_2$, 50 μM PMS, 5 mM ^{32}P-labeled orthophosphate, 1 mM ADP, and thylakoids corresponding to 25 μg of chl/mL. The specific activity of the incubation mixture was 25 μCi/mL. After 30 s of photoactivation, the sample was denatured by addition of $HClO_4$ at a final concentration of 0.3 M. ATP synthesis was measured according to Sugino and Myoshi (*19*). (A) Dependence of ATP synthesis on the concentration of orthophosphate with different amounts of bound ANPP. The ANPP/CF_1 ratios are 0 (closed squares), 0.6 (open squares), 1 (diamonds), 1.3 (triangles), and 1.72 mol/mol (circles). (B) V_{max} values at each ANPP concentration, calculated from double-reciprocal plots of the data shown in panel A, are plotted as a function of the amount of bound ANPP.

ANPP resulted specifically from binding to CF_1. Interestingly, the data of Figure 3B indicate a clear linear correspondence between the extent of inhibition of ATP synthesis and the amount of ANPP covalently incorporated into CF_1. Extrapolation of the data to zero ATP synthesis indicated that complete inhibition of activity occurs when close to 2 mol of ANPP is bound per mol of CF_1. A similar relationship was observed between the amount of bound ANPP and the ATPase activity of the enzyme isolated from thylakoid membranes following ANPP treatment (Figure 4). In this case, however, near maximum inhibition was observed when less than 1.5 mol of ANPP was bound per mol of CF_1.

To identify the ANPP labeling site on the α-subunit, CF_1 was isolated following treatment of membranes with ANPP, and the subunits were separated by SDS gel electrophoresis. The α-subunit was electroeluted from the gels and subjected to limit trypsin digestion. These experiments were initially attempted using ^{32}P-labeled ANPP, but it was found that a

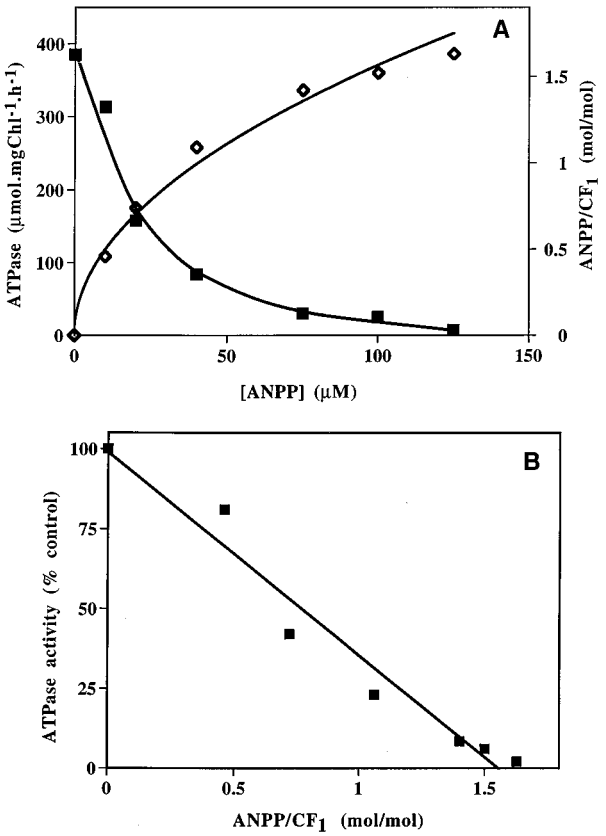

FIGURE 4: Correlation between the amount of covalently bound ANPP and the inhibition of ATPase activity. Photolabeling of the membrane-bound CF_1 was performed as described in the legend to Figure 2. Thylakoid membranes were washed three times as described in the legend to Figure 3. The final pellet was resuspended in 30 mM HEPES−HCl buffer, pH 8.0, containing 50 mM NaCl, 0.5 mM $MgCl_2$, 50 μM PMS, and thylakoids corresponding to 35 μg of chl/mL. After 2 min of illumination with white light (100 mW/cm^2) [γ^{32}-P]ATP at a final concentration of 0.5 mM was added at the onset of the dark period. ATP hydrolysis was measured as described by Schumann (*31*). (A) ANPP concentration dependence for inhibition of ATPase activity versus the amount of ANPP bound per CF_1. (B) ATPase activity is plotted as a function of bound ANPP.

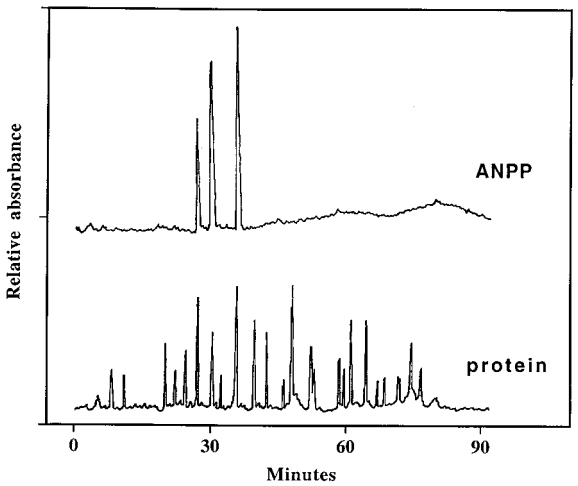

FIGURE 5: HPLC analysis of trypic peptides of α-ANPP. Gel electrophoresis was performed using 10% acrylamide tube gels according to Laemmli (*16*), modified as described by Schaegger et al. (*17*) in order to visualize protein bands during their separation. CF_1 subunits were electroeluted using a Biometra Elucon E71 extractor (Biometra, Goettingen). The isolated α-subunit was subjected to 16 h of trypsin digestion at 35 °C and subsequent reverse-phase HPLC following the protocol of Cross et al. (*18*). Protein and ANPP were detected by their absorbance at 210 and 247 nm, respectively.

Table 3: Labeling Patterns of CF_1-Derived Polypeptides on SDS Gels

peptide[a]	% total label	tryptic peptide
I	40	His256 to Arg272
II	30	Gly217 to Arg253
III	20	Gln173 to Arg216

[a] The three peptide peaks shown in Figure 5 and resulting from tryptic digestion of the α-subunit were collected and dried under nitrogen. The peptides were given to the core protein facility at the University of Hannover for identification by Edman degradation.

significant proportion of the radioactive phosphate was liberated from the bound ANPP during subunit isolation, coincident with the appearance of radioactivity at the gel front and in the electrode buffer. It was subsequently found that ANPP could be more reliably detected during chromatography by monitoring the absorbance of labeled peptides at 247 nm, the peak absorbance of the azidonitrophenyl group. The amount of ANPP that was attached to CF_1 determined using this method remained constant. A typical HPLC profile is shown in Figure 5. More than 90% of the bound ANPP was located in three peptides. The labeled peptides were collected and sequenced (Table 3).

DISCUSSION

Our data have identified a phosphate binding site on the α-subunit of the chloroplast ATP synthase. This site exhibits saturable ANPP binding leading to covalent incorporation of a maximum of 1 mol of ANPP/mol of CF_1. At the same time and within the same range of ANPP concentration, another site on one of the three β-subunits becomes labeled. This site is assumed to be the site identified earlier by Pougeois et al. (*9*), which specifically binds ANPP on isolated CF_1 since its ANPP binding characteristics are indistinguishable from those of the β site on the soluble enzyme. Inorganic phosphate and ATP effectively compete with ANPP for binding to both the α and β sites. The amount of protection afforded against ANPP labeling of either α- or β-subunits is approximately the same at any given concentration of P_i or ATP. In contrast, ADP is only partially protective (ca. 35% at 2 mM, Table 2). The weak competition afforded by ADP suggests that ANPP binds primarily to the site normally occupied by the γ-phosphate of ATP within the nucleotide binding pockets on both α- and β-subunits.

Complete inactivation of ATP synthesis by CF_1 was obtained under conditions where 2 mol of ANPP was covalently bound per mol of enzyme. This result was surprising. Since the extent of labeling of the β-subunit was consistently higher than that of the α-subunit at the lower ANPP concentrations (Table 1) and since with isolated CF_1 complete inhibition coincides with ANPP binding to a single site on one of the three β-subunits as predicted by the alternating sites hypothesis, complete inhibition was expected to occur when less than 2 ANPP was bound per CF_1. The result instead suggests that full inhibition of ATP synthesis requires ANPP binding to both sites. We have, at this time, no satisfactory explanation for this observation. In contrast, full inhibition of ATP hydrolysis by CF_1 isolated from the

ANPP-treated membranes extrapolated to binding of less than 1.5 mol of ANPP bound/mol of CF_1 (Figure 4B). This is consistent with complete inhibition resulting from binding of ANPP to one of the three β-subunits as observed when labeling was performed on the isolated enzyme (*9*).

That ANPP does indeed bind in the nucleotide binding pocket was confirmed by inspection of the atomic coordinates of the beef heart mitochondrial F_1 (*5*). The three peptides of the α-subunit of the mitochondrial enzyme, which are analogous to the three CF_1 peptides that were labeled by ANPP (Figure 5 and Table 3), are all adjacent to the nucleotide binding pocket and within about 9 Å from the γ-phosphate group of bound ATP. The maximum calculated length of ANPP is 9.4 Å (*22*). ANPP would thus fit snugly between the γ-phosphate site and the region of α containing the labeled stretches of amino acids. Interestingly, the labeled peptides project away from the phosphate binding site in the opposite direction to the adenine binding site. The nitrophenyl moiety of ANPP, therefore, is not likely to associate directly with the adenine binding site. This observation explains why the sites labeled by ANPP had not been labeled using photoaffinity nucleotide analogues with photoreactive groups bound either to the 2 position of the base or on the sugar moiety (*23*), which are known to bind in an analogous way to ATP. A parallel situation exists for the location of ANPP bound to the β-subunit. The most frequently labeled amino acid residue is Tyr 311 in mitochondrial F_1 (*23*) and the analogous Tyr 328 in CF_1 (*24*). This residue is located in an equivalent position to the peptides labeled by ANPP on the α-subunit, that is, about 6 Å from the γ-phosphate of bound ATP in the opposite direction to the adenine binding site. ANPP probably binds in a very similar orientation to the nucleotide analogue 8-azido-ATP, which labels similar peptides to those labeled by ANPP when occupying catalytic and noncatalytic sites on the mitochondrial enzyme (*25*).

Why the α-subunit is labeled on the membrane-bound CF_1 but not on the isolated enzyme is intriguing. A similar situation may exist for the mitochondrial enzyme in which a band corresponding to the α-subunit was labeled with ANPP while the enzyme was attached to submitochondrial particles (*10*). The same band was not labeled in the isolated enzyme. It is possible that the procedures used to isolate the F_1 enzymes from the membranes altered the enzymes in some way such that the α-subunit could no longer bind ANPP. For example, it is possible that the presence of tightly bound ADP or ATP may influence ANPP binding and that the content of bound nucleotides changes during enzyme isolation. One particularly interesting possibility is that ANPP only binds with high (low micromolar) affinity to CF_1 at sites that are already occupied by tightly bound ADP. Consistent with this possibility is a recent observation in our laboratory in which it was shown that preillumination of thylakoid membranes reduced the maximum extent of ANPP labeling of CF_1 from ~1.8 to ~0.8 mol/mol. It is well-known that preillumination of thylakoids results in the release of ADP (~1 mol/mol) from CF_1 (*11*, *26*). At this time we do not know if the treatment specifically decreases labeling of either the α- or β-subunits or whether it decreases labeling of both subunits. This is currently being investigated.

A requirement for bound ADP for ANPP labeling could also explain some observed effects of ADP in competition experiments. Pougeois et al. (*9*) demonstrated that the extent of inhibition by ADP of ANPP binding to β was significantly reduced in the presence of magnesium ions. CF_1 isolated by the methods employed in their experiments is known to have between one and two sites occupied by tightly bound ADP (*27*). One of the two sites contains MgADP whereas the second site contains variable amounts of ADP only. Incubation of the enzyme with MgADP results in the complete filling of the second site (*27*). So the decreased sensitivity to ADP may have resulted from binding of additional MgADP to CF_1, thus enhancing the extent of ANPP binding. Differences in nucleotide binding site occupancy could also explain the marked differences observed in the maximum extent of ANPP labeling of the mitochondrial (~0.5 mol/mol; *10*, *23*), *E. coli* (~0.5 mol/mol; *24*), thermophilic bacterial (~0.8 mol/mol; *24*), and chloroplast (~1 mol/mol; *9*) enzymes.

Creczynski-Pasa et al. (*28*) recently showed that detergent-isolated CF_oF_1 has very similar nucleotide binding properties to the isolated CF_1. After treatment with ATP in the presence or absence of magnesium ions, two of the three noncatalytic sites (on α-subunits) are occupied with tightly bound ATP and between one and two of the three catalytic sites (on β-subunits) are occupied either with tightly bound ATP or ADP. The other two sites do not bind nucleotides with high enough affinity to remain bound after three successive gel filtration steps. This study very clearly confirms the existence of asymmetry among both catalytic and noncatalytic sites on CF_1 and indicates that this asymmetry persists in the intact CF_0F_1 complex. The absence of ADP bound in a noncatalytic site on CF_oF_1 is, at first glance, inconsistent with the hypothesis that ANPP binding to a noncatalytic site depends on having ADP bound at that site. There are, however, two possible explanantions for this discrepancy. First, the presence of continuous illumination during isolation of the CF_oF_1 complex may have led to release of bound ADP from the third α site that remains unavailable to nucleotides in the absence of a proton gradient. Alternatively, this site may simply have too low an affinity for its nucleotide to retain it through the desalting steps.

In summary, it may be concluded that ANPP is a bona fide phosphate analogue that specifically targets the γ-phosphate binding site of both α- and β-subunits of CF_1. The fact that the α-subunit is only labeled in membrane-bound CF_1 indicates that conformational differences exist between the isolated and membrane-bound forms of the enzyme and that the three α-subunits of the CF_oCF_1 are, like the β-subunits, nonidentical. These differences may reflect, as suggested elsewhere (*29*, *30*), the possibility that ATP synthesis is not simply a reversal of ATP hydrolysis but may involve a different or modified catalytic pathway than that operating in isolated CF_1. Clearly, considerably more work is needed to identify the catalytic pathway for ATP synthesis and to determine the role that the α-subunits play in this process.

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