Relationship of Tightly Bound ADP and ATP to Control and Catalysis by Chloroplast ATP Synthase†

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ABSTRACT: Whether the tightly bound ADP that can cause a pronounced inhibition of ATP hydrolysis by the chloroplast ATP synthase and F_1 ATPase (CF_1) is bound at catalytic sites or at noncatalytic regulatory sites or both has been uncertain. We have used photolabeling by 2-azido-ATP and 2-azido-ADP to ascertain the location, with Mg^{2+} activation, of tightly bound ADP (a) that inhibits the hydrolysis of ATP by chloroplast ATP synthase, (b) that can result in an inhibited form of CF_1 that slowly regains activity during ATP hydrolysis, and (c) that arises when low concentrations of ADP markedly inhibit the hydrolysis of GTP by CF_1. The data show that in all instances the inhibition is associated with ADP binding without inorganic phosphate (P_i) at catalytic sites. After photophosphorylation of ADP or 2-azido-ADP with [^{32}P]P_i, similar amounts of the corresponding triphosphates are present on washed thylakoid membranes. Trials with appropriately labeled substrates show that a small portion of the tightly bound 2-azido-ATP gives rise to covalent labeling with an ATP moiety at noncatalytic sites but that most of the bound 2-azido-ATP gives rise to covalent labeling by an ADP moiety at a catalytic site. We also report the occurrence of a 1–2-min delay in the onset of the Mg^{2+}-induced inhibition after addition of CF_1 to solutions containing Mg^{2+} and ATP, and that this delay is not associated with the filling of noncatalytic sites. A rapid burst of P_i formation is followed by a much lower, constant steady-state rate. The burst is not observed with GTP as a substrate or with Ca^{2+} as the activating cation.

It is widely recognized that the presence of ADP and Mg^{2+} can cause potent inhibition of the ATPase activity of chloroplast thylakoids, associated with a tight binding of the ADP (Carmeli & Lifshitz, 1972; Strotmann & Bickel-Sandkötter, 1977; Shoshan & Selman, 1979; Malyan, 1981; Bar-Zvi & Shavit, 1982; Bickel-Sandkötter, 1983; Anthon & Jagendorf, 1984; Schumann, 1987; Strotmann et al., 1987). The tightly bound ADP exchanges very slowly with medium ADP in the dark. Release of the ADP accompanies the reactivation of the ATPase when the thylakoids are exposed to light or to a pH gradient (Smith & Boyer, 1976; Strotmann et al., 1976; Schlodder & Witt, 1981). Although early evidence suggested that the released ADP came from catalytic sites (Smith & Boyer, 1976), a frequent interpretation has been that the inhibition results from ADP binding to noncatalytic regulatory sites. However, it has been recognized that the same binding site might show either regulatory or catalytic behavior [for example, see Bickel-Sandkötter (1983) and Malyan and Vitseva (1983)]. Good evidence has been presented that ADP inhibits mitochondrial F_1 ATPase when the ADP is bound without concomitant inorganic phosphate (P_i)[1] at a catalytic site (Drobinskaya et al., 1985).

Both tightly bound ADP and ATP are present on CF_1 as usually isolated (Harris, 1978). Considerable evidence has accrued that tightly bound ADP may be present at both catalytic and noncatalytic sites [see Feldman and Boyer (1985) and references cited therein]. The tight binding of 2-azido-ADP at separate catalytic and noncatalytic sites on CF_1 has recently been demonstrated (Xue et al., 1987a,b). The isolated CF_1 ATPase like the intact synthase shows a potent inhibition by ADP. When the enzyme with ADP tightly bound in the presence of EDTA is exposed to Mg^{2+} or Ca^{2+}, an inhibited form arises that is only slowly reactivated by the presence of MgATP, accompanied by release of the bound ADP (Carmeli et al., 1981; Feldman & Boyer, 1985). It has been suggested that part or most of such tightly bound ADP appears to be at catalytic sites (Feldman & Sigman, 1982; Feldman & Boyer, 1985; Leckband & Hammes, 1987).

For these and other related studies on the role of bound nucleotides, clarification could be provided if a means were available for determining the location of tightly bound nucleotides on the enzymes. Such a means has now been made available by the demonstration that the 2-azido-ADP or 2-azido-ATP, when bound at separate catalytic or noncatalytic sites, labels specific tyrosine residues in adjacent tryptic peptides from the β subunits of the enzyme from mitochondria (Cross et al., 1987), chloroplasts (Xue et al., 1987b), or *Escherichia coli* (Wise et al., 1987). The tryptic peptides can be readily identified by their separation on ion-exchange and reversed-phase HPLC columns. We report here the application of this approach to the chloroplast ATP syntase and the isolated CF_1 to determine the location of the tightly bound ADP that is correlated with enzyme inhibition. The results show that the inhibitory behavior mentioned above, and other related inhibitions by ADP, results from Mg^{2+}-induced changes in properties of the enzyme associated with conversion of an

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[1] Abbreviations: CF_1, ATPase portion of the ATP synthase from chloroplasts; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; P_i, inorganic phosphate; Tris, tris(hydroxymethyl)aminomethane; PP_i, inorganic pyrophosphate; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography.

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ADP bound at a catalytic site to a slowly exchangeable form. We also report observations about a burst in activity of CF_1 that occurs before the onset of the Mg^{2+}-induced inhibition, and some clarifications of the site location, the labeling, and the role of tightly bound ATP on chloroplast thylakoid membranes.

Experimental Procedures

Materials. Previously described methods were used for preparation of CF_1 (Lien & Racker, 1971), chloroplast thylakoids (Rosen et al., 1979), and 2-azido-ADP and 2-azido-ATP (Melese & Boyer, 1985; Xue et al., 1987b). ATP synthase concentrations were estimated as 1.3 nmol of synthase/mg of chlorophyll (Strotmann et al., 1973).

Photophosphorylation. The reactions were carried out in 50 mM Tricine, 50 mM KCl, 5 mM $MgCl_2$, 50 μM phenazine methosulfate, and 0.2 mg/mL chlorophyll at pH 8 and room temperature. Concentrations of P_i and ADP or 2-azido-ADP were as indicated in the text. Illumination was done with a Kodak slide projector and a 300-W lamp with an amber filter to remove ultraviolet light.

Covalent Modification of CF_1 on the Membrane. Photophosphorylation was stopped by the addition of NH_4Cl to 250 mM and Tris to 80 mM final concentration at pH 8. Thylakoid membranes were collected by centrifugation at 12000*g* for 2 min. The pellet was washed twice with 50 mM Tris/150 mM NH_4Cl, pH 8, and resuspended in the same buffer to 0.1 mg of chlorophyll/mL. Ultraviolet irradiation was with a 200-W Mercury Light Source SP-200 ultraviolet lamp for 30 min under constant stirring. The distance between the lamp and the surface of the solution was 4 cm.

Release of the Modified CF_1 from the Membrane. After ultraviolet irradiation, thylakoid membranes were washed twice with 10 mM $NaPP_i$, pH 8, and centrifuged at 12000*g* for 2 min. The pellet was then resuspended in 2 mM Tricine/Tris and 0.3 M sucrose, pH 8, to 0.05 mg of chlorophyll/mL and stirred at room temperature for 30 min. The suspension was centrifuged at 48000*g* for 30 min, and the supernatant was concentrated with an Amicon concentrator to 1 mL. This was then mixed with carrier CF_1 that had been modified with nonradioactive 2-azido-ATP at both catalytic and noncatalytic sites (Xue et al., 1987a).

Perchloric acid precipitation, trypsin digestion, and HPLC isolation of peptides were done essentially as described by Wise et al. (1987).

Results

Location of the Bound ADP That Inhibits ATP Hydrolysis in Chloroplast Thylakoids. When chloroplast thylakoids carry out photophosphorylation with 2-azido[β-^{32}P]ADP, and the light is turned off, ATPase activity declines, and a tightly bound 2-azido[β-^{32}P]ADP is found. This is analogous to the behavior with ADP. To find which sites were labeled when ATPase inhibition sets in, thylakoid membranes were exposed under photophosphorylating conditions to 100 μM 2-azido-[β-^{32}P]ADP in the presence of 1 mM P_i for 1 min. The samples were then photolyzed by exposure to ultraviolet light for 30 min. The CF_1 was released from the membrane and separated as described under Experimental Procedures. This radiolabeled CF_1 was mixed with 2 mg of CF_1 that had been covalently derivatized with unlabeled 2-azido-ADP and 2-azido-ATP at both catalytic and noncatalytic sites (Xue et al., 1987b). The CF_1 derivatized by unlabeled azido nucleotides provided carrier peptides to allow identification of peaks by the absorption at 215 nm in the HPLC separation of tryptic peptides.

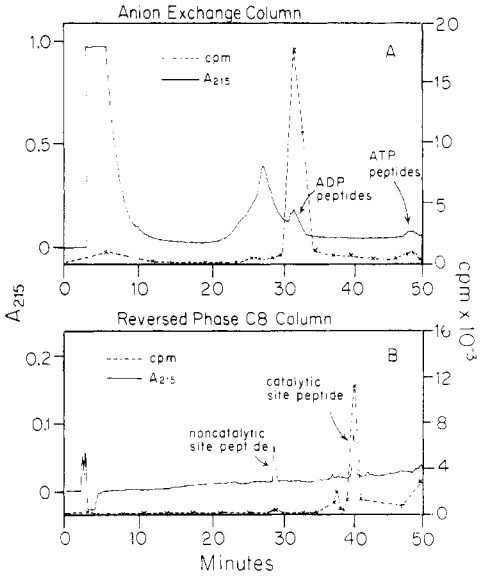

FIGURE 1: (A) Separation of labeled peptides after photophosphorylation with 2-azido[β-^{32}P]ADP and photolysis. The ^{32}P-labeled CF_1 and added carrier CF_1 were digested with trypsin and the peptides formed separated on an HPLC ion-exchange column. Most peptides readily pass through the column. Radioactive ADP-labeled peptides, detected by absorption at 215 nm, appear as a shoulder on the larger peak of other peptides (Xue et al., 1987b). (B) Separation of the [^{32}P]ADP-labeled peptides and the catalytic and noncatalytic site peptides. The ADP-labeled peptides (A) were separated on a reversed-phase C-8 HPLC column (Xue et al., 1987b) and the fractions containing radioactivity determined.

The separation on an anion-exchange column of the ADP- and ATP-labeled tryptic peptides that contained radioactivity is shown in Figure 1A. The subsequent separation of the ADP-peptides on a reversed-phase C8 column is shown in Figure 1B. Very little ^{32}P was present in the noncatalytic peptide[2] area. In contrast, most of the ^{32}P incorporated was present as a [^{32}P]ADP moiety on a catalytic site peptide.

In other experiments, thylakoid membranes were incubated under the same photophosphorylating conditions as used for Figure 1 but for 0.5 min with 20 μM 2-azido[β,γ-^{32}P]ATP instead of 2-azido[β-^{32}P]ADP. Over 95% of the ^{32}P incorporation was as an ADP moiety at catalytic sites. With an increase of the exposure time to 100 min with 100 μM 2-azido[β,γ-^{32}P]ATP, more labeling of a noncatalytic peptide by a [^{32}P]ATP moiety was detected. About 10% of the total ^{32}P nucleotide incorporated was present as an ATP moiety on a noncatalytic site peptide and 90% as an ADP moiety on both noncatalytic (about 15%) and catalytic site peptides (about 85%).

These results show that the inhibition of the ATPase activity of the thylakoid membranes is clearly associated with tight binding of an ADP moiety at a catalytic site.

Labeling of a Tightly Bound ATP on Thylakoid Membranes. As reported in the preceding section, some ATP binding at noncatalytic sites is obtained when thylakoids are incubated under photophosphorylating conditions but with 2-azido[β,γ-^{32}P]ATP instead of 2-azido[β-^{32}P]ADP. The near absence of noncatalytic site labeling after a short time of exposure of thylakoid membranes to the 2-azido-ATP under photophosphorylation conditions means that any noncatalytic

[2] Peptides labeled from 2-azido nucleotides bound at catalytic or noncatalytic sites are called catalytic site and noncatalytic site peptides for convenience, with full recognition that only the side chains of the labeled tyrosines might be in the vicinity of the bound nucleotide. Other portions of the peptides may or may not be adjacent to the bound substrates.

sites on the synthase were already largely filled. Even after 1 h of incubation, only limited exchange of tightly bound nucleotides at noncatalytic sites with medium 2-azido-ATP had occurred. Likely, most of the light-promoted exchange of tightly bound nucleotides on thylakoid membranes that has been noted occurs with nucleotides bound at catalytic sites.

Additional exploration of tight ATP binding on chloroplast thylakoids was undertaken in view of observations made in Shavit's laboratory (Bar-Zvi & Shavit, 1982; Aflalo & Shavit, 1982). They reported that after photophosphorylation with [^{32}P]P_i their washed thylakoid membrane preparations contained about 0.15–0.2 tightly bound ATP per CF_1 that did not readily turn over when photophosphorylation was continued with unlabeled P_i. We prepared and exposed thylakoid membranes to ADP, [^{32}P]P_i, and light using the conditions they reported. We obtained labeling of about 0.1 tightly bound [^{32}P]ATP per CF_1 on the washed thylakoid membranes. Similar labeling was obtained with either ADP or 2-azido-ADP and [^{32}P]P_i as substrates.

Exposures with 2-azido-ADP were followed by a 30-min photolysis of the washed thylakoid membranes to obtain covalent derivatization. The labeled CF_1 was isolated and mixed with 2-azido nucleotide-labeled carrier CF_1, and the distribution of radioactivity in tryptic peptides was determined as described earlier. When 2-azido-ADP and [^{32}P]P_i were used, only a small amount (0.005–0.01 mol/mol of CF_1) of ^{32}P nucleotide was covalently inserted, and this was nearly all as a [^{32}P]ATP moiety associated with the noncatalytic peptide. In contrast, when 2-azido[β-^{32}P]ADP and unlabeled P_i were used as substrates, much more (about 1 mol/mol of CF_1) tightly bound ^{32}P nucleotide was found, and after photolysis, nearly all the ^{32}P was present as an ADP moiety associated with the catalytic peptide. As mentioned earlier, when thylakoids were illuminated in the presence of 20 μM 2-azido-[β,γ-^{32}P]ATP, nearly all the covalent labeling obtained was as an ADP moiety at catalytic sites. Such results show that the tightly bound 2-azido-ATP at catalytic sites, initially derived from medium 2-azido-ADP or 2-azido-ATP, hydrolyzes during the isolation, photolysis, and separation procedure to give rise to a covalently inserted ADP moiety. The observations make it probable that the portion of the tightly bound [^{32}P]ATP formed from [^{32}P]P_i that was not readily replaced in studies from Shavit's laboratory was likely at noncatalytic sites. Washed thylakoid preparations may have some noncatalytic sites empty, and, if so, these can readily bind MgATP tightly.

Bound ADP on CF_1 Associated with the Mg^{2+}-Induced Inhibition. When 5 μM CF_1 is exposed to 200 μM 2-azido-[β-^{32}P]ADP in 40 mM Tricine and 2 mM EDTA at pH 8 and room temperature for 2 h, and the solution passed through a Sephadex centrifuge column (Penefsky, 1979), 1.1–1.2 mol of bound 2-azido[^{32}P]ADP per mole of enzyme is found. These exposure conditions are like those used in Hammes' laboratory for labeling the site they designate as the nondissociable ADP site (Bruist & Hammes, 1982). The behavior of the enzyme with tightly bound 2-azido-ADP is quite similar to that observed with tightly bound [^{3}H]ADP (Feldman & Boyer, 1985; Leckband & Hammes, 1987). Exposure of the enzyme with bound 2-azido-ADP to 5 mM Mg^{2+} gives an inhibited form that requires many minutes of exposure to MgATP to regain activity and release the labeled ADP. If Mg^{2+} and ATP are added simultaneously, part of the bound 2-azido-ADP is rapidly replaced.

The enzyme, after incubation with 2-azido-ADP and EDTA as outlined above, before or after an ATP chase, was photolyzed and the sites of labeling determined as described earlier (Xue et al., 1987b). In the chase, the enzyme was exposed at room temperature to 5 mM ATP, 2 mM Mg^{2+}, and 40 mM Tricine at pH 8.0 for 2 min. For the experiment reported in Table I, 83% of the bound 2-azido[β-^{32}P]ADP could be rapidly replaced. The distributions of label (Table I) show only a small amount of labeling of the noncatalytic site and that most of the label before or after the chase was at catalytic sites. The observations demonstrate that both the rapidly and slowly replaced bound 2-azido[^{32}P]ADP occupied a catalytic site. We conclude that the formation of the inhibited enzyme induced by exposure to Mg^{2+} is accompanied by conversion of ADP bound at a catalytic site to a very slowly replaced form. The minor, scattered labeling at other locations reported in Table I could represent some labeling of other amino acids in the catalytic or noncatalytic peptides, the labeling of other peptides, and incomplete trypsin digestion, or decomposition that releases ^{32}P. Labeling of other regions of the β subunit of mitochondrial F_1 by 2-azido-ADP has been noted by Lunardi et al. (1987).

ADP and ATP Inhibition of GTP Hydrolysis by CF_1. Low concentrations of added ADP are known to markedly inhibit the rate of hydrolysis of ATP by chloroplast ATPase (Carmeli

Table I: Site Labeling of Heat-Activated CF_1 after Prolonged Exposure to 2-Azido[β-^{32}P]ADP and EDTA

ATP chase	distribution of recovered radioactivity (%): noncatalytic site peptide	catalytic site peptide	other	total[b] (%)
no	2.0	63	35[c]	100
yes	0.4	10.3	7[d]	17.7

[a] Conditions for labeling and chase are given in the text. Covalent modification and peptide isolation conditions are described under Experimental Procedures. [b] Total covalent labeling without chase was 0.8 mol of ADP moiety/mol of CF_1. [c] Includes an unknown peak with 8% of the radioactivity and a longer elution time than both catalytic and noncatalytic site peptide peaks, and 27% of the widely distributed base-line radioactivity. [d] Includes 2% from the unknown peak described above and 5% of the base-line radioactivity.

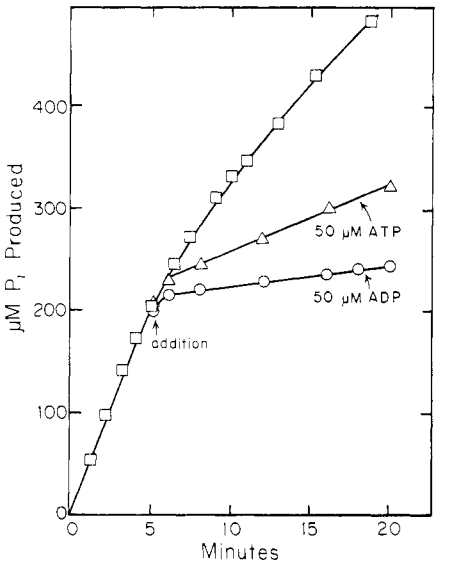

FIGURE 2: Inhibition of GTP hydrolysis by addition of low concentrations of ADP or ATP. The reaction mixture contained 40 mM Tricine, 5 mM GTP, 2 mM $MgCl_2$, and 25 nM heat-activated CF_1 at pH 7.8 and room temperature. Additions were as indicated in the figure. When ATP was used, phosphoenolpyruvate and pyruvate kinase were included in the addition so that the final concentrations in the reaction mix were 3 mM and 100 μg/mL, respectively.

Table II: Site Labeling of Heat-Activated CF_1 with 2-Azido[β-^{32}P]ADP and 2-Azido[β,γ-^{32}P]ATP during GTP Hydrolysis[a]

addition	ADP-labeled peptide: fraction of total recovered radioact. (%)	ADP-labeled peptide: distribution (%) noncatalytic	ADP-labeled peptide: distribution (%) catalytic	ATP-labeled peptides, fraction of total recovered radioact. (%)	other,[b] fraction of total recovered radioact. (%)	total peptides,[c] distribution (%) noncatalytic	total peptides,[c] distribution (%) catalytic
2-N_3-ADP	73	2	98	0	27	2	98
2-N_3-ATP	61	1	99	10	29	8	92

[a] Reaction conditions were the same as in Figure 2 except that azido[β-^{32}P]ADP and 2-azido[β,γ-^{32}P]ATP were used rather than ADP and ATP. [b] Includes 5–13% column flow-through and 14–25% of the base-line radioactivity. [c] Total covalent labeling was 0.14 mol of ADP moiety/mol of CF_1 for 2-azido-ADP and 0.18 mol of ADP moiety/mol of CF_1 for 2-azido-ATP. Calculation of the distribution assumes that all the ATP-labeled peptide is noncatalytic site peptide and takes into account that the ATP-labeled peptide has twice the specific radioactivity of the ADP-labeled peptide.

& Lifshitz, 1972; Bar-Zvi & Shavit, 1982). The occurrence of marked inhibition of GTP hydrolysis by CF_1 following the delayed addition of ADP or ATP is shown in Figure 2. When ATP was added, pyruvate kinase and phosphoenolpyruvate were also present to trap any ADP formed. The sharp inhibition by 50 μM ADP is readily evident, as is the somewhat weaker inhibition resulting from the presence of 50 μM ATP. Similar inhibitions were noted with 2-azido[β-^{32}P]ADP or 2-azido[β,γ-^{32}P]ATP. The distributions of the binding at catalytic and noncatalytic sites that result when the azido nucleotides were used are shown in Table II. One important observation is that when the enzyme was inhibited by 2-azido[β,γ-^{32}P]ATP, the covalent labeling was still largely by an ADP moiety. Nearly all of the inserted [β-^{32}P]ADP moiety was at a catalytic site whether the inhibition resulted from the binding of medium 2-azido[β-^{32}P]ADP or 2-azido[β,γ-^{32}P]-ATP. Earlier findings (Feldman & Boyer, 1985) showed that high medium P_i concentrations can help overcome the inhibition of CF_1 by Mg^{2+} and tightly bound ADP and that tightly bound ATP thus formed can be hydrolyzed in a kinetically competent manner (Feldman & Sigman, 1982). The onset of inhibition of mitochondrial F_1 ATPase after binding of ATP is correlated with the release of bound P_i, leaving ADP at a catalytic site (Drobinskaya et al., 1985). It thus seems probable that the pronounced inhibition of GTP hydrolysis by ADP or ATP depends on the presence at catalytic sites of ADP bound from the medium or formed from medium ATP after binding.

Also evident in Figure 2 is a short delay in the onset of the inhibition after the addition of the adenine nucleotides. This is documented further in the next section.

Delay in the Onset of the Mg^{2+}-Induced Inhibition of ATP Hydrolysis by CF_1. The pronounced inhibition of the activity of CF_1 by excess Mg^{2+} is well recognized [for example, see Hochman and Carmeli (1981) and Malyan (1981)]. The occurrence of this inhibition, and its absence under other conditions, is shown in Figure 3. Little or no inhibition is seen with GTP as a substrate or with Ca^{2+} as the activating cation, or with Mg^{2+} and ATP if methanol and sulfite are present. An important characteristic not previously reported and shown in this figure is the delay in onset of the Mg^{2+}-induced inhibition. This is illustrated by the curve for 5 mM ATP and 2 mM Mg^{2+}. Actually, the curve is triphasic—a short lag of 1–2 s, as shown previously (Carmeli et al., 1981; Leckband & Hammes, 1987), precedes the rapid phase, and then the pronounced inhibition sets in, leading to a lowered steady-state rate. Results in Figure 3 cover only a 60-s time period. In assays under the same conditions for longer time periods, a constant steady-state rate is attained after 1–2 min (data not shown). The attainment of a steady-state rate with lower ATP concentrations and with 2-azido-ATP hydrolysis is shown in Figure 4.

If Mg^{2+} is in excess of ATP (curve for 2 mM ATP and 5 mM Mg^{2+}), the initial burst is not seen, but a very slow hydrolysis rate is observed. The rate attained during the burst with 2 mM Mg^{2+} and 5 mM ATP is higher than that attained at any time with 2 mM Mg^{2+} and 5 mM GTP or with 2 mM Ca^{2+} replacing Mg^{2+} as the activating cation. The highest activity shown in the figure is with methanol and sulfite present, conditions that are known to promote release of tightly bound ADP (Anthon & Jagendorf, 1984).

These results and other findings prompted exploration of whether the burst in activity was related to the filling of vacant

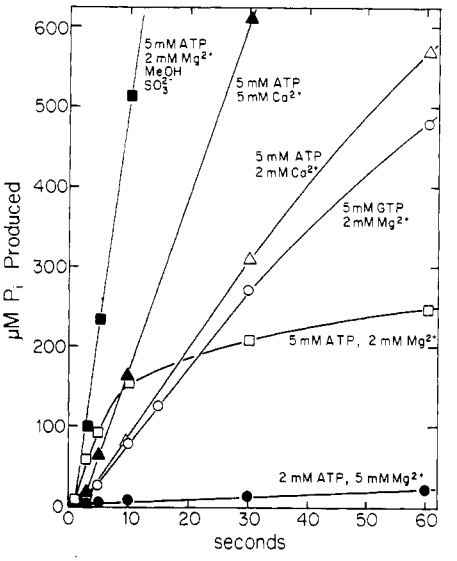

FIGURE 3: Delayed inhibition of CF_1 by Mg^{2+} and ADP and the inhibition or lack of inhibition under other conditions. Additions were as indicated in the figure. Other conditions were as given with Figure 2 except 0.3 μM heat-activated CF_1 was used.

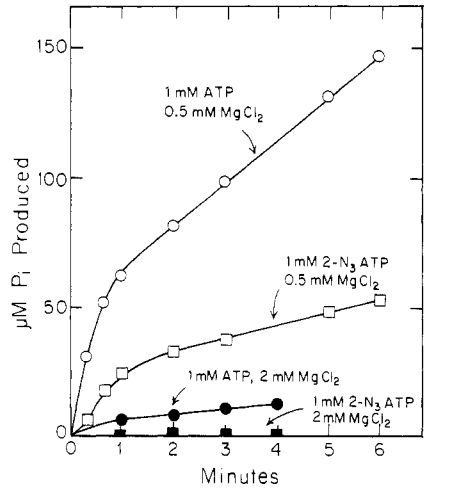

FIGURE 4: Appearance of a burst with CF_1 that has the noncatalytic sites filled. Additions were as given in the figure. Other conditions were the same as in Figure 2 except 0.13 μM heat-activated CF_1 which had been incubated first with ATP and Mg^{2+} and then EDTA, as indicated in the text, was used.

Table III: Distribution of Radioactive Label after Preloading the Noncatalytic Site of the Heat-Activated CF_1 with ATP, Followed by Exposure to 2-Azido[β,γ-^{32}P]ATP, or after Preloading with 2-Azido[β,γ-^{32}P]ATP, Followed by Exposure to ATP[a]

	ADP-labeled peptides			ATP-labeled peptides				total peptides,[c] distribution (%)	
	fraction of total	distribution (%)		fraction of total	distribution (%)		other,[b]		
preloaded nucleotide	recovered radioact. (%)	noncatalytic	catalytic	recovered radioact. (%)	noncatalytic	catalytic	fraction of total recovered radioact. (%)	noncatalytic	catalytic
2-N_3-ATP	12	23	77	75	100	0	13	82	18
ATP	70	7	93	22	94	6	8	19	81

[a] Incubation conditions are given in the text. Conditions for covalent modification and peptide isolation are the same as in Table I. [b] Base-line radioactivity widely distributed throughout. [c] Total covalent labeling corresponds to 0.5 mol of nucleotide/mol of CF_1 for preloading with 2-N_3-ATP and to 0.8 mol of nucleotide/mol of CF_1 for preloading with ATP. Calculation of the distribution takes into account that the ATP-labeled peptide has twice the specific radioactivity of the ADP-labeled peptide.

noncatalytic sites by MgATP. The vacant noncatalytic site on heat-activated CF_1, as reported earlier (Xue et al., 1987b), corresponds to the tight MgATP site demonstrated in Hammes' laboratory [see Bruist and Hammes (1981)]. To test whether the burst phenomenon was related to the filling of this site, conditions were sought where the tight MgATP site would be filled, but the enzyme would not be in the partially inhibited form as when the steady-state rate is reached, or in the markedly inhibited form, as when added to a solution with 5 mM Mg^{2+} and 2 mM ATP (Figure 3). Exploration of various conditions led to the following procedure. The tight MgATP site was filled by exposing 5 μM enzyme to 200 μM [^{3}H]ATP or 2-azido[β,γ-^{32}P]ATP and 2 mM Mg^{2+} for 2 min in 40 mM Tricine buffer at pH 8.0 and room temperature. The enzyme was then separated on a Sephadex centrifuge column containing 40 mM Tricine and 2 mM EDTA at pH 8.0. After 20 min in this solution, the enzyme still retained the [^{3}H]ATP or 2-azido[β,γ-^{32}P]ATP at a noncatalytic site. Importantly, the enzyme when then exposed to 1 mM ATP or to 1 mM 2-azido-ATP and 0.5 mM Mg^{2+} still showed a burst as reported in Figure 4. Both the burst and the steady-state rate were lower with the 2-azido[^{32}P]ATP than with ATP as a substrate. Because the burst phenomenon is seen with the 2-azido-ATP, the covalent labeling patterns can be taken as also representing sites of ATP binding.

After the treatment described in Figure 4, enzyme preparations were separated on Sephadex centrifuge columns, photolyzed, and digested with trypsin, and the catalytic- and noncatalytic-derivatized peptides were separated by HPLC. The distributions of labeled species under the different conditions are given in Table III. When vacant noncatalytic sites were preloaded with ATP, the subsequent hydrolysis of 2-azido[β,γ-^{32}P]ATP resulted in nearly exclusive labeling of catalytic sites with an ADP moiety. In contrast, when vacant noncatalytic sites were preloaded with 2-azido[β,γ-^{32}P]ATP followed by ATP hydrolysis, over 80% of the covalently incorporated nucleotide was as an ATP moiety at noncatalytic sites. As anticipated from earlier findings, the noncatalytic site retained its 2-azido-ATP during the subsequent ATP hydrolysis. These findings demonstrate that the occurrence of the burst is not the result of binding to a noncatalytic site that might have a control function but is correlated with a tight binding of ADP at catalytic sites. The factors governing the delayed onset of inhibition and determining the steady-state rate are currently under investigation.

Discussion

The results give strong support to the hypothesis that the reported inhibitory control of the ATPase activity of chloroplast ATP synthase and of the isolated CF_1 is associated with tight binding of ADP at catalytic sites. ADP effects are observed with Mg^{2+} but not with Ca^{2+} as the activating cation nor with GTP as a substrate. However, the potent inhibition of GTP hydrolysis by ADP results from catalytic site binding of the ADP.

The conversion to the inhibited form requires Mg^{2+}, as shown earlier by Carmeli et al. (1981), Malyan (1981), and Feldman and Boyer (1985). As noted by Bar-Zvi and Shavit (1982), GDP does not give a similar inactivation of the chloroplast ATPase and protects from the ADP inactivation. The ability to give the inactive form in the presence of Mg^{2+} appears to be relatively specific for ADP. The conversion of CF_1 to the inactive form occurs with the enzyme that has an ADP bound at a catalytic site without bound P_i (Feldman & Boyer, 1985). A similar behavior appears likely for the membrane-bound enzyme.

The conversion of an enzyme to an inhibited form in the presence of a metal ion and one bound substrate at a catalytic site is not an expected property. Such behavior, particularly the very slow exchange or replacement of the ADP once the inactive form has been attained, has been regarded by some as evidence against the binding change mechanism for ATP synthesis. The present findings eliminate such objections. In the binding change mechanism, during ATP cleavage tightly bound ADP and P_i are transient intermediates. At low ATP concentrations, insufficient to readily fill an alternate catalytic site, the ADP and P_i remain bound for a more prolonged period and interconvert with bound ATP to give rise to the phosphate oxygen exchanges observed [see Kohlbrenner and Boyer (1983) and O'Neal and Boyer (1984)]. During steady-state ATP hydrolysis, both bound ADP and P_i are considered to depart after the conformation at the catalytic site has been changed to loosen both of their binding. During steady-state photophosphorylation, the loosely bound ADP and P_i at a catalytic site are converted to tightly bound forms and to tightly bound ATP. When net ATP formation ceases, bound ATP is hydrolyzed to ADP and P_i, and the condition is attained so that P_i dissociates preferentially, leaving the enzyme with tightly bound ADP at the catalytic site. This is not an enzyme form that participates in either steady-state synthesis or hydrolysis with ample substrates present. It is the form that is very susceptible to conversion to an inhibited enzyme if free Mg^{2+} is present (Hochman & Carmeli, 1981).

In the experiments on photophosphorylation with chloroplast thylakoids reportd here, we obtained only a small amount of 2-azido[γ-^{32}P]ATP tightly bound to the washed thylakoid membranes. Under conditions as used in Shavit's laboratory, we observed covalent labeling of only a noncatalytic site by an ATP moiety. The site likely corresponds to the tight MgATP binding site on isolated CF_1 that has been well characterized in Hammes' laboratory [see Bruist and Hammes (1981)], and confirmed by us to be a noncatalytic site by 2-azido[β,γ-^{32}P]ATP binding (Xue et al., 1987a). Detection of a tightly bound ATP at catalytic sites, that is a transient intermediate during photophosphorylation, was shown in the studies of Rosen et al. (1979) and Smith et al. (1983) with

the ATP synthase. Similarly, when CF_1 actively hydrolyzing ATP is separated from nucleotides, a tightly bound ATP is present at the catalytic site (Wu & Boyer, 1986). With both the synthase and CF_1, over time the bound ATP is converted to bound ADP and P_i, the P_i dissociates, and only tightly bound ADP remains. Such behavior is in accord with the earlier observations of Magnusson and McCarty (1976) showing that [^{14}C]ATP that became tightly bound to the synthase in the light was present as bound [^{14}C]ADP on the isolated CF_1. Our data shown that their bound ADP was very likely at a catalytic site. In contrast, ATP or 2-azido-ATP bound at the tight MgATP site remains as such. Thus, after photophosphorylation with [^{32}P]P_i under the conditions used in experiments reported from Shavit's laboratory (Bar-Zvi & Shavit, 1982; Aflalo & Shavit, 1982), some [^{32}P]ATP binding at noncatalytic sites may occur, but, because of loss of the ^{32}P-labeled γ-phosphoryl group, little or no ^{32}P nucleotide binding at catalytic sites would be expected. Their interpretation that a tightly bound ATP at a catalytic site is not an intermediate in ATP synthesis, and that the major role of tightly bound ATP is in the modulation of the ATP synthase activities, does not seem appropriate in view of the findings reported here, as well as other evidence for a tightly bound ATP at catalytic sites as a transient catalytic intermediate. That portion of the tightly bound ATP that Shavit and coworkers noted to be slowly replaced was quite likely at noncatalytic sites and was probably not an intermediate in photophosphorylation. Similarly, the lack of replacement of tightly bound ATP on chromatophores during brief photophosphorylation (Harris & Baltscheffsky, 1979) probably reflects a noncatalytic site location and is thus not inconsistent with the binding change mechanism.

A tightly bound ADP on chloroplast thylakoids labeled under the conditions used in Strotmann's laboratory (Shavit & Strotmann, 1980) has also been studied by various investigators. The labeling is by exposure to [^{3}H]ADP for 5–20 s under photophosphorylation conditions but without P_i present. The results presented in this paper show that we obtained nearly exclusive labeling of catalytic sites under these conditions. However, as mentioned above, it is possible that some thylakoid preparations may have some of the noncatalytic sites vacant. If so, upon exposure to medium nucleotides and Mg^{2+}, the sites will be occupied by either ADP or ATP depending upon conditions. The important point is that the conditions of Shavit and Strotmann will result in prominent and perhaps nearly exclusive labeling of tight ADP on catalytic sites after photophosphorylation ceases and the membranes are washed. In related studies, Anthon and Jagendorf interpreted their results on the effect of methanol on the tightly bound [^{3}H]-ADP, labeled as mentioned above, as arguing against any model in which the tightly bound ADP inhibits by binding to catalytic sites (Anthon & Jagendorf, 1984). They recognized, however, that their results could be consistent with a catalytic site binding of the inhibitory ADP without bound P_i if the methanol-induced release of bound ADP were approximately balanced by a methanol inactivation of the enzyme to give the approximate linearity of P_i formation with elapsed time.

An inhibition associated with ADP binding to mitochondrial F_1 ATPase, delayed in onset or in recovery after ATP addition, has been widely observed (Harris et al., 1981; Di Pietro et al., 1980; Thomassen & Klungsoyr, 1983; Vasilyeva et al., 1982), and a delayed onset of a Mg^{2+} inhibition, slowly reversible by ATP, has been noted (Hackney, 1979). Mg^{2+} can produce an inhibited form without ADP addition; the converse does not appear to occur. The role of the Mg^{2+} obviously needs more clarification. The basis for these phenomena may be slow conformational changes between active and less active or inactive forms. Such slow changes likely underlie the delayed onset of inhibitions noted in Figures 2 and 4 of this paper. When a bound ADP is involved, earlier results, together with those in this paper, and the 2-azido-ATP labeling data with the mitochondrial enzyme (Cross et al., 1987), make it likely that the inhibitory ADP is at catalytic sites without bound P_i. Such behavior of the mitochondrial enzyme is in harmony with the interpretation of Drobinskaya et al. (1985), but not with the suggestion of Milgrom and Murataliev (1986) of ADP release and rebinding in a noncatalytic site.

Comment is also appropriate about another implication of our observations that the tightly bound 2-azido-ADP at catalytic sites arising from a bound 2-azido-ATP that is an intermediate in catalysis derivatizes the same tyrosine as the tightly bound ADP present in the Mg^{2+}-inhibited form. This demonstrates that both ADPs are bound to the same region of the enzyme but does not necessarily mean that the conformations of the site under the two binding conditions are identical. There could be important differences in the binding such that catalysis is favored or blocked, yet the nitrene formed on photolysis could have a location or a sufficient mobility that allows insertion in the favorably reactive tyrosine.

A final comment is that other studies on the nature and properties of the nucleotide binding sites on CF_1, such as the studies of Girault et al. (1982), or on the ATP synthase, as in the recent studies of Schumann (1987), need to be interpreted with the clear recognition of the properties of the catalytic and noncatalytic sites that can both bind nucleotides tightly, and of the conditions that lead to their labeling.

Registry No. ATPase, 9000-83-3; ADP, 58-64-0; ATP, 56-65-5; ATP synthase, 37205-63-3; Mg, 7439-95-4.

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NMR Signal Assignments of Amide Protons in the α-Helical Domains of Staphylococcal Nuclease

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ABSTRACT: We report complete assignments of the amide proton signals in the three long d_{NN} connectivity sequences observed in the NOESY spectrum of deuteriated staphylococcal nuclease (Nase) complexed with thymidine 3',5'-bisphosphate (pdTp) and Ca^{2+}, M_r 18K. The assignments are made by comparing NOESY spectra with ^1H-^{15}N and ^1H-^{13}C heteronuclear multiple-quantum shift correlation (HMQC) spectra of Nase samples containing ^{15}N- and ^{13}C-labeled amino acids. The assignments show that the residues which are linked by the d_{NN} connectivity sequences are located in the three α-helical domains of Nase. Our results indicate that by combining NOESY and HMQC spectra of appropriately labeled samples it should be possible to delineate and study α-helical domains in soluble proteins having molecular weights that are greater than 18K.

Staphylococcal nuclease, Nase,[1] is a well-characterized enzyme that has been the subject of many structure–function studies (Tucker et al., 1978, 1979a,b,c). The recent success in expressing the structural gene of Nase in *Escherichia coli* (Shortle, 1983) has generated much new interest in understanding the activity and structure of the protein (Calderon et al., 1985; Shortle & Lin, 1985; Fox et al., 1986; Evans et al., 1987; Hibler et al., 1987; Serpersu et al., 1987). Although two-dimensional proton nuclear magnetic resonance, NMR, spectroscopy is a powerful method for determining the solution structures of small proteins (Wuethrich, 1986), spectra of

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[1] Abbreviations: Nase, staphylococcal nuclease; pdTp, thymidine 3',5'-bisphosphate; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, two-dimensional NOE spectroscopy; HMQC, two-dimensional heteronuclear multiple-quantum shift correlation spectroscopy.