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Guanosine and formycin triphosphates bind at non-catalytic nucleotide binding sites of CF_1 ATPase and inhibit ATP hydrolysis

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Guanosine triphosphate and formycin triphosphate (FTP) in the presence of excess Mg^{2+} can bind to empty non-catalytic sites of spinach chloroplast ATPase (CF_1). This results in a greatly reduced capacity for ATP hydrolysis compared to the enzyme with non-catalytic sites filled with ATP. With two GTP bound at non-catalytic sites the inhibition is about 90%; with two FTP bound about 80% inhibition is obtained. Binding and release of the nucleotides from the non-catalytic sites are relatively slow processes. Exposure of CF_1 with one or two empty non-catalytic sites to 5–10 μM FTP or GTP for 15 min suffices for about 50% of the maximum inhibition. Reactivation of CF_1 after exposure to higher FTP or GTP concentrations requires long exposure to 2 μM EDTA. The findings show that, contrary to previous assumptions, GTP can bind tightly to non-catalytic sites of CF_1. They suggest that the presence of adenine nucleotides at non-catalytic sites might be essential for high catalytic capacity of the F_1 ATPases.

Spinach chloroplast adenosine triphosphatase (CF_1 ATPase); Non-catalytic site; Formycin triphosphate; Guanosine triphosphate

1. INTRODUCTION

The F_1 ATPases from various sources are regarded as having 6 nucleotide binding sites ([1–4], see [5] for review). Three of these sites appear to bind and release nucleotides rapidly during catalysis, and are considered to be catalytic nucleotide binding sites [1,5–7]. Nucleotides shown in early studies to be slowly replaced by medium nucleotides [8] are bound at non-catalytic sites. The function of nucleotides bound at the non-catalytic sites has been obscure. The sites could play a role in enzyme assembly (see [9] for such a suggestion) and have often been regarded as having a regulatory function even in the absence of supportive evidence. Preference for adenine nucleotide binding at these sites has also been observed [10–12].

Convincing evidence for the promotion of GTP hydrolysis by the binding of ATP at the non-catalytic sites has recently been obtained [13]. The filling of the non-catalytic nucleotide binding sites by adenine nucleotides markedly accelerated the rate of MgGTP hydrolysis by the F_1 ATPase isolated from spinach chloroplast membranes (CF_1). We now report experiments which show that both GTP and FTP bind slowly and tightly to the non-catalytic sites of CF_1 in the presence of excess Mg^{2+} and that such binding can strongly inhibit ATP or GTP hydrolysis. Both GDP and FDP can also bind and inhibit activity to a lesser extent. The binding of GTP to non-catalytic sites was not expected because of reports [10–12,14] indicating that tight binding at the non-catalytic sites occurred only with adenine nucleotides or with adenine nucleotide analogues.

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2. EXPERIMENTAL

CF_1, prepared as described previously [17], was heat-activated [18] in the presence of either ATP or ADP as described. CF_1, activated in the presence of the different nucleotides, will be referred to as either ATP-, or ADP-heat-activated enzyme, respectively. Formycin triphosphate was either obtained from Calbiochem or was synthesized as described [19] from formycin monophosphate (Sigma). Radiolabeled [β,γ-^{32}P]FTP and [β-^{32}P]FDP were synthesized and purified as described [18]. [8-^{3}H]GTP was purchased from ICN Radiochemicals.

ATPase assays were usually performed at pH 8 and room temperature in 50 mM Tricine buffer with 5 mM ATP, 2 mM Mg^{2+} or Ca^{2+}, and an ATP regenerating system consisting of 1 mM phosphoenolpyruvate and 100 μg/ml pyruvate kinase. Protein was determined by the Lowry method [19] with defatted bovine serum albumin as a standard. Conversion factors were based on an A_{277} at 1 mg/ml = 0.483 [20] for CF_1 and a molecular mass of 400 kDa [21] and an A_{280} for serum albumin at 1 mg/ml of 0.667 [22]. Rates of nucleotide hydrolysis were monitored either by a catalyzed P_i assay [23] or by the disappearance of NADH in a coupled assay [24].

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3. RESULTS

3.1. *CF_1ATPase is strongly inhibited if some non-catalytic sites are filled with FTP or GTP*

FTP, a close structural analogue of ATP [25], is a poor substrate for CF_1 [17]. We tested this after all the non-catalytic sites were filled with adenine nucleotide by exposing ATP-heat-activated CF_1 to 1 mM Mg^{2+} and 0.5 mM ATP for 5 min followed by Sephadex-centrifuge separation. Hydrolysis rates with 1 mM FTP and 0.5 mM Mg^{2+} were about 10% of those observed for MgATP hydrolysis under similar conditions.

To assess possible effects of nucleotide binding at non-catalytic sites, CF_1 containing partially empty non-catalytic sites was used. When CF_1 is heat-activated in the presence of ADP and passed through a Sephadex-centrifuge column, at least two of the 3 non-catalytic sites remain empty and one may be partially filled with ADP. With ATP-heat-activated enzyme only about one non-catalytic site remains empty and two are filled with ATP [13]. Complete filling of the non-catalytic sites by ATP is achieved by a short exposure to ATP in the presence of excess Mg^{2+} [13]. Table I reports the effect of exposure to Mg^{2+} and FTP or GTP on the activity of either ADP- or ATP-heat-activated enzymes. If the Mg^{2+} and FTP or GTP exposure is preceded by an exposure to Mg^{2+} and ATP, as described above, to fill all the non-catalytic sites, FTP does not inhibit ATP hydrolysis. However, strong inhibition is observed if CF_1 that has non-catalytic sites vacant is first exposed to Mg^{2+} and FTP. Similar but even more prominent inhibition occurs with GTP; binding at about two non-catalytic sites gave 90–95% inhibition.

Exposure of ADP-heat-activated CF_1 to FDP or GDP in trials similar to the above resulted in only about 7 and 20% inhibition of MgATPase, respectively. Other experiments (data not given) showed that the binding of either FTP or GTP at the non-catalytic sites decreases V_{max} but does not appreciably change the apparent K_m values for MgATP.

3.2. *Concentration and time dependence of GTP and FTP inhibitions*

The binding of FTP and GTP to non-catalytic sites is relatively slow but nearly complete binding occurs with low concentrations. For example, with 5–10 μM concentrations of FTP or GTP and 1 mM Mg^{2+} binding nears completion in 15 min and results in about 50% final inhibition of both ADP- and ATP-heat-activated enzymes.

CF1 that has non-catalytic sites loaded with either MgFTP or MgGTP remains inhibited for a long time (>5 h) after medium nucleotides are removed and if 1 mM Mg^{2+} is present. Enzyme activity is slowly regained if Mg^{2+} is omitted and 2 mM EDTA is present. For ADP-heat-activated CF_1 reactivation requires about 3 h, whereas for ATP-heat-activated CF_1 full reactivation is achieved in about 30 min in the presence of 2 mM EDTA.

Table I

Effect of binding of FTP or GTP to empty non-catalytic nucleotide binding sites of CF_1 on subsequent MgATP hydrolysis

Non-adenine nucleotides at non-catalytic sites	Activity % of control
None	100
~1 FTP	47 ± 2
~1 GTP	37 ± 2
~2 FTP	20 ± 5
~2 GTP	6 ± 3

ADP- or ATP-heat-activated CF_1 were exposed to 100 μM FTP or GTP and 1 mM Mg^{2+} for 5 min before removing free FTP by a Sephadex-centrifuge column containing 1 mM Mg^{2+} in the column buffer (50 mM Tricine pH 8). The activity of CF_1 that was exposed to Mg^{2+} and ATP to fill the non-catalytic sites (see text) was used as the control. Averages were calculated from at least 3 experimental trials with different enzyme preparations. The control specific activities were 1.3–1.6 units/mg

3.3. *Quantitation of FTP and GTP binding to CF_1*

The binding of FTP, as noted by Shoshan et al. [17], and of GTP requires Mg^{2+}. ADP- or ATP-heat-activated enzyme was exposed to 100 μM [β,γ-^{32}P]FTP or [8-^{3}H]GTP and 1 mM Mg^{2+} for 5 min, either with or without the prior exposure to MgATP, then free nucleotides removed by a Sephadex-centrifuge column containing 1 mM Mg^{2+} in the buffer. Data for the binding are shown in Table II.

Close to one FTP or GTP binds to the ATP-heat-activated enzyme, in harmony with earlier data [13] showing that one non-catalytic site is empty. The binding of over 2 FTP per F_1 with the ADP-heat-activated enzyme means that over two non-catalytic sites were originally empty or some replacement of ADP at a non-catalytic site has occurred. Binding of FDP was considerably weaker. With ADP-heat-activated CF_1 and [β-^{32}P]FDP, less than 1 FDP was retained in the non-catalytic sites (0.7 mol FDP/mol CF_1).

Table II

FTP and GTP binding to non-catalytic sites of CF_1

CF_1 preparation	Nucleotide incorporated (mol/mol CF_1)	
	FTP	GTP
ADP-heat-activated	2.51	1.86
ATP-heat-activated	1.07	0.80

Measurements were obtained as described in the text. Values are averages for 2 or 3 trials. Total FTP binding was corrected for about 0.26 mol bound to catalytic sites as assessed by binding not prevented by prior exposure to Mg^{2+} and ATP. Total binding of GTP was corrected for about 0.3 mol bound to catalytic sites as measured by GTP release by an ATP chase

Table III

Effect of FTP at non-catalytic sites on CaATP or MgFTP hydrolysis

Non-adenine nucleotides at non-catalytic sites	Activity (% of control)	
	MgFTP hydrolysis	CaATP hydrolysis
None	100	100
~1 FTP	53	–
~2 FTP	–	17

The control specific activity for MgFTP hydrolysis was 0.21 units/mg and for CaATP hydrolysis was 3.5 units/mg.

A chase with 1 mM MgATP in the presence of 1 mM excess Mg^{2+} for 2 min does not change the amount of FTP bound to non-catalytic sites but removes about 0.3 mol of guanine nucleotide/mol CF_1.

3.4. *The presence of MgFTP at the non-catalytic sites of ADP-heat-activated CF_1 enhances MgGTP hydrolysis by 50%*

The loading of non-catalytic sites with FTP gave higher rates of MgATP hydrolysis than when the non-catalytic sites were loaded with GTP. We thus measured the effect of non-catalytic site loading with MgFTP during MgGTP hydrolysis. ADP-heat-activated CF_1 was exposed to either MgGTP or MgFTP as previously described, free nucleotides were removed and MgGTPase activity were measured in the presence of 60 mM bicarbonate. MgGTP hydrolysis rates were 50% higher with MgFTP bound at the non-catalytic sites compared with MgGTP bound at the sites.

3.5. *Inhibition of MgFTP and CaATP hydrolysis, and of MgATP hydrolysis with bicarbonate activation*

The effects of FTP loading of the non-catalytic nucleotide binding sites on MgFTP and CaATP hydrolysis were measured and are shown in Table III. Hydrolysis of MgFTP by CF_1, with all non-catalytic sites loaded with ATP instead of FTP was over twice as fast as when one site contained FTP. The presence of GTP, instead of ATP at about two non-catalytic sites decreases CaATP hydrolysis rates by 83%.

The rate of MgATP hydrolysis is considerably enhanced in the presence of bicarbonate or other activating anions. As shown in Table IV even though higher steady state rates are obtained with 60 mM bicarbonate, the degree of inhibition by GTP bound at non-catalytic sites is the same.

Table IV

Effect of bound MgGTP at non-catalytic sites on MgATP hydrolysis in the presence of 60 mM bicarbonate

Non-adenine nucleotides at non-catalytic sites	Activity (% of control)
None	100
~1 GTP	41
~2 GTP	7

The control specific activity in the presence of 60 mM bicarbonate was 3.6 units/mg.

4. DISCUSSION

The results show that nucleotides other than adenine nucleotides can bind tightly at non-catalytic sites of CF_1 and can markedly influence the ability of CF_1 to hydrolyze ATP. Binding of FTP or GTP to two sites causes greater inhibition than when only one non-catalytic site is filled. The binding of GTP causes more inhibition than FTP binding.

ATP readily binds to any empty non-catalytic sites when added to CF_1 in the presence of Mg^{2+}. Thus, our results are not sufficient to show whether ATP hydrolysis may occur readily with empty non-catalytic sites, or if the filling with nucleotide is essential for ATP hydrolysis. The latter seems possible, in view of earlier results showing that filling of non-catalytic sites with ATP markedly promotes GTP hydrolysis [13].

To what extent the behavior observed with CF_1 applies to other F_1 ATPases is uncertain. Nucleotide depleted F_1 from *E. coli* was found to couple ATP cleavage to proton pumping and oxidative phosphorylation of GDP in membrane vesicles without tight GDP or GTP binding to non-catalytic sites being detected [26]. However, bound guanine nucleotides may dissociate more readily from non-catalytic sites than adenine nucleotides and might have been removed by the extensive washing procedure used. With the beef heart mitochondrial F_1 [27] binding of FTP or iso-GTP does not affect ATPase activity. The binding likely occurs at non-catalytic sites and the FTP-or iso-GTP-like ATP at non-catalytic sites may serve for full activation of the mitochondrial enzyme. Further study is obviously needed to characterize the effects of filling of non-catalytic sites on various F_1 ATPases by adenosine or other nucleoside di- or triphosphates.

The observed activity inhibitions of CF_1 by GTP or FTP at non-catalytic sites could result if the nucleotides interacted directly with catalytic site nucleotides or from indirect conformational effects. The possibility of direct interaction needs consideration because 2-azido-ATP bound at catalytic or non-catalytic sites labels tyrosines only 25 residues apart [6,7,28]. This evidence and the inherent adenylate kinase-like activity of CF_1 [29] could reflect close proximity of the catalytic and non-catalytic nucleotides.

Indirect conformational effects by nucleotide binding at the non-catalytic sites could modulate the ATP cleavage reaction or the positive catalytic site cooperativity characteristic of the F_1 ATPases. There is abundant evidence associating adenine nucleotide binding with conformational changes, including demonstrations of pronounced conformational

changes accompanying ATP binding to the isolated α and β subunits and the intact F_1 ATPase [30–32].

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