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Interaction of Sulfite with the Noncatalytic and Catalytic Sites of Chloroplast Coupling Factor CF_1

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Abstract—The interaction between sulfite, an efficient Mg^{2+}-dependent F_1-ATPase activator, and chloroplast CF_1-ATPase was studied. The sulfite anion was shown to inhibit ADP and ATP binding to the noncatalytic sites of CF_1. The stimulating activity of sulfite persists when all noncatalytic sites are nucleotide-occupied. Phosphate, a competing candidate for binding to CF_1 catalytic sites, suppresses this activity. These results support the suggestion that the stimulation of Mg^{2+}-dependent ATPase activity of CF_1 is caused by sulfite binding to its catalytic sites.

Key words: coupling factor CF_1, ATPase, ATP synthase, sulfite, chloroplasts, oxyanions

Chloroplast coupling factor CF_1, the catalytic part of chloroplast ATP synthase, is an enzymatic complex that phosphorylates ADP driven by electrochemical transmembrane proton gradient. Like coupling factors of mitochondria and bacteria, CF_1 consists of five types of subunits designated as α to ε in descending order of size that have stoichiometry of 3 : 3 : 1 : 1 : 1. At the interfaces between the α- and β-subunits there are three catalytic and three noncatalytic nucleotide binding sites. Unlike the catalytic sites, the noncatalytic sites (NS) are capable of retaining nucleotides for a rather long time and exhibit no catalytic properties. After incubating chloroplasts in low ionic strength media, CF_1 dissociates into the aqueous solution and displays properties of latent ATPase. To unmask its latent activity, CF_1 is incubated in thiol-containing media for several hours. Increasing the temperature to 65°C allows the incubation time to be decreased to a few minutes and yields higher CF_1 activity, but then the CF_1 loses its ability to bind to the membrane part of the ATP-synthase complex and to catalyze photophosphorylation. The unmasked CF_1 exhibits Ca^{2+}-dependent and only slightly Mg^{2+}-dependent ATPase activity. The low value of the latter is caused by reversible inactivation of CF_1 due to reaction product (MgADP) binding to one of its catalytic sites [1, 2]. The F_1-ATPase of mitochondria and photosynthetic bacteria display a similar though less pronounced property [3, 4]. The MgADP-induced inactivation is reduced by anions of weak polybasic acids; the most efficient of them is sulfite ion [5-7]. According to one current hypothesis, the stimulating effect of sulfite is caused by its interaction with one of the three catalytic sites of CF_1 [2]. This interaction results in loosening of MgADP binding to another catalytic site and subsequent nucleotide dissociation. This suggestion is supported by competition between phosphate and sulfite that occurs during photophosphorylation [7-9]. However, an analog of sulfite ion, the sulfate ion, can bind to CF_1 noncatalytic sites [10]. As reported, NS must be ATP-filled to display catalytic activity and to reverse MgADP-dependent inactivation of mitochondrial and bacterial coupling factors [10-12]. It is suggested that, similar to ATP to NS binding, sulfite binding to noncatalytic sites may accelerate MgADP dissociation from catalytic sites [13].

The current study is focused on the interaction of sulfite with the noncatalytic sites of the chloroplast coupling factor. It is shown that sulfite suppresses nucleotide binding to NS at the initial stage of their interaction. However, the number of nucleotide-filled NS resulting from a long-term incubation remains unaffected. Chloroplast coupling factor having all NS filled with nucleotides preserves its ability of stimulating Mg^{2+}-dependent ATPase activity with sulfite. These results are consistent with the suggestion that the stimulating activity of sulfite is caused by its binding to the catalytic sites of CF_1.

Abbreviations: DTT) dithiothreitol; NS) noncatalytic sites.

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METHODS OF INVESTIGATION

Spinach chloroplast coupling factor isolated according to Binder et al. [14] was purified by HPLC using G4000SW and DEAE 5PW columns and stored in 2 M ammonium sulfate in the presence of 1 mM ATP, 1 mM EDTA, and 50 mM Tris-SO_4. Nucleotides and ammonium sulfate were removed by forced gel filtration using a fine Sephadex G-50 column equilibrated with 50 mM Tris-HCl, pH 8.0, and 50 mM KCl. The resulting preparation contained one mole of ADP per mole of CF_1 and was ATP-free. It was activated in the presence of 2 μM thioredoxin and 2 mM dithiothreitol (DTT) at room temperature for 30-60 min. The protein concentration was determined according to Bradford [15] using a coefficient (1.25) for the low sensitivity of this method compared with that by Lowry et al. [16]. The molecular weight of CF_1 was assumed to be 400 kD [17]. The binding of nucleotide to CF_1 was performed in 50 μl of medium containing [^{3}H]ATP or [^{3}H]ADP, 2 mM $MgCl_2$, 50 mM Tris-HCl, pH 8.0, and 50 mM KCl. To maintain the [^{3}H]ATP concentration, the incubation medium also contained pyruvate kinase and phosphoenolpyruvate. Cessation of binding of labeled nucleotides and their selective dissociation from catalytic sites were performed using the "chase" method developed by Boyer and colleagues [18]. For this purpose, 1.5 mM "unlabeled" ATP and 50 mM K_2SO_3 were added to the reaction mixture; 15 sec later a 50 μl aliquot was applied onto a fine Sephadex G-50 column equilibrated with 50 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, and 50 mM KCl. CF_1 concentration was determined taking into account the presence of pyruvate kinase in the resulting fraction. After denaturation of the protein at 100°C for 1.5 min, the fraction was subjected to centrifugation (2 min at 14,000*g*) and HPLC using a 0.5 × 7.5 cm DEAE 5PW column. The eluent contained 80 mM KH_2PO_4 and 120 mM KCl. The nucleotide content was counted from radioactivity of the fractions.

ATPase activity was estimated by the rate of phosphate release [19] during incubation of CF_1 with 5 mM ATP, 6 mM $CaCl_2$, 50 mM Tris-HCl, pH 8.0, and 1 mM EDTA at 37°C. Pre-steady-state kinetics of ATP hydrolysis was studied at 22°C according to Pullman et al. [20].

RESULTS AND DISCUSSION

The isolated and purified coupling factor has rather low ATPase activity [21]. CF_1 is activated by reduction of the disulfide bond of the γ-subunit either by short incubation of the CF_1 in the presence of a small amount of DTT at an elevated temperature (65°C) [22] or by its long-term incubation (14 h) at high DTT concentrations at ambient temperature [23]. In both cases ATP is used to stabilize the CF_1. As a result, nucleotides bind to noncatalytic sites as early as at the step of ATPase activity unmasking, thus making difficult the study of their interactions. The current study utilized the ability of thioredoxin to accelerate DTT-induced reduction of the γ-subunit disulfide bond [24]. As seen from Fig. 1, in the presence of 2 μM thioredoxin and 2 mM DTT the maximal activation of CF_1-ATPase is achieved after 30 min, whereas it takes more than 3 h at high DTT concentrations. The shortened procedure of activation in the presence of thioredoxin eliminates the requirement for CF_1-stabilizing nucleotides, and therefore in the resulting CF_1 all three noncatalytic nucleotide binding sites are vacant. At room temperature, the noncatalytic sites of DTT-activated CF_1 bind nucleotides less tightly than those of heat-activated CF_1 [10]. Therefore, it was necessary to determine to what extent the noncatalytic site-bound nucleotide quantification technique developed for heat-activated CF_1 [18] is applicable in the case of thioredoxin-activated CF_1. To answer this question, thioredoxin-activated CF_1 was incubated with [^{3}H]ATP and Mg^{2+} for 40 min and then subjected to gel filtration either immediately or after addition of surplus unlabeled ATP and sulfite. The sample applied on a column immediately after incubation contained about 3.5 moles of tightly bound ^{3}H-labeled nucleotides per mole of CF_1. Approximately the same quantity of nucleotides was bound by heat-activated CF_1 [10, 25]. As follows from Fig. 2, addition of ATP and sulfite to the reaction medium initiated a decrease in radioactivity caused by exchange of labeled for unlabeled nucleotides. About 0.4 mole of nucleotide is removed during the first 15 sec of

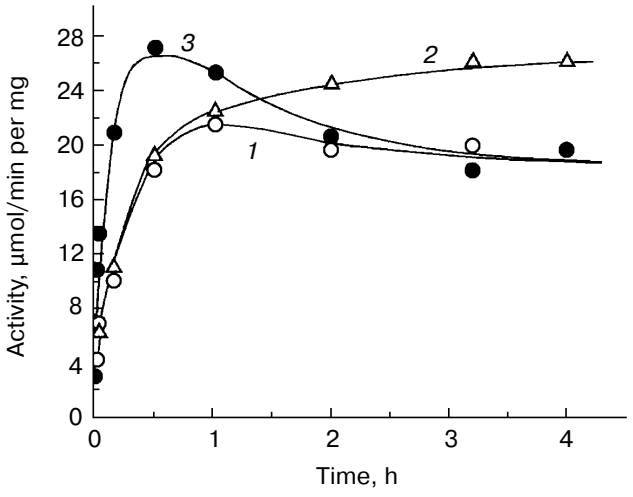

Fig. 1. Activation of latent chloroplast CF_1-ATPase. CF_1 (1-2 mg/ml) was incubated at 22°C with 50 mM Tris-HCl, pH 8.0, and 50 mM KCl in the presence of 40 mM DTT (*1*), 40 mM DTT and 1 mM ATP (*2*), 2 mM DTT and 2 μM thioredoxin (*3*).

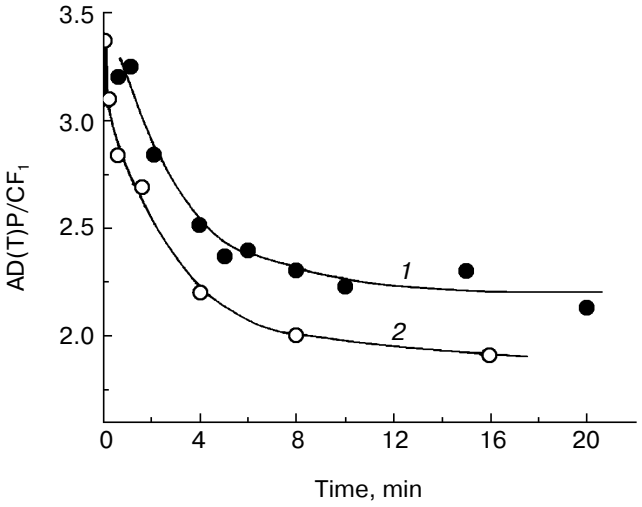

Fig. 2. Effect of sulfite on dissociation of tightly bound nucleotides. CF_1 (0.72 mg/ml) was incubated for 40 min with 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 30 μM [^{3}H]ATP, 2 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 0.2 mg/ml pyruvate kinase, and added 1.5 mM ATP (*1*) or 1.5 mM ATP and 50 mM K_2SO_3 (*2*). At indicated intervals, 50 μl aliquots were applied onto a Sephadex G-50 column and the quantity of tightly bound nucleotides was determined.

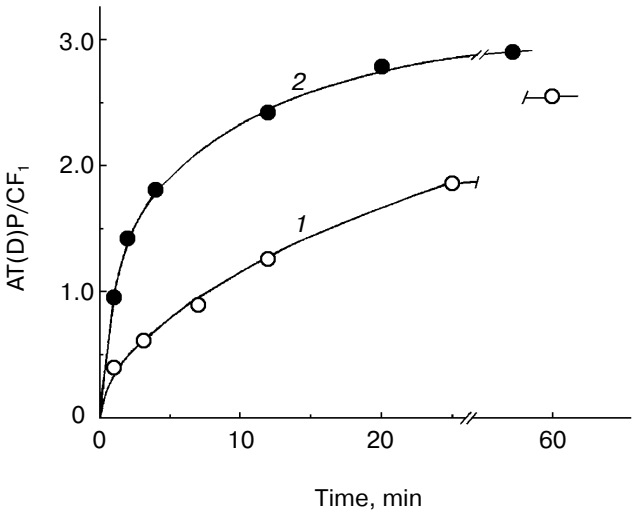

Fig. 3. Effect of sulfite on kinetics of nucleotide binding to CF_1 noncatalytic sites. CF_1 (0.26 mg/ml) activated with thioredoxin and DTT was incubated with 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 2 mM $MgCl_2$, 13 μM [^{3}H]ATP, 1 mM phosphoenolpyruvate, and 0.13 mg/ml pyruvate kinase in the presence (*1*) or absence (*2*) of 5 mM K_2SO_3. At indicated intervals, 1.5 mM ATP and 50 mM K_2SO_3 were added to the medium; 15 sec later an aliquot was applied onto a Sephadex G-50 column and the quantity of nucleotides tightly bound to noncatalytic sites was determined.

incubation. Then the process slows down, and after 8 min about 2 mol of labeled nucleotide still remains bound to the CF_1. In the absence of sulfite, which stimulates catalytic activity many-fold, the phase of rapid radioactivity decrease was not observed. This suggests that the rapid phase occurs due to displacement of labeled nucleotides from the catalytic sites by unlabeled nucleotides. CF_1 activity measured in the control and amounting to 150 rpm supports this suggestion. For further experiments, it was assumed that 15 sec incubation with 1.5 mM unlabeled ATP and 50 mM sulfite was sufficient for selective removal of nucleotides from the catalytic sites of CF_1.

Figure 3 presents the kinetics of ATP binding to noncatalytic sites. It reveals a rapid initial phase within 1-2 min and a subsequent slow phase during which nucleotide binding to all noncatalytic sites is accomplished. The presence of 5 mM sulfite makes nucleotide binding much slower; however, the sulfite effect decreases with increasing incubation time. After 1 min, the inhibition was 60%, after 20 min 46%, and after 60 min only 14%. The dependence (in reverse coordinates) of the quantity of nucleotides bound after 1 min on ATP concentration is approximated by linear functions (Fig. 4). This approximation requires that only a minor fraction of the ATP sites are filled within the ATP concentration range of 5-22 μM. Extrapolation of these plots shows that, irrespective of the presence of sulfite, high ATP concentrations provide for binding at three noncatalytic sites. The data can be explained by the suggestion that at the initial stage ATP competes with sulfite for reversible binding to noncatalytic sites. According to the experiment, the apparent ATP dissociation constant and sulfite-induced inhibition constant are 25 μM and 2 mM, respectively.

Figure 5 illustrates the effect of 10 min preincubation of CF_1 with 0.1 mM MgATP on the stimulating effect of sulfite. As shown by chromatography, this preincubation results in binding ~3 moles of nucleotides, where ATP amounts to 2.5 moles and ADP to 0.5 mole. As seen from Fig. 5, the maximal activation of CF_1-ATPase is achieved within 9 sec, whereas $t_{1/2}$ of nucleotide dissociation from the most labile noncatalytic site amounts to about 4 min (Fig. 2). This means that the stimulating activity of sulfite remains unaffected by ATP binding at all NS. However, with only some NS occupied by ATP, a possibility cannot be ruled out that the stimulation is caused by sulfite binding specifically to an NS region interacting with ATP γ-phosphate and remaining free during ADP binding. To obtain more accurate results, we studied the effect of preincubation of CF_1 with MgATP on the stimulating effect of quercetin, which is similar to adenosine in structure [26]. As shown previously, quercetin stimulates Mg^{2+}-dependent activity of CF_1-ATPase at Mg^{2+}/ATP = 0.2-0.3 and competitively inhibits it at higher values of this ratio [27]. Taking into account the ability of quercetin to interact with compo-

nents of the ATP-regenerating system, pyruvate kinase and lactate dehydrogenase, ATPase activity was estimated from released inorganic phosphate [19]. Addition of 25 μM quercetin to the reaction mixture was found to induce identical (1.7-fold) increases in ATPase activity for CF_1 preincubation with 1 mM Mg^{2+} and with 1 mM Mg^{2+} plus 100 μM ATP. These results indicate that stimulation of ATP hydrolysis is independent of the interaction between stimulators and noncatalytic sites of CF_1. Competition of sulfite with inorganic phosphate during photophosphorylation was a basis for the previous suggestion [8, 9] on a possible interaction of sulfite with catalytic sites of CF_1. To shed light on the origin of the CF_1 sites responsible for stimulation of the reaction, we studied the effect of inorganic phosphate on sulfite activation. In the presence of phosphate, the ATP hydrolysis rate remained almost unchanged, though stimulation of the reaction was markedly lower (Fig. 5, curve *3*). This supports the idea that the sulfite-induced stimulation of ATP hydrolysis results from an interaction of sulfite with catalytic sites of CF_1. Additional supporting evidence is stimulation of the reaction with millimolar concentrations of ATP [2], as well as its competitive inhibition by a calcium derivative of quercetin [27]. This mechanism is adequately accommodated by the earlier proposed scheme of reversible CF_1-ATPase inactivation by MgADP, according to which inactivation is accompanied by concerted changes in properties of all catalytic sites

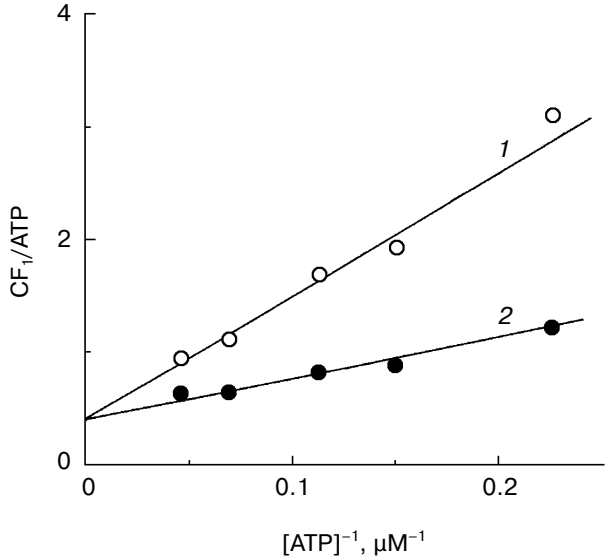

Fig. 4. Adenosine triphosphate binding at noncatalytic sites as dependent on ATP concentration in the presence (*1*) or absence (*2*) of 5 mM K_2SO_3. CF_1 (0.18 mg/ml) activated with thioredoxin and DTT was incubated for 1 min with 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 2 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 0.12 mg/ml pyruvate kinase, and indicated amounts of [^{3}H]ATP. Then the quantity of nucleotides tightly bound to noncatalytic sites was determined.

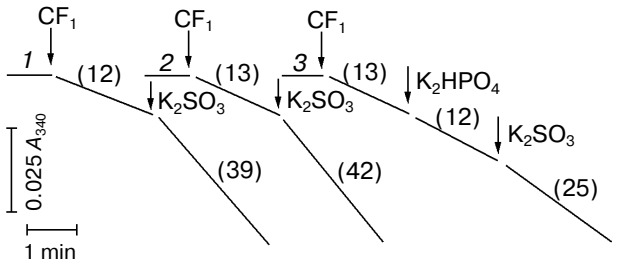

Fig. 5. Effects of CF_1 preincubation with MgATP and of the presence of phosphate on sulfite-induced stimulation of Mg^{2+}-dependent CF_1-ATPase activity. *1*) ATP hydrolysis without preincubation of CF_1 with MgATP; *2*, *3*) ATP hydrolysis after 10 min incubation of CF_1 with 0.1 mM MgATP. The medium contained 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 10 mM $MgCl_2$, 1.5 mM ATP, 1 mM phosphoenolpyruvate, 6 units of pyruvate kinase, 8 units of lactate dehydrogenase, and 0.064 mg/ml CF_1. The times of addition of CF_1, K_2SO_3 (2.5 mM), and phosphate (5 mM) are indicated by arrows. Activity values (nmol/min per mg) are given in brackets.

[2]: one of them tightly binds MgADP, another binds MgATP, and the third becomes vacant due to a significant decrease in its affinity for nucleotides. This distribution of nucleotides among catalytic sites was confirmed by X-ray analysis of the spatial structure of mitochondrial ATPase at 250 μM of a non-hydrolyzable ATP analog [28]. According to a proposed scheme [2], during CF_1-ATPase reactivation (stimulation) the site that was vacant in inactivated CF_1 binds HSO_3^-, while two other sites alternatively participate in ATP binding, hydrolysis, and in dissociation of reaction products. This mechanism ensures a high activity of CF_1 in two-site catalysis. As reported in [29], at optimal sulfite concentrations the activity is as high as 77 μmol/min per mg, this corresponding to a turnover of ~500 sec^{-1}. A high ATPase activity was reported for two-site catalysis of mitochondrial ATPase [30]. According to the rotation catalysis scheme [31], binding and dissociation of the stimulating anion can occur consecutively with binding and conversion of ATP, the process involving all the three catalytic sites. Their participation in stimulation of CF_1 activity does not rule out involvement of noncatalytic sites. It can be easily suggested that CF_1-ATPase reactivation, like MgATP-dependent inactivation, requires concerted changes in conformation of the CF_1 β-subunits. The effect of nucleotide binding to NS on stimulation of F_1-ATPase reported earlier [11, 12] is probably determined by the role of NS in cooperative functioning of the β-subunits [32, 33].

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