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INFLUENCE OF SULFITE ON ATPase ACTIVITY OF COUPLING FACTOR CF_1 ISOLATED FROM SPINACH CHLOROPLASTS

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The present study of sulfite participation in the regulation of the chloroplast ATP-synthase complex can help to understand the mechanisms of acid rains toxic effects on the plant photosynthetic apparatus. The aim of the work was to study the effect of sulfite on Ca^{2+}- and Mg^{2+}-dependent ATP hydrolysis catalysed by coupling factor CF_1, exposed to preliminary short-term acid treatment. CF_1 was isolated from spinach chloroplasts (Spinacia oleracea L.). The latent ATPase activity of CF_1 was activated by sodium sulfite addition into incubation media. The rate of ATP hydrolysis was determined by the release of inorganic phosphate. In the presence of 25 mM exogenous sulfite the Mg^{2+}-dependent hydrolytic activity of CF_1 was increased 7-fold and Ca^{2+}-dependent activity 3-fold compared to the control. Carbonic anhydrase inhibitors acetazolamide or ethoxyzolamide eliminated sulfite-induced stimulation of ATP hydrolysis. The sulfite stimulating effect on Ca^{2+}- and Mg^{2+}-ATPase activity was increased dramatically after incubation (5 min) of isolated CF_1 in a medium with pH 3.5 and its subsequent transition to the alkali medium (pH 8.0). In this case, 1 mM sulfite-induced a 10-fold acceleration of ATP hydrolysis. Carbonic anhydrase specific inhibitors (50 μM) removed the sulfite effect. These data suggest that sulfite was able to replace bicarbonate in the CF_1 structure after the release of bound HCO_3^- during acid treatment.

K e y w o r d s: spinach chloroplasts, CF_1-ATPase, ATP hydrolysis, pH-dependence, sulfite, acetazolamide, ethoxyzolamide.

The H^+-ATP synthase complex of chloroplasts (CF_oCF_1) is a reversible ATP synthase/ATPase (EC 3.6.3.14) [1]. Its catalytic part – the coupling factor CF_1 –consists of 5 types of subunits (α, β, γ, δ, ε) in stoichiometry of 3: 3: 1: 1: 1 [1-3]. *In vivo*, the hydrolytic activity of CF_1 is blocked in the dark but it can grow under reduction of the disulfide bond of the γ-subunit due to light energization of chloroplasts [4]. As the high energy state dissipates, the ATPase activity of CF_1 disappears after turning off the light. Obviously, the blocking of the hydrolytic activity of the enzyme prevents unproductive energy loss in the dark [5]. After separation of the CF_1 from the thylakoid membrane, its ATPase activity remains very low and is stimulated under heating, limited trypsin proteolysis, treatment with alcohols, detergents or oxyanions [3, 6]. Among the latter, sulfite is the most effective stimulator of the hydrolytic activity of membrane-bound and isolated CF_1 [5-7].

Significant stimulation of ATP hydrolysis rate at the addition of exogenous sulfite was observed in many organisms, including archaebacteria [8], cyanobacteria [9], *Rhodospirillaceae* [10], and mammalian mitochondria [11, 12]. As the sulfite concentration increases from 0 to 100 mM, V_{max} of the hydrolytic reaction increases, while K_M for MgATP remains unchanged, indicating a gradual transition of enzymes pool with low activity to a highly active state [13].

It has been proven that the blocking of the catalytic activity of isolated F_1-ATPases is caused by the tight binding of ADP and Mg^{2+} ions in the regulatory centers located on the border of the α and β subunits

of the enzyme [3]. A total of 6 nucleotide binding sites were characterized in CF_1, three of which do not directly participate in the catalytic stages of ATP hydrolysis, but play a regulatory role [1-3].

It is believed that in result of sulfite treatment bound ADP and Mg^{2+} is removed from the regulatory centers, which block the enzymatic activity of CF_1, and the reaction rate increases to values characteristic of the membrane-bound enzyme [3].

Recently, we have studied the effect of bicarbonate anions on CF_1 reactivation and showed [14], that the hydrolytic activity of CF_1-ATPase is stimulated by much lower concentrations of bicarbonate and the level of activation increased after a short acid shock. Since bicarbonate stimulation of ATP hydrolysis was completely eliminated by specific carbonic anhydrase inhibitors, it was suggested that carbonic anhydrase activity [15] was involved in the regulation of CF_1-ATPase. Considering the structural similarity of sulfite and bicarbonate anions, as well as the fact that sulfite is a more effective stimulator of CF_1-ATPase compared to bicarbonate [16, 17], the purpose of this work was to determine the effect of sulfite on the Ca^{2+}- and Mg^{2+}-ATPase activity of CF_1 after short-term acid treatment and comparison of these data with the effects of bicarbonate, studied previously.

Materials and Methods

Chloroplasts were isolated from fresh spinach leaves (*Spinacia oleracea* L.), as described previously [18]. Chloroplast pellet was resuspended in medium containing 400 mM sorbitol, 10 mM NaCl, 10 mM KCl, 2.5 mM $MgCl_2$ and 50 mM Tris-HCl (pH 7.8) and centrifuged for 1 minute at 800 g to remove aggregates and large cell fragments. After 10 minutes of osmotic shock in a hypotonic medium containing 10 mM Tris-HCl (pH 7.8) and 10 mM $Na_4P_2O_7$, the thylakoid membranes were washed three times from the stromal components by centrifugation (10 min, 10,000 g) and resuspension in the same solution. The coupling factor CF_1 was isolated by Schtrotmann's method [19] with some modifications. In particular, 1 mM $MgCl_2$ was added to washing medium for more complete removal of RuBP-carboxylase. All operations on the isolation of thylakoids and CF_1 were performed at 4 °C. The resulting preparation of CF_1 was desalted by passage through two consecutive Sephadex G-50 centrifuge columns equilibrated with 50 mM Tris-HCl (pH 7.8), 50 mM NaCl and 2 mM EDTA. The protein was loaded on to a DEAE A-50 column, equilibrated with 20 mM Tris-HCl (pH 7.8), 10 mM NaCl and washed with 20 mM Tris-HCl (pH 7.8), 100 mM NaCl. The CF_1 fraction was eluted with a solution of 20 mM Tris-HCl (pH 7.8), 400 mM NaCl [20]. Purity was evaluated by an electrophoretic method with a published protocol [15]. The protein concentration in the coupling factor preparations was determined according to Lowry [21].

The acid-base transition was carried out by mixing solutions A and B. Solution A contained 10 mM sodium succinate and 10 μg of isolated protein. Solution B contained 50 mM Tris-HCl (pH 8.5). The pH of solution A was adjusted to 3.5 with subsequent incubation for 5 min. After that, the calculated amount of the solution B was added to the solution A to a final pH of 8.0 and the mixture was incubated for 5 min at room temperature.

ATPase activity was determined at 37 °C by the amount of inorganic phosphorus formed in the reaction solution containing 1 mM ATP, 50 mM Tris-HCl (pH 7.8), 10 mM $CaCl_2$ (or $MgCl_2$), 1-25 mM Na_2SO_3 and was expressed in μmol $P_i \cdot (mg\ protein)^{-1} \cdot min^{-1}$. The amount of P_i in the sample was determined by the method of Lowry and Lopez in the modification Skulachev [22].

In the work, salts and trichloroacetic acid of the reagent grade were used. ATP (adenosine triphosphate acid disodium salt), acetazolamide (N-[5-(Aminosulfonyl)-1,3,4-thiadiazol-2-yl]acetamide (AA), ethoxyzolamide (6-ethoxybenzothiazol-2-sulfonamide (EA) were acquired in the company Sigma (USA).

Experiments were performed in triplicate and repeated three times with similar results. The mean values and their standard deviations were determined. When comparing samples, Student's *t*-test was used, the differences were considered significant at $P \leq 0.05$.

Results and Discussion

Fig. 1 shows the dependence of the rate of ATP hydrolysis on the concentration of exogenous sodium sulfite. It can be seen that both Mg^{2+}- and the Ca^{2+}-dependent reaction are significantly stimulated by the addition of 10-25 mM of Na_2SO_3. The positive effect of sulfite on the rate of Mg^{2+}-dependent hydrolysis was significantly greater compared with the Ca^{2+}-dependent reaction.

In the presence of 25 mM Na_2SO_3, the rate of Mg^{2+}-ATPase reaction exceeded Ca^{2+}-ATPase reac-

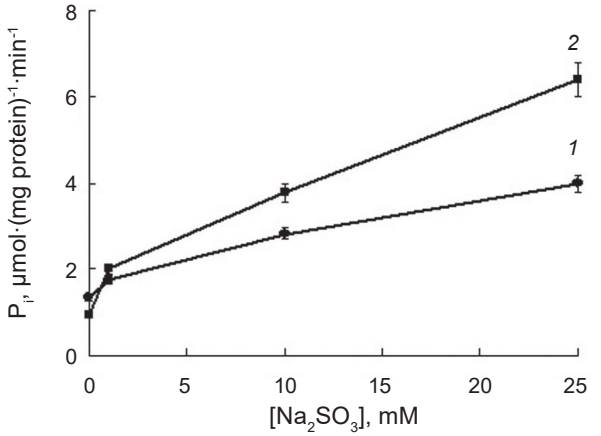

Fig. 1. Dependence of the rate of ATP hydrolysis by isolated CF_1 on the concentration of exogenous sodium sulfite: 1 – Ca^{2+}-dependent hydrolysis; 2 – Mg^{2+}-dependent hydrolysis. Composition of the reaction mixture: 1 mM ATP, 50 mM Tris-HCl (pH 7.8), 10 mM $CaCl_2$ (or $MgCl_2$), 1-25 mM Na_2SO_3, and 10 μg of isolated protein. The mixture was incubated for 30 min at 37 °C

tion rate almost twice. Such a sulfite effect is consistent with the results obtained by Malyan [3], who showed that in the presence of sulfite, ADP- and Mg^{2+}-dependent inactivation of CF_1-ATPase is eliminated, whereas Ca^{2+}-dependent activity is stimulated much less.

The effect of specific carbonic anhydrase inhibitors is shown in Fig. 2. It can be seen that increasing concentrations of water-soluble acetazolamide and lipophilic ethoxyzolamide inhibit Ca^{2+}-ATPase activity of isolated CF_1. The effect of EA was more pronounced at low concentrations (from 1 to 5 μM). Adding 1 μM EA caused a sharp decrease in ATP hydrolysis rate, and at higher concentrations, the effects of both inhibitors were comparable.

As we found earlier, when studying the effects of bicarbonate on CF_1-ATPase activity, the rate of catalysis increased significantly after a short-term incubation of the enzyme preparation in weakly acid media [14]. pH-dependent stimulation was removed when carbonic anhydrase inhibitors were present in the reaction medium. To compare the effects of bicarbonate and sulfite on the enzymatic activity of CF_1, we investigated the effects of acid pre-incubation on Mg^{2+}- and Ca^{2+}-ATPase activity as well as the effects of carbonic anhydrase inhibitors.

The data presented in Fig. 3 (*A* and *B*) show that short-term incubation of isolated CF_1 at pH 3.5 with subsequent transfer to a buffer medium with pH 8.0 resulted in stimulation of the activity of Ca^{2+}- and Mg^{2+}-ATPase 4-6 times, respectively.

In the presence of carbonic anhydrase inhibitors at a final concentration of 50 μM in the reaction medium, ATPase reaction was not accelerated compared with the control (latent) activity of CF_1. If 1 mM of sodium sulfite was added to the medium, the rate of the Ca^{2+}-dependent ATPase reaction increased 7-fold, and the Mg^{2+}-ATPase activity increased almost 10-fold compared to the control (pH 8.0). The addition of 50 μM AA or EA removed the stimulation with sulfite, and the reaction rate decreased to values that were obtained without sulfite.

According to the literature [12, 17], sulfite is the most effective activator of F_1-ATPase, and its stimulating effect develops in the concentration range from 0 to 100 mM [13, 16]. In the presence of exogenous sulfite, Mg^{2+}-ATPase is stimulated to a greater degree, than Ca^{2+}-dependent activity [3]. To activate Ca^{2+}- and Mg^{2+}-dependent hydrolysis in this work, we used 25 mM sodium sulfite. This concentration caused a 7-fold and almost 4-fold acceleration of Mg^{2+}- and Ca^{2+}-dependent CF_1-ATP, respectively (Fig. 1). Stimulation of the hydrolytic activity of CF_1 was completely eliminated by the addition of carbonic anhydrase inhibitors to the reaction medium – acetazolamide or ethoxyzolamide. This result sug-

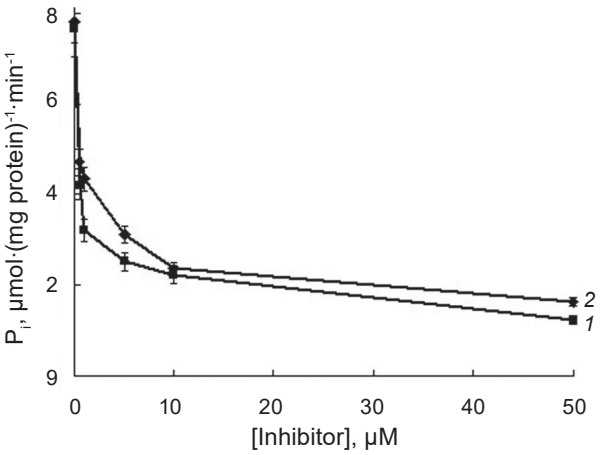

Fig. 2. Effect of carbonic anhydrase inhibitors ethoxyzolamide (1) and acetazolamide (2) on the rate of Ca^{2+}-dependent ATP hydrolysis by CF_1 under activation with sodium sulfite. The composition of the reaction medium: 1 mM ATP, 50 mM Tris-HCl (pH 7.8), 10 mM $CaCl_2$, 25 mM Na_2SO_3, and 10 μg of isolated protein. The mixture was incubated for 30 min at 37 °C

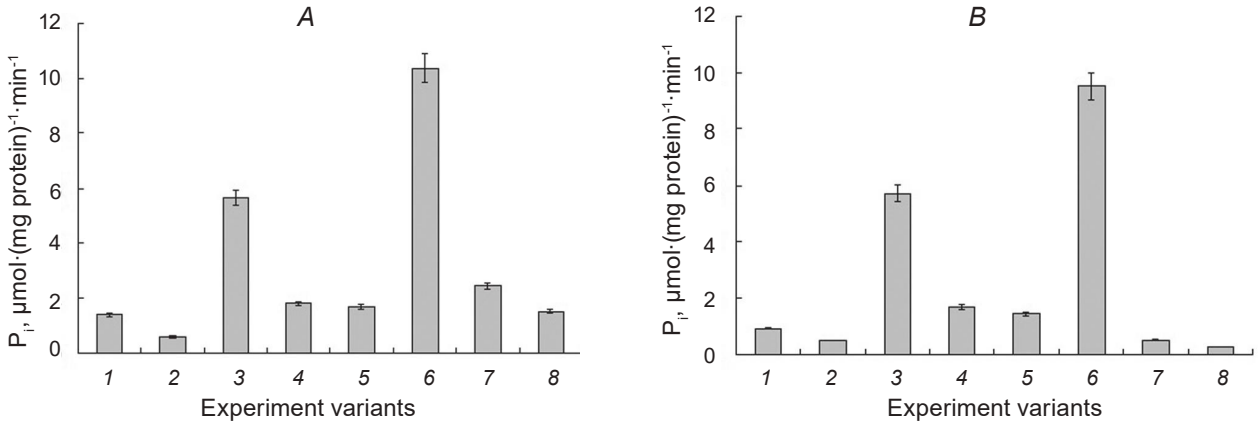

*Fig. 3. Effect of sulfite and carbonic anhydrase inhibitors on Ca^{2+}-ATPase (**A**) and Mg^{2+}-ATPase (**B**) activity of isolated CF_1 after a short-term incubation at pH 3.5 and subsequent transfer to the medium with pH 8.0. Acid-base transition was carried out as described in the Materials and methods. When activated by sulfite, the reaction medium contained: 1 mM ATP, 50 mM Tris-HCl (pH 7.8), 10 mM $CaCl_2$ (or $MgCl_2$) and 1 mM Na_2SO_3. The mixture was incubated for 30 min at 37 °C. The designations here: 1 – pH 8.0 (control); 2 – pH 3.5; 3 – pH-transition 3.5→8.0; 4 – pH-transition 3.5→8.0 + 50 μM AA; 5 – pH-transition 3.5→8.0 + 50 μM EA; 6 – pH-transition 3.5→8.0 + 1 mM Na_2SO_3; 7 – pH-transition 3.5→8.0 + 1 mM Na_2SO_3 + 50 μM AA; 8 – pH-transition 3.5→8.0 + 1 mM Na_2SO_3 + 50 μM EA*

gests that their suppression of sulfite stimulation of ATP hydrolysis may be linked to the involvement of carbonic anhydrase activity associated with CF_1 [15], this way sulfite is able to bind to the putative bicarbonate centers of the enzyme.

This assumption is supported by the data obtained from studying the effect of acid shock on the character of the sulfite effect. Immediately after preincubation in a medium with pH 3.5, the level of latent ATPase activity was 2-2.5 times lower than the control (pH 8.0), that is, acid incubation caused the enzyme inhibition (Fig. 3). In the presence of 1 mM sodium sulfite in the reaction medium during pH-transition 3.5→8.0, the hydrolytic activity of CF_1 increased (Fig. 3) and significantly exceeded the maximum value measured for untreated enzyme in the presence of 25 mM of this anion (Fig. 1). Thus, a short acid shock not only did not inhibit the potential enzymatic activity of CF_1, but also increased its sensitivity to the stimulatory effect of sulfite.

Previously, a similar effect was observed in the examination of acid shock effect on the sensitivity of CF_1-ATPase to stimulation by bicarbonate anions [14]. Based on the data obtained in the cited paper, an assumption was made about the existence bound bicarbonate in the CF_1 structure which is involved in the regulation of enzymatic activity. The putative bound bicarbonate can participate in the protonation/deprotonation of substrates during the synthesis/hydrolysis of ATP. Its equilibrium with various forms of carbonic acid (CO_3^{2-}, HCO_3^-, H_2CO_3, CO_2), constantly present in water solutions, is under control of carbonic anhydrase activity of CF_1. During acid treatment, bicarbonate in its binding sites is protonated to form unstable carbonic acid which decomposes into CO_2 and H_2O released from the medium. After depletion from bound bicarbonate, the sensitivity of free from bound bicarbonate CF_1-ATPase to stimulating action of anions , including the sulfite anion, increased significantly as evidenced by the results of this and previous works. Herewith, the stimulation with sulfite of ATPase activity is completely eliminated by the addition of carbonic anhydrase inhibitors.

Thus, the results of the work show that sulfite, as well as bicarbonate [14], can completely restore the ATPase activity of CF_1 after acid treatment of the enzyme.

In nature, sulfite is formed by the interaction of sulfur oxide (SO_2), which is emitted in large quantities into the atmosphere during the combustion of coal and wood. SO_2 is present in significant quantities in exhaust gases of automobiles, in industrial gases – emissions from metallurgical enterprises and thermal power plants. Reacting with the water of the atmosphere, this oxide turns into a solution of sulphurous acid, and as a part of rain or snow falls to the ground.

Acid rain has a very negative effect on plants, leading to characteristic symptoms of leaf damage, biochemical, ultrastructural and functional changes in the photosynthetic apparatus [23], the mechanisms of which have not yet been fully studied. Since ATP hydrolysis in chloroplasts is a process in which energy stored at photosynthesis unproductively dissipates the increase in hydrolytic activity of the catalytic part of the chloroplast H^+-ATP synthase (factor CF_1) under the action of sulfite from acid rainfall may be one of the mechanisms of the negative impact of such an anthropogenic phenomenon as acid rain.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

ВПЛИВ СУЛЬФІТУ НА АТРазну АКТИВНІСТЬ ЧИННИКА СПРЯЖЕННЯ CF_1, ІЗОЛЬОВАНОГО ІЗ ХЛОРОПЛАСТІВ ШПИНАТУ

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Проведені дослідження з розкриття механізмів участі сульфіту в регуляції АТР-синтазного комплексу хлоропластів є актуальними з огляду впливу кислотних дощів на фотосинтетичний апарат рослин. Метою роботи було вивчення дії сульфіту на Ca^{2+}- і Mg^{2+}-залежний гідроліз АТР чинником спряження CF_1 із хлоропластів шпинату, які піддавали попередній кислотній інкубації. CF_1 ізолювали із хлоропластів шпинату (*Spinacia oleracea* L.). Латентну (приховану) АТРазну активність CF_1 активували додаванням у середовище сульфіту натрію. Швидкість гідролізу АТР визначали за звільненням неорганічного фосфату. За наявності 25 мМ екзогенного сульфіту Mg^{2+}-залежна гідролітична активність CF_1 зростала у 7 разів, а Ca^{2+}-залежна – у 3 рази порівняно з контролем. Інгібітори карбоангідрази ацетазоламід або етоксизоламід усували стимуляцію гідролізу АТР сульфітом. Спричинена сульфітом стимуляція Ca^{2+}- і Mg^{2+}-АТРазної активності значно посилювалась після інкубації (5 хв) ізольованого CF_1 за pH 3,5 із наступним переведенням у середовище з pH 8,0. За таких умов 1 мМ сульфіт спричиняв 10-кратне прискорення гідролізу АТР. Специфічні інгібітори карбоангідраз (50 мкМ) знімали стимулювальний ефект сульфіту. Одержані дані дозволяють припустити, що сульфіт здатен заміщувати бікарбонат у структурі CF_1 після вивільнення зв'язаного HCO_3^- внаслідок кислотної інкубації.

Ключові слова: хлоропласти шпинату, CF_1-АТРаза, гідроліз АТР, pH-залежність, сульфіт, ацетазоламід, етоксизоламід.

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