UDC 577.23 + 547.327 doi: https://doi.org/10.15407/ubj92.04.096

# SULFONAMIDES INFLUENCE ON THE ACTIVITY OF THYLAKOID ATPase ISOLATED FROM SPINACH CHLOROPLASTS

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**Received:** 13 September 2019; **Accepted:** 15 May 2020

*The effect of two water-soluble sulfonamides: aromatic 4-aminobenzenesulfonamide (SA) and aliphatic trifluoromethylsulfonamide (TFMSA) on the latent and stimulated ATPase activity of the catalytic part of the thylakoid ATP synthase isolated from spinach (Spinacia oleracea L.) chloroplasts was studied. Three methods of ATPase activity stimulation were applied in the work: brief heating of the enzyme at 62 °C; incubation with 20% methanol; 25 mM sodium sulfite adding to the reaction medium. TFMSA effectively inhibited the ATPase activity of the enzyme at all types of activation.* $I_{50}$ *(TFMSA) value for was found to be about 5 μM in the case of enzyme heat treatment and 1 μM in the case of enzyme activation with sulfite or methanol. SA did not affect ATPase activity at stimulation with sulfite and inhibited with* $I_{50}$ *about 50 μM at stimulation by heating or methanol. No effect of SA on latent enzyme activity was observed, while TFMSA was shown to activate latent ATPase by about 6 times. The data obtained showed that both sulfonamide compounds, known as carbonic anhydrase inhibitors, are able to modify the activity of chloroplast ATPase.*

*Keywords: spinach chloroplasts,* $CF_1$*-ATPase, ATP hydrolysis, sulfonamides, trifluoromethanesulfonamide, 4-aminobenzenesulfonamide.*

Sulfonamide preparations are an important class of compounds that became the first antibacterial agents for systemic use, starting the antibiotic revolution in medicine [1]. Due to their low cost, low toxicity, and excellent activity against bacterial diseases, they are still widely used as antimicrobial agets [1-3]. The mechanism of the bactericide action of sulfonamides is based on the similarity of their structure with p-aminobenzoic acid (PABA), competing with which they inhibit the synthesis of tetrahydrofolate acid, necessary for the synthesis of pyrimidine bases of DNA and RNA. Among the 15,000 sulfanilic acid derivatives synthesized to date, there are compounds with anticancer, antimalarial, antiviral, anticonvulsant effects [4], the chemotherapeutic effect of which is associated with their ability to inhibit carbonic anhydrase, the multiple forms of which are present in various tissues and organs of animals and humans [5]. So, popular sulfonamide diuretics (diacarb, furosemide, indapamide), ophthalmic drugs (brinzolamide, dorzolamide), some anticancer agents are inhibitors of carbonic anhydrases [4]. Carbonic anhydrase in multiple forms is also present in the cells of higher plants. Their role and localization have not yet been fully elucidated [6].

When studying the pH-dependent activation of an isolated $Mg^{2+}$-ATPase of chloroplasts, we found that sulfonamide carbonic anhydrase inhibitors, acetazolamide and ethoxysolamide, almost completely suppress the activity of this enzyme [7, 8]. This unexpected fact may confirm the earlier hypothesis about the functional relationship of ATP synthase of chloroplasts with an unknown form of carbonic anhydrase [7, 9]. Given the high degree of structural similarity between chloroplast, bacterial, and animal mitochondrial ATPases [10], a deeper study of this effect may be important in establishing the mechanisms of action of sulfonamide drugs on living organisms. In this work, we tested 2 water-soluble preparations - aromatic 4-aminobenzenesulfonamide (SA) and aliphatic trifluoromethanesulfonamide (TFMSA). SA, known as streptocide was one of the first compounds of this class, which was used in the treatment of bacterial diseases. TFMSA is a powerful inhibitor of human carbonic anhydrase, which is not used in therapeutic practice due to its high toxicity [5]. Based on the discovery of the high gametocidal activity of TFMSA in relation to the male gametophyte of corn and sorghum [11, 12] without negative effects for the plant as a whole, its use in breeding is considered promising.

The aim of this work was to study the effect of 4-aminobenzenesulfonylamide and trifluoromethanesulfonamide on the latent and stimulated ATPase activity of the catalytic part of the thylakoid ATP synthase isolated from spinach chloroplasts.

## Materials and Methods

Chloroplasts were isolated from fresh spinach leaves (*Spinacia oleracea* L.) mainly as described previously [13]. After removing fragments of cell walls and large aggregates, chloroplasts were subjected to osmotic shocking at 4 °C for 10 min in a hypotonic medium containing 10 mM Tris-HCl (pH 7.2) and 10 mM NaCl. Thylakoid membranes were washed three times from RBP-carboxylase, which is the main soluble chloroplast protein. For this, the membranes were resuspended in a solution of 10 mM NaCl, 10 mM Tris-HCl (pH 7.2) and 2 mM $MgCl_2$, each time precipitating the membranes at 7000 g for 10 min. After the last washing, the precipitate was suspended at room temperature in 250 mM sorbitol, 10 mM Tris-HCl (pH 7.2), 1 mM EDTA, 2 mM ATP at a chlorophyll concentration of 2.5 $mg^{-1}\cdot ml^{-1}$. ATPase (factor $CF_1$) was isolated as previously described by the method of Zakharov et al. [14] by treating the membranes with chloroform at a 1: 2 ratio of chloroform to thylakoid suspension. The suspension and chloroform were transferred to a separatory funnel and shaken vigorously for 30 sec. To completely separate the phases, the emulsion was centrifuged for 5 min at 1000 g. The upper phase was taken using a siphon and centrifuged for 30 min at 15,000 g. The yellowish supernatant containing the isolated ATPase of thylakoids was concentrated by precipitation with ammonium sulfate and stored at 4 °C in 2 M $(NH_4)_2SO_4$. The $CF_1$-ATPase was purified according to [15]. For this, the protein precipitate in ammonium sulfate was shaken, transferred to a centrifuge tube and precipitated at 5000 g for 10 min. The precipitate was dissolved in a minimal amount of a buffer solution containing 2 mM EDTA, 1 mM ATP, and 20 mM tricine-NaOH (pH 8.0) and applied to a column (1.5x80 cm) with Sephadex G-200, which was equilibrated with the same buffer. The fractions containing ATPase were combined and concentrated by adding dry ammonium sulfate to a concentration of 2 M. The preparation was stored in a refrigerator at 4 °C about for three months without loss of activity. The purity of the enzyme was evaluated using electrophoretic analysis according to the published protocol [16]. The protein concentration in preparations of isolated ATPase was determined according to the Lowry method [17]. Before the experiments, the obtained $CF_1$ preparation was desalted sequentially on two centrifuge columns with Sephadex G-50, which was balanced with 50 mM Tris-HCl (pH 7.8), 50 mM NaCl and 2 mM EDTA [18]. The ATPase activity was determined in the reaction solution containing 1 mM ATP, 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$ at 37 °C and expressed as μmol $P_i$ (mg protein) - 1 $min^{-1}$. The amount of $P_i$ in the sample was determined by the Lowry and Lopez method in the Skulachev's modification as in previous publications [7, 8].

The activation of Mg-dependent ATPase activity was carried out 1) by brief heating (4 min) at 62 °C according to [19]; 2) by the treatment with 20% methanol as in [20]; 3) by adding 25 mM sodium sulfite, as described previously [7]. Salts and trichloroacetic acids of laboratory grade qualification, ATP (adenosine triphosphoric acid (disodium salt), sulfonamide (SA) and trifluoromethanesulfonamide (TFMSA) purchased from Sigma (USA) were used.

The studies were carried out in 3-4 single replications. The mean values and their standard deviations were determined. When comparing the samples, Student *t*-test was used, the differences were considered significant at $P \leq 0.05$.

## Results and Discussion

The catalytic part of the ATP synthase complex of chloroplasts ($CF_1$, coupling factor) after separation from thylakoid membranes loses the ability to synthesize ATP, but retains hydrolytic activity. Regulation of ATPase activity is complex and is controlled by several mechanisms. The most conservative is the non-competitive inhibition by the MgADP complex (ADP inhibition). When ADP binds to a catalytic site without phosphate, the enzyme can undergo conformational changes that block ADP in the bound state, which leads to inactivation of the enzyme. Oxyanions (for example, bicarbonate or sulfite) can displace bound ADP, in result of which the ATPase activity of the enzyme increases. The most effective activator of ATPase is sodium sulfite [21]. Previously, we found that after enzyme activation in the presence of stimulating concentrations of $Na_2SO_3$, the rate of ATP hydrolysis sharply decreased with the addition of sulfonamide inhibitors of ethoxyzolamide, or acetazolamide with an $I_{50}$ of about 1 µM [7]. Fig. 1 shows the effect of increasing concentrations of sulfonamides, which were studied in this work - SA and TFMSA on the rate of $Mg^{2+}$-dependent ATP hydrolysis after activation of $CF_1$-ATPase by sodium sulfite.

It can be seen that in the presence of SA, the rate of ATP hydrolysis remained consistently high, whereas with the addition of TFMSA, the hydrolytic activity decreased sharply and reached the level of the latent enzyme at a concentration of TFMSA of 50-200 µM. The concentration of the inhibitor, when added, the activity of the enzyme was halved ($I_{50}$) was about 1 µM. Thus, the dependence of the rate of ATP hydrolysis on the concentration of TFMSA was very similar to the dependence of this reaction on the concentration of ethoxyzolamide or acetazolamide under conditions of activation of $CF_1$-ATPase by sodium sulfite [7]. The data of Fig. 1 show that another sulfonamide - SA did not affect the hydrolytic activity of thylakoid ATPase activated by sodium sulphate.

The sulfonamides studied in this work affected the ATPase activity of $CF_1$ after its activation by brief heating at 62 °C somewhat differently than in the case of sulfite-activated ATPase [19]. The mechanism of heat activation of $CF_1$-ATPase is correlated with a change in the conformational state or position of the ε-subunit, the smallest subunit of the catalytic part of the ATP synthase of chloroplasts, which performs regulatory functions. In particular, it is capable of selectively inhibiting ATP hydrolysis. Several experimental studies show that this regulation is achieved due to large conformational changes in the α-helical C-terminal domain of the ε-subunit [22]. The effect of SA and TMFSA on the activity of heat-activated $CF_1$-ATPase is shown in Fig. 2.

TFMSA was found to be a more effective inhibitor of ATPase, in the presence of which its activity of the heat-activated enzyme decreased to the level of the latent one at a concentration of 50 µM, and $I_{50}$ (TFMSA) was about 5 µM. SA inhibited the ATP-ase reaction much weaker: $I_{50}$ (SA) was 50 µM. But, it should be noted that, unlike the enzyme activated by sodium sulfite, $CF_1$-ATPase became sensitive to SA inhibition after the heat activation.

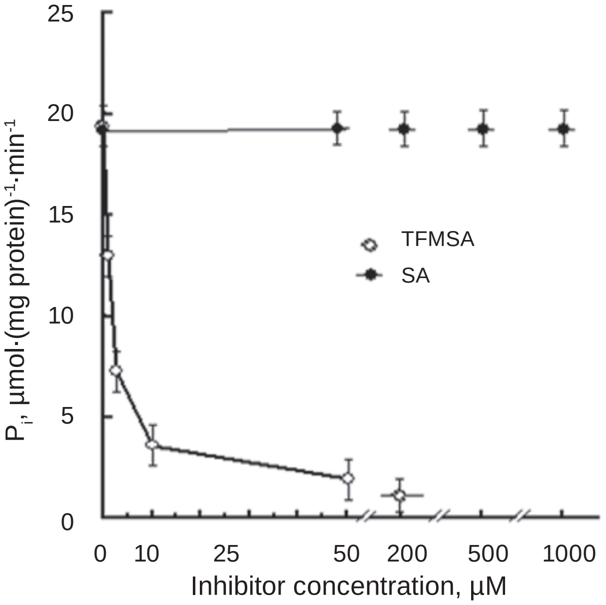


*Fig.1. The effect of sulfonamides on the rate of $Mg^{2+}$-dependent ATP hydrolysis under sodium sulfite activation of $CF_1$-ATPase. Composition of the reaction mixture: 1 mM ATP, 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 25 mM $Na_2SO_3$ and 10 µg of isolated $CF_1$-ATPase. The mixture was incubated for 30 min at 37 °C*

Activation of the chloroplast ATPase is also observed when treated with methanol, ethanol, and other short-chain alcohols [20]. Both soluble and thylakoid-bound [20] $CF_1$ exhibits high rates of ATP hydrolysis if 20-35% methanol is added to the reaction medium. The main effect of methanol in the activation of ATPase is to suppress the ε-subunit or at least to lose its ability to inhibit ATPase. Prolonged exposure to alcohols can lead to other irreversible changes, even loss of the ε-subunit and fixation of ATPase in activated form. The displacement of the ε-subunit from $CF_1$-ATPase by the action of alcohols was shown in [23]. The effect of sulfonamide SA and TMPSA inhibitors on the rate of ATP hydrolysis in the presence of $CF_1$-ATPase activated by 20% methanol is shown in Fig. 3. It can be seen that, compared to the heat-activated enzyme, the $CF_1$ hydrolase activity increases twice after the addition of methanol (Fig. 2).

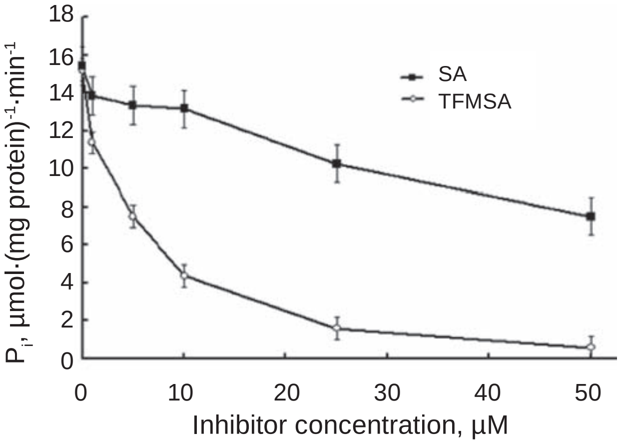


*Fig. 2. Influence of sulfonamide inhibitors on the rate of Mg$^{2+}$-dependent ATP hydrolysis by heat-activated isolated spinach chloroplast CF$_1$-ATPase. The enzyme activation was performed at 62 °C for 4 min*

As in the case of heat activation, both inhibitors suppress the ATPase reaction to different degrees. TFMSA inhibits the rate of ATP hydrolysis to the level of latent enzyme at concentrations of 100-150 μM, and I$_{50}$ (TFMSA) is about 1.5 μM. The inhibitory effect of SA is significantly weaker than TFMSA. 50% inhibition of ATP hydrolysis was observed with the addition of approximately 100 μM of SA. Thus, for all types of activation studied, the hydrolytic activity of CF$_1$-ATPase is effectively suppressed by TFMSA, while SA affects ATPase only after treatments that modify the state of the inhibitory ε-subunit.

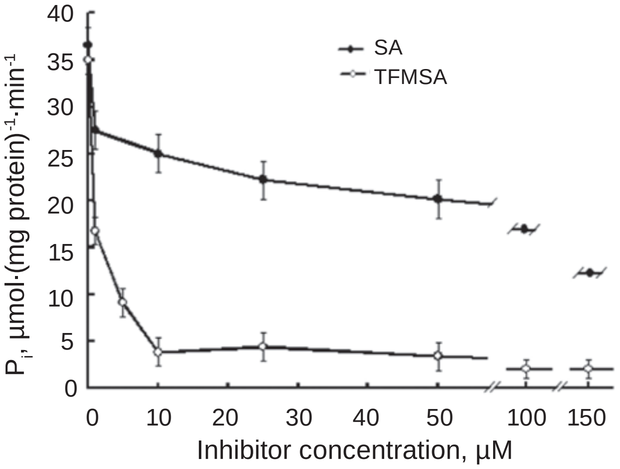


*Fig. 3. The effect of carbonic anhydrase inhibitors on the Mg$^{2+}$-ATPase activity of isolated CF$_1$ under conditions of activation with 20% methanol*

It is known that some organic solvents and nonionic detergents such as, for example, octyl glucoside are capable, like alcohols, of activating latent ATPase [10]. To test the effect of sulfonamides SA and TFMSA on the level of enzymatic activity of latent CF$_1$, we determined the dependence of the rate of the ATPase reaction on the concentration of these drugs. The data presented in Fig. 4 show that the low rate of hydrolytic reaction did not change with the addition of SA at a concentration of 1 to 300 μM. The dependence of the rate of ATP hydrolysis on the concentration of TFMSA was nonmonotonic, but in general, starting from a concentration of 1 μM,

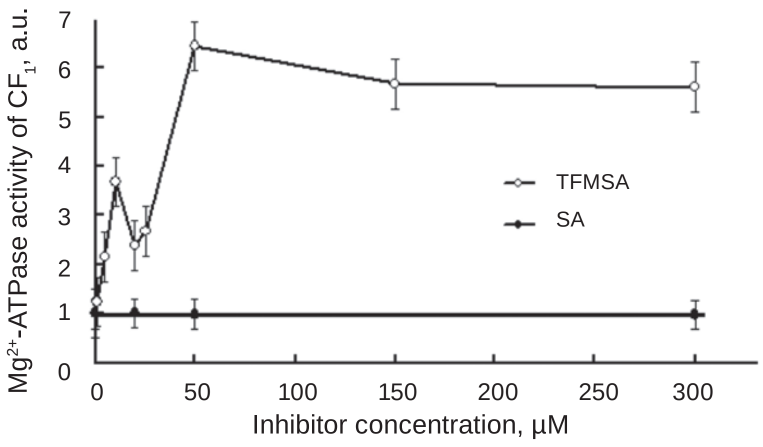
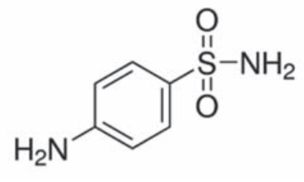
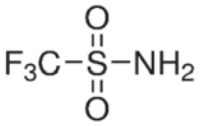


*Fig. 4. The effect of water soluble sulfonamide inhibitors on Mg$^{2+}$-ATPase activity of isolated latent CF$_1$. The latent enzyme activity ranged from 0.8 to 1.1 μmol P$_i$ (mg protein)$^{-1}$·min$^{-1}$. Its average value is taken on the graph as 1, and the value of enzymatic activity is provided in arbitrary units with respect to the averaged activity of the latent enzyme*



4-aminobenzenesulfonamide (SA)



$F_3C-\overset{O}{\underset{O}{S}}-NH_2$

trifluoromethanesulfonamide (TFMSA)

*Fig. 5. Structural formulas of sulfonamide inhibitors studied in this work*

this sulfonamide stimulated ATPase activity: in the presence of 50-300 µM of TFMSA, it exceeded the activity level of the latent enzyme by about 6 times. Thus, the results show that that water-soluble sulfonamides are capable of both stimulating and inhibiting the ATPase activity of isolated $CF_1$ depending on the reaction conditions.

TFMSA is known as one of the most potent inhibitor of several human α-carbonic anhydrases [5] and, according to the results obtained, it effectively suppresses the rate of ATP hydrolysis in the presence of isolated $CF_1$ activated by heat, methanol, or sodium sulfite (Fig. 2-4). The mechanisms that ensure the activation of latent ATPase during these treatments are not the same, but it is obvious that as a result of molecular rearrangements of the enzyme under the influence of these treatments, some functionally important parts of the molecule become accessible for interaction with TFMSA. Notably, when TFMSA is added to the latent enzyme, the hydrolytic reaction is noticeably stimulatei and reaches a 6-fold acceleration at drug concentrations of 50-300 µM. SA, in contrast to TFMSA (Fig. 5), either did not affect at all, or influenced the activity of the isolated chloroplast ATPase much weaker and only under the conditions of the modified state of the ε-subunit.

The inhibitory effect of sulfonamides on the activity of carbonic anhydrase depends on their solubility in water, pKa, and pH of the medium, since these inhibitors are active only in ionized form [24]. Therefore, the proportion of ionized molecular forms with $pKa < 6.0$ at physiological pH values significantly exceeds the relative amount of ionized inhibitors with $pKa > 8$. In contrast to SA ($pKa = 10.43$, $pKb = 11.63$) [25], TFMSA has the lowest $pKa \approx 5.8$ among sulfonamides and it dissolves in water (> 500 mM) better than SA [26]. The acidity of the sulfonamide group, which is very high for compounds of this class, is associated with the negative induction effect of the $CF_3$-group and its proximity to the sulfonamide residue. Structural analysis showed that the method of binding this inhibitor to the active site of carbonic anhydrase is significantly different from that for classical aromatic / heterocyclic sulfonamides [5].

Inhibitory analysis is an important approach to studying the functioning of enzymes, the use of which allows obtaining information about the structure of both active and allosteric centers of enzymes. Upon binding of the effectors to the allosteric center, the tertiary and often also quaternary structure of the molecule and, accordingly, the configuration of the active center can change, which causes a decrease or increase in enzymatic activity. According to the results of this and previous works [7, 8], we can state that sulfonamide compounds, known as carbonic anhydrase inhibitors, are able to modify the activity of the chloroplast ATPase, a multisubunits enzyme, which has several active and regulatory centers [10], and can be a useful tool when studying the features of the catalytic process in membrane ATP synthases of various origins. The data of this and previous works [7, 8] show for the first time that some compounds of the sulfonamide class are agents aimed at ATP synthase, which cause growing interest during the development of new methods of treating human and animal diseases and agricultural applications [27, 28].

*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.

*Funding sources.* This work was funded by the Project № 0112U002315 (01/2017-12/2021) “Coordination of bioenergetic processes in plants under conditions of increasing atmospheric $CO_2$ content.”

## ВПЛИВ СУЛЬФАНІЛАМІДІВ НА АКТИВНІСТЬ ТИЛАКОЇДНОЇ АТРази, ІЗОЛЬОВАНОЇ ІЗ ХЛОРОПЛАСТІВ ШПИНАТУ

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Вивчалася дія двох водорозчинних сульфаніламідів ароматичного 4-амінобензолсульфоніламіду (СА) і аліфатичного трифторметилсульфоніламіду (ТФМСА) на латентну і стимульовану АТРазну активність каталітичної частини тилакоїдної АТР-синтази, ізольованої з хлоропластів шпинату. У роботі застосовували три методи стимуляції АТРазної активності: нетривалий прогрів ензиму при 62 °С; інкубація з 20%-им метанолом; додаванням до реакційного середовища 25 мМ сульфіту натрію. ТФМСА ефективно пригнічував АТРазну активність ензиму за всіх типів активації. У разі теплової обробки $I_{50}$ (ТФМСА) становила ~ 5 мкМ, а за активації сульфітом або метанолом ~ 1 мкМ. СА зовсім не впливав на АТРазну активність за наявності у середовищі сульфіту, а у випадку активації ензиму нагріванням або метанолом інгібував її значно слабше, ніж ТФМСА: величина $I_{50}$ (СА) ~ 50 мкМ. Додавання досліджуваних інгібіторів до реакційного середовища з латентним ензимом не спричиняло ніякого ефекту в разі застосування СА і призводило до активації АТРазної реакції приблизно у 6 разів за додавання ТФМСА. Одержані результати показують, що обидва сульфаніламіди, відомі як інгібітори карбоангідраз, здатні також модифікувати АТРазу хлоропластів.

Ключові слова: хлоропласти шпинату, $CF_1$-АТРаза, гідроліз АТР, сульфаніламіди, трифторметансульфонамід, 4-амінобензолсульфоніламід.

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