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REVERSIBLE pH-DEPENDENT ACTIVATION/INACTIVATION OF CF_1-ATPase OF SPINACH CHLOROPLASTS

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The aim of the work was to study the reverse pH-dependent regulation of the enzymatic activity of the catalytic part of ATP synthase (EC 3.6.3.14) of chloroplast – coupling factor CF_1. It was shown that the short-term incubation of isolated CF_1 in the media with pH 4.5 or 3.5 leads to inactivation of Ca^{2+}-ATPase, which is rapidly ($t_{1/2}$ ~ 1 min) restored in the medium containing 0.5-10 mM bicarbonate at pH 7.8. After acid treatment, the rate of Mg^{2+}-ATPase reaction was also stimulated in the presence of 1 mM bicarbonate (pH 7.8; 37 °C). The increase in Ca^{2+}- and Mg^{2+}-ATP activity of CF_1 associated with the addition of $NaHCO_3$ solution was completely eliminated after the introduction of 50 mM acetazolamide – a specific inhibitor of carbonic anhydrase. The obtained results suggest the existence of the bound bicarbonate in the CF_1 structure, which apparently participates in proton transfer.

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

ATP synthase (EC 3.6.3.14) is a membrane enzymatic complex which synthesis and hydrolysis of ATP coupled with the transmembrane proton transfer in chloroplasts, mitochondria and bacteria. It consists of a hydrophobic part F_0, which functions as a proton channel and a hydrophilic part – a coupling factor F_1, which contains several nucleotide binding sites and performs catalytic function [1, 2]. Proton ATP synthases from various organisms have similar structure. This is largely characteristic of chloroplast ATPase catalytic part – the CF_1 factor, which is water-soluble enzyme and consists of five types of polypeptides in stoichiometric ratio α:β:γ:δ:ε ~ 3:3:1:1:1 [2-4]. The central position of the ATP synthase complex in the energy supply of a living cell is determined by the need of precise regulation of its functioning and coordinated with the physiological state of an organism and its demand for energy. To date, several mechanisms have been identified that are involved in the regulation of ATP synthase. After separation from the membrane, the catalytic part of the complex, the isolated coupling factor CF_1, loses the ability to catalyse the ATP synthesis, but retains ATPase activity. In this case, the isolated CF_1 is a latent (hidden) ATPase and catalyses the hydrolysis of ATP only after activation by heat [5] or as a result of treatment with redox reagents, alcohols and some detergents [2]. A significant activation of ATP hydrolysis is also achieved when adding oxyanion – bicarbonate, sulphite, phosphate, etc., to the reaction medium. [2, 5, 6].

The enzymatic activity of membrane-bound ATP synthases is primarily controlled by the value of the transmembrane proton gradient and realized during proton transfer through polypeptides of the complex during protonation/ deprotonation of certain amino acid groups [6, 7]. The protonation of specific groups in the isolated CF_1 factor can simulate the energization process. Analysis of the pH-dependence of Mg^{2+}-ATPase reaction catalyzed by the isolated factor CF_1 made it possible to estimate the dissociation constant of functionally important residues (pK 5.8-6.7) [6], herewith it was shown that the kinetic behavior of CF_1-ATPase is controlled by the degree of protonation of these groups. According to the data of Malyan [8], the activity of Mg^{2+}-ATPase increases significantly after incubation of the isolated coupling factor CF_1 at pH 5.5 and is in-

hibited again when CF_1 is transferred to a weakly alkaline medium that indicates the existence of at least two functional states of the coupling factor, reversible transitions between them occurring with pH changes.

The aim of this work was to study the pH-dependent inactivation/reactivation of Ca^{2+}- and Mg^{2+}-ATPase activity of the isolated coupling factor CF_1 of spinach chloroplasts and the effect of specific inhibitor of carbonic anhydrase – acetazolamide on these processes.

Materials and Methods

Chloroplasts were isolated from fresh spinach (*Spinacia oleracea* L.) leaves as described previously in [9] and disrupted for 10 min in hypotonic medium that contained 5 mM Tris-HCl (pH 7.8) and 10 mM NaCl. The thylakoids were washed twice with a hypotonic solution, reprecipitated for 10 min at 15,000 g and used to isolate the preparation of the coupling factor CF_1 by the method of [10] with some modifications. All isolation operations of thylakoids and CF_1 were performed at 4 °C. The chlorophyll concentration in the preparations of the thylakoid membranes was determined according to Arnon [11], the protein concentration – according to Lowry [12]. The purity of the obtained CF_1 preparation was assessed by the results of electrophoresis with charge shift as described in [13].

The latent isolated CF_1-ATPase was activated by heating; to do this the preparation (1.5-2.0 mg) was added to 1 ml of a solution containing 10 mM ATP, 25 mM Tris-HCl (pH 7.9), and 10 mM $CaCl_2$ (or $MgCl_2$). The mixture was placed on a 37 °C water bath and incubated for 30 min, after that it was transferred to a container with water (26 °C). ATPase reaction was stopped by adding 1 ml of 8% trichloroacetic acid (TCA) solution to the mixture. ATPase activity was determined by measuring concentrations of released inorganic phosphorus (P_i) in the reaction medium that contained 15 mM Tris-HCl, pH 7.9, 5 mM ATP and 5 mM $CaCl_2$ (or $MgCl_2$) (at 26 °C or 37 °C) and expressed as μmol $P_i \cdot (\text{mg protein})^{-1} \cdot h^{-1}$. The amount of P_i in the sample was determined by the Lowry and Lopez method in the Skulachev modification [14].

To determine the dependence of ATPase activity on pH, the CF_1 solution was incubated for 5 min in the media which contained 10 mM succinate and 50 mM Tris-HCl with pH of 3.5; 4.5; 5.4 and 7.8. After a 5-minute incubation, CF_1-ATPase was activated by heating and the rate of Ca^{2+} or Mg^{2+}-dependent ATP hydrolysis was determined.

For ATPase reactivation after 5-minute acid incubation at pH 3.5; 4.5 or 5.4, the calculated amount of 1 M NaOH solution was added to the medium so that the final pH of the solution was 7.8. ATPase activity of the enzyme in this series of experiments was determined after 10 min incubation at pH 7.8.

The salts of laboratory grade (NaCl, $CaCl_2$, $MgCl_2$, $NaHCO_3$, Na_2SO_3, $CuSO_4$, CH_3COONa, $(NH_4)_2MoO_4$) and TCA were purchased from the company Synbios (Ukraine). ATP and acetazolamide (N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)acetamide, N-(5-[aminosulfonyl]-1,3,4-thiadiazol-2-yl) acetamide) from the company Sigma (USA) were used in the work.

All studies were performed in triplicate. Student's *t*-test was used to compare the samples, the differences were considered reliable at $P < 0.05$. The figures and the table show the mean values and their standard errors.

Results and Discussion

Table 1 shows the results of determination of Ca^{2+}- and Mg^{2+}-dependent ATPase activity after 5 min of incubation of isolated CF_1 at different pH. It is evident that Ca^{2+}-ATPase CF_1, previously incubated under physiological pH (≈ 7.8), increased significantly during the 30-minute exposure at 37 °C.

At all pH values, the Ca^{2+}-dependent ATPase activity at 26 °C was noticeably lower than the measurements made at 37 °C, that agrees with [2, 5]. It should also be noted that Ca^{2+}-ATPase activity of the isolated coupling factor CF_1 after preliminary incubation at low pH values (3.5, 4.5 or 5.4) was significantly inhibited compared to the control and at pH 7.8 both at 26 °C and at 37 °C.

In contrast to Ca^{2+}-ATPase, Mg^{2+}-ATPase activity was practically unchanged during the 30-minute incubation of the preparations at 37 °C, preincubated at reduced pH (Table 1). At the same time, there was a more than triple increase in the rate of Mg^{2+}-dependent ATP hydrolysis in samples incubated at pH 3.5 in comparison with the control (pH 7.8). However it should be noted that the level of Mg^{2+}-ATPase activity remained low for all pH and temperatures studied.

The question of whether the inactivation of CF_1-ATPase at low pH was irreversible was solved in the following series of experiments (Table 2). First, the solution of desalted isolated coupling factor CF_1

Table 1. Ca^{2+}- and Mg^{2+}-dependent ATPase activity after 5 min of CF_1 incubation at various pH (M ± m, n = 3)

pH of incubation medium	ATPase activity of isolated CF_1, μmol·(mg protein)$^{-1}$·h^{-1}			
	Ca^{2+}-ATPase activity		Mg^{2+}-ATPase activity	
	26 °C	37 °C	26 °C	37 °C
3.5	13.47 ± 2.34	53.39 ± 4.17	21.18 ± 0.98	15.43 ± 1.03
4.5	45.06 ± 2.38	52.41 ± 1.77	17.63 ± 0.74	21.67 ± 2.74
5.4	–	55.96 ± 0.22	12.00 ± 0.81	25.84 ± 3.12
7.8	50.82 ± 4.62	195.68 ± 4.48	6.24 ± 0.33	23.88 ± 2.73

was incubated at pH 3.5 for 5 min, then the solution pH was increased to 7.8. In the variants indicated (Table 2) 1 mM $NaHCO_3$ or 50 μM of specific carbonic anhydrase inhibitor acetazolamide (AZ) was added to the mixture.

The data of Table 2 show, that Ca^{2+}-ATP activity of the preparation pre-treated at pH 3.5 increases after the subsequent 10 min incubation in the reaction solution with pH 7.8, exceeding almost twice the activity of Ca^{2+}-ATPase determined immediately after the acidic incubation. This indicates the reversibility of the acid inactivation of the enzyme and the possibility of its reactivation in the case of transfer to a slightly alkaline environment. A similar effect was previously described by Malyan [6, 8] in the study of the pH dependence of the Mg^{2+}-ATPase activity of isolated CF_1.

In our experiments, the rate of ATP hydrolysis increased significantly, when 1 mM $NaHCO_3$ was added to the medium (pH 7.8) in the final incubation (Table 2). The addition of 50 μM acetazolamide, a specific inhibitor of carbonic anhydrase, to the reaction mixture had practically no effect on the rate of ATP hydrolysis but eliminated the stimulating effect of bicarbonate on Ca^{2+}- and Mg^{2+}-ATPase. Previously, we observed [4] the elimination of bicarbonate stimulation of photosynthetic phosphorylation in isolated thylakoids after addition of acetazolamide.

The results of Table 2 indicate a similarity in the character of the reactivation of Ca^{2+}- and Mg^{2+}-ATPases after the acid treatment of CF_1. The activity in both cases increased with incubation in a slightly alkaline medium (pH 7.8), and when the bicarbonate anion was added, Mg^{2+}-ATPase was stimulated almost 4 times, and Ca^{2+}-ATPase – 2 times. The effective concentration of the anion (1 mM) was much lower than the published value for activation of Mg^{2+}-ATPase [15, 16]. In our experiments the sensitivity to the bicarbonate anion was increased for factor CF_1 treated at low pH, and the difference between Mg^{2+}-dependent and Ca^{2+}-dependent ATPase activities decreased (Table 2).

Fig. 1 and 2 show the dependence of Ca^{2+}- i Mg^{2+}-ATPase activity of CF_1, treated at pH 3.5 on concentration of $NaHCO_3$ at the final stage of incubation (pH 7.8).

It can be seen that the rate of ATP hydrolysis depended on temperature. Ca^{2+}-ATPase activity was stimulated with bicarbonate both at 26 °C and at 37 °C (Fig. 1). Addition of 0.1-1 mM of $NaHCO_3$ led

Table 2. The effect of bicarbonate and acetazolamide (AZ) on the reactivation of Ca^{2+}- and Mg^{2+}-ATPase activity of the isolated factor CF_1 (M ± m, n = 3)

Additives during incubation, 30 min, pH 7.8, 37 °C	Ca^{2+}-ATPase activity, μmol·(mg protein)$^{-1}$·h^{-1}	Mg^{2+}-ATPase activity, μmol·(mg protein)$^{-1}$·h^{-1}
–	95.27 ± 7.91	44.57 ± 7.31
50 μM of AZ	110.33 ± 8.9	50.57 ± 3.53
1 mM $NaHCO_3$	180.13 ± 12.01	144.37 ± 30.3
1 mM $NaHCO_3$ + 50 μM AZ	100.66 ± 6.74	40.65 ± 3.82

Note: pH of initial incubation was 3.5

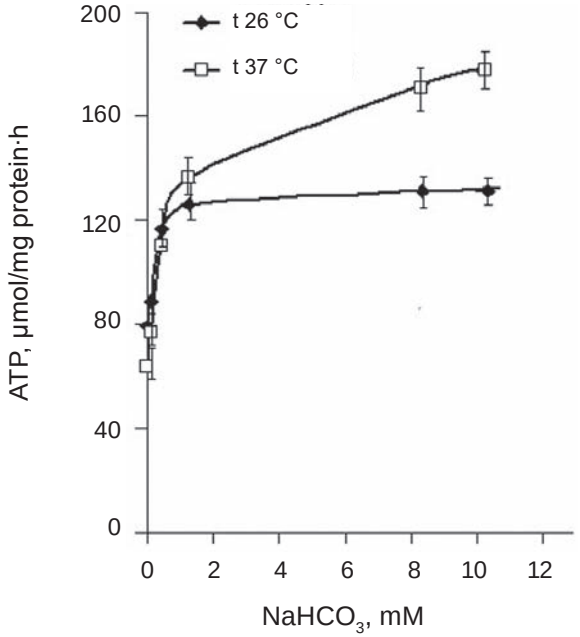

Fig. 1. Ca^{2+}-ATPase activity dependence on $NaHCO_3$ concentration at pH 7.8 after acid inactivation of CF_1-ATPase

to a sharp increase in the ATP hydrolysis rate, and maximum stimulation was observed at 37 °C with bicarbonate in concentrations exceeding 1 mM.

Mg^{2+}-ATPase activity was stimulated with bicarbonate only at 37 °C, whereas at 26 °C bicarbonate did not affect the rate of ATPase reaction (Fig. 2).

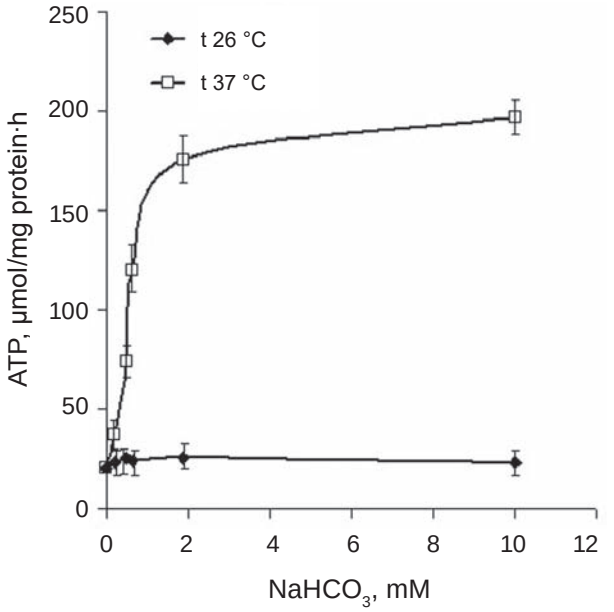

Fig. 2. Mg^{2+}-ATPase activity dependence on $NaHCO_3$ concentration at pH 7.8 after acid inactivation of CF_1-ATPase

The dynamics of the development of Ca^{2+}-and Mg^{2+}-ATPase activity at 37 °C in the presence of 1 mM $NaHCO_3$ in the medium is shown in Fig. 3.

Samples of isolated CF_1 after a 5-minute incubation at pH 3.5 were transferred to the medium with pH 7.8, they were incubated for 30 seconds, 1, 2, 5 and 10 min and added to the reaction mixture containing 1 mM $NaHCO_3$, ATPase reaction was activated by heating. It can be seen that half-maximal activation of Ca^{2+}-and Mg^{2+}-ATPase was achieved after approximately 1.5 and 2 min, respectively. According to Malyan [6], the reactivation of CF_1 after incubation with pH of 5.5 was much slower and required 11 min to achieve half-maximal activity.

The results obtained in the present work show that the enzymatic activity of the catalytic part of the ATP synthase of chloroplasts – factor CF_1 in the reaction of ATP hydrolysis is inhibited after its incubation in a medium with pH <5.5. The inhibition is reversible and partially eliminated, when the enzyme is transferred to a medium with pH of 7.8. If bicarbonate is present in the solution, the reactivation of ATPase to the control level of enzymatic activity occurs much faster.

Bicarbonate is a known stimulator of Mg^{2+}-ATPase activity of CF_1 [6, 15, 16]. However, but ac-

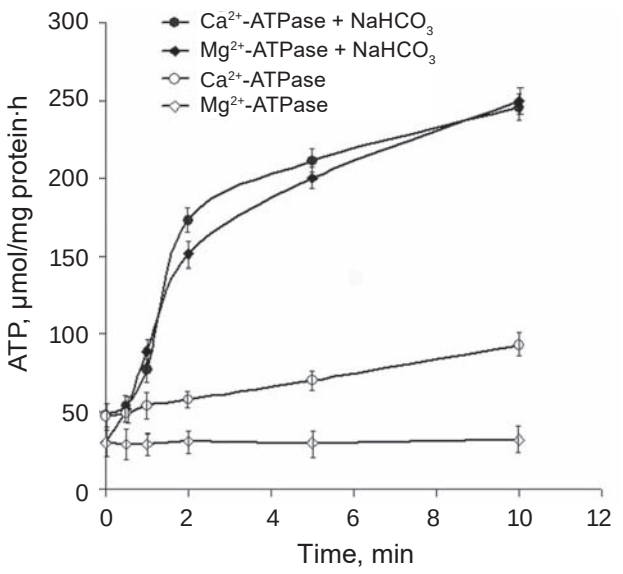

Fig. 3. Dynamics of reactivation of Ca^{2+}- i Mg^{2+}-ATPase after acid inactivation of CF_1-ATPase at pH 3.5 and transfer to the medium with pH 7.8 in the presence of 1 mM $NaHCO_3$

cording to the cited studies, its effective concentration is more than an order of magnitude higher than bicarbonate concentration, which was effective in the reactivation of CF_1-ATPase after acid treatment in our work (Fig. 2 and 3).

Previously, we reported the presence of carbonic anhydrase activity in isolated coupling factor CF_1 [13, 19]. The results of this study suggest that CF_1-carbonic anhydrase is involved in the process of reactivation of ATPase activity after acid inhibition. This is evidenced by data on the negative effect of acetazolamide, the inhibitor of carbonic anhydrases, on the process of reactivation of the ATPase reaction (Table 2).

The results of the work suggest the existence in the structure of the isolated coupling factor CF_1 a bound bicarbonate, which, possibly, participates in providing proton transfer. Incubation in the medium with pH <5.5 promotes the release of the bound bicarbonate in the form of CO_2. When exogenous bicarbonate is added to the secondary incubation medium with pH 7.8, it re-binds to CF_1, whereby ATP activity increases to its original level. Thus, the presence of bound bicarbonate can control the functional state of CF_1.

ОБОРОТНА pH-ЗАЛЕЖНА АКТИВАЦІЯ/ІНАКТИВАЦІЯ CF_1-АТРази ХЛОРОПЛАСТІВ ШПИНАТУ

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Метою роботи було вивчення оборотної pH-залежної регуляції ензиматичної активності каталітичної частини АТР-синтази (3.6.3.14) хлоропластів – чинника спряження CF_1. Показано, що нетривала інкубація ізольованого CF_1 у середовищах із pH 4,5 або 3,5 призводила до інактивації Ca^{2+}-АТРази. Активність ізольованого чинника спряження CF_1 швидко ($t_{1/2} \approx 1$ хв) відновлювалася в середовищі з pH 7,8, до якого додавали розчин $NaHCO_3$ в діапазоні концентрацій від 0,5 до 10 мМ. Mg^{2+}-АТРаза також активувалася внаслідок кислотної інкубації CF_1 за наявності 1 мМ $NaHCO_3$ (pH 7,8; 37 °C). Зростання Ca^{2+}- і Mg^{2+}-АТРазної активності CF_1, пов'язане з додаванням розчину $NaHCO_3$, повністю усувалося після введення 50 мкМ ацетазоламіду – специфічного інгібітора карбоангідраз. Одержані результати дозволяють припускати існування в структурі CF_1 зв'язаного бікарбонату, який бере участь у забезпеченні протонного перенесення.

Ключові слова: хлоропласти шпинату, CF_1-АТРаза, активація/інактивація, гідроліз АТР, pH-залежність, ацетазоламід.

ОБРАТИМАЯ pH-ЗАВИСИМАЯ АКТИВАЦИЯ/ИНАКТИВАЦИЯ CF_1-АТРазы ХЛОРОПЛАСТОВ ШПИНАТА

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Целью работы было изучение обратимой pH-зависимой регуляции энзиматической активности каталитической части АТР-синтазы (3.6.3.14) хлоропластов – фактора сопряжения CF_1. Показано, что непродолжительная инкубация изолированного CF_1 в средах с pH 4,5 или 3,5 приводила к инактивации Ca^{2+}-АТРазы. Энзиматическая активность быстро ($t_{1/2} \approx 1$ мин) восстанавливалась в среде с pH 7,8, в которую добавляли раствор $NaHCO_3$ в диапазоне концентраций от 0,5 до 10 мМ. Mg^{2+}-АТРаза также активировалась в присутствии 1 мМ бикарбоната (pH 7,8; 37 °C) после кислотной обработки CF_1. Стимулирующий эффект $NaHCO_3$ полностью устранялся при введении в среду 50 мкМ ацетазоламида – специфического ингибитора карбоангидраз. Полученные результаты позволяют предполагать существование в структуре CF_1 связанного бикарбоната, участвующего в обеспечении протонного переноса.

Ключевые слова: хлоропласты шпината, CF_1-АТРаза, активация/инактивация, гидролиз АТР, pH-зависимость, ацетазоламид.

References

1. Junge W, Nelson N. ATP synthase. *Annu Rev Biochem*. 2015; 84: 631-657.
2. Malyan AN. Noncatalytic nucleotide binding sites: properties and mechanism of involvement in ATP synthase activity regulation. *Biochemistry (Mosc)*. 2013; 78(13): 1512-1523.
3. Groth G, Pohl E. The structure of the chloroplast F1-ATPase at 3.2 A resolution. *J Biol Chem*. 2001; 276(2): 1345-1352.
4. Tiedge H, Lünsdorf H, Schäfer G, Schairer HU. Subunit stoichiometry and juxtaposition of the photosynthetic coupling factor 1: Immunoelectron microscopy using monoclonal antibodies. *Proc Natl Acad Sci USA*. 1985; 82(23): 7874-7878.
5. Bakker-Grunwald T, van Dam K. On the mechanism of activation of the ATPase in chloroplasts. *Biochim Biophys Acta*. 1974; 347(2): 290-298.
6. Malyan AN, Vitseva OI, Gubanova ON. Mg^{2+}-dependent inactivation/H^{+}-dependent activation equilibrium of chloroplast F1-ATPase. *Photosynth Res*. 1998; 57(3): 297-303.
7. Zolotareva EK, Gasparyan ME, Yaguzhinsky LS. Transfer of tightly-bound tritium from the chloroplast membranes to CF1 is activated by the photophosphorylation process. *FEBS Lett*. 1990; 272(1-2): 184-186.
8. Malyan AN. A possible mechanism of coupling ATP synthesis with proton transfer in the enzymatic complex of chloroplast CF1. *Rep Acad Sci USSR*. 1986; 291: 1015-1018. (In Russian).
9. Zolotareva EK, Tereshchenko AF, Dovbysh EF, Onoiko EB. Effect of alcohols on inhibition of photophosphorylation and electron transport by N,N'-dicyclohexylcarbodiimide in pea chloroplasts. *Biochemistry (Moscow)*. 1997; 62(6): 631-635.
10. Zakharov SD, Sytnik SK, Mal'yan AN, Makarov AD. Isolation and properties of CF1 ATPase chloroplasts with changed submolecular structure. *Biokhimiia*. 1978; 43(5): 887-891. (In Russian).
11. Arnon DI. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris. *Plant Physiol*. 1949; 24(1): 1-15.
12. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951; 193(1): 265-275.
13. Semenihin AV, Zolotareva EK. Identification of carbonic anhydrase activity associated with protein complexes of photosynthetic membranes of spinach chloroplasts. *Rep Nat Acad Sci Ukr*. 2014; 9: 141-145. (In Ukrainian).
14. Nikulina GN. A review of the methods for the colorimetric determination of phosphorus by the formation of molybdenum blue. M.-L.: Nauka, 1965. 45 p. (In Russian).
15. Murataliev MB, Boyer PD. The mechanism of stimulation of MgATPase activity of chloroplast F1-ATPase by non-catalytic adenine-nucleotide binding. Acceleration of the ATP-dependent release of inhibitory ADP from a catalytic site. *Eur J Biochem*. 1992; 209(2): 681-687.
16. Guerrero KJ, Xue ZX, Boyer PD. Active/inactive state transitions of the chloroplast F1 ATPase are induced by a slow binding and release of Mg^{2+}. Relationship to catalysis and control of F1 ATPases. *J Biol Chem*. 1990; 265(27): 16280-16287.
17. Onoiko EB, Polishchuk AV, Zolotareva EK. Stimulation of photophosphorylation in spinach isolated chloroplasts by exogenous bicarbonate: role of carbonic anhydrase. *Rep Nat Acad Sci Ukr*. 2010; 10: 160-165. (In Russian).
18. van Rensen JJ. Role of bicarbonate at the acceptor side of Photosystem II. *Photosynth Res*. 2002; 73(1-3): 185-192.
19. Semenihin AV, Zolotareva OK. Carbonic anhydrase activity of integral-functional complexes of thylakoid membranes of spinach chloroplasts. *Ukr Biochem J*. 2015; 87(3): 47-56.
20. Fabre N, Reiter IM, Becuwe-Linka N, Genty B, Rumeau D. Characterization and expression analysis of genes encoding alpha and beta carbonic anhydrases in Arabidopsis. *Plant Cell Environ*. 2007; 30(5): 617-629.
21. Rudenko NN, Ignatova LK, Fedorchuk TP, Ivanov BN. Carbonic anhydrases in photosynthetic cells of higher plants. *Biochemistry (Mosc)*. 2015; 80(6): 674-687.

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