

CARBONIC ANHYDRASE ACTIVITY OF INTEGRAL-FUNCTIONAL COMPLEXES OF THYLAKOID MEMBRANES OF SPINACH CHLOROPLASTS

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Isolated thylakoid membranes were disrupted by treatment with nonionic detergents digitonin or dodecyl maltoside. Solubilized polypeptide complexes were separated by native gel charge shift electrophoresis. The position of ATP-synthase complex and its isolated catalytic part (CF_1) within gel was determined using the color reaction for ATPase activity. Due to the presence of cytochromes, the red band in unstained gels corresponded to the cytochrome b6f complex. Localization of the cytochrome b6f complex, ATP synthase and coupling CF_1 in the native gel was confirmed by their subunit composition determined after SDS-electrophoretic analysis. Carbonic anhydrase (CA) activity in polypeptide zones of PS II, cytochrome b6f complex, and ATP-synthase CF_1 was identified in native gels using indicator bromothymol blue. CA activity of isolated CF_1 in solution was determined by infrared gas analysis as the rate of bicarbonate dehydration. The water-soluble acetazolamide, an inhibitor of CA, unlike lipophilic ethoxzolamide inhibited CA activity of CF_1 . Thus, it was shown for the first time that ATP-synthase has a component which is capable of catalyzing the interconversion of forms of carbonic acid associated with proton exchange. The data obtained suggest the presence of multiple forms of carbonic anhydrase in the thylakoid membranes of spinach chloroplasts and confirm their involvement in the proton transfer to the ATP synthase.

Key words: thylakoid membranes, ATP-synthase, coupling factor CF_1 , carbonic anhydrase, proton transport.

The light-dependent reactions of photosynthesis take place on sheets of inner thylakoid membranes of chloroplasts containing 4 functional multiprotein complexes: photosystems I and II, cytochrome b6f, and ATP-synthase. These complexes participate in absorption and transformation of light energy to satisfy the metabolic demands of plant cells [1-3]. These complexes, together with other components of thylakoid membranes, carry out photosynthetic electron transfer and proton transfer coupled with it. This process creates transmembrane proton gradient that is the driving force of ATP synthesis. There is a couple of ways by which the protons may enter into thylakoid lumen. The first happens in plastoquinone protonation-deprotonation cycle, which acquires protons from the outer side of the membrane in Q_B center of photosystem II and releases them after oxidation reaction in cytochrome b₆ center. The second occurs when water is decomposed in photosystem II complex. Protons leave thylakoids via proton transport of ATP-synthase coupled with synthesis of ATP [4]. Thus, at least three out of

four main multiprotein complexes of photosynthetic membranes are involved in light-dependent proton exchange. According to analysis [5] the required rate of proton transport from sites where they are generated on thylakoid membrane to ATP-synthase cannot be maintained by free diffusion. Intracellular proton transport is significantly accelerated through facilitated diffusion with dissolved buffers, like bicarbonate, which is maintained by carbonic anhydrase (CA). This allows assuming the existence of proteins with carbonic anhydrase activity within membrane polypeptide complexes in addition to carbonic anhydrases associated with photosystem II.

CA (carbonate hydrolyase, EC 4.2.1.1) is a zinc-containing metalloenzyme that catalyzes interconversion of CO_2 and HCO_3^- . CA accelerates the rate of the equilibrium in this reaction by tens of thousand times. Without CA in solution, the equilibrium is attained very slowly. There are both soluble and membrane-bound forms of this enzyme in cells of higher plants [6]. It has been demonstrated that CA molecules in thylakoid membranes of chloroplasts

are associated with at least three different components of electron-transport chain. Two proteins with carbonic anhydrase activity have been identified in monomeric and dimeric forms of photosystem II (PS II) [7]. It has been also demonstrated that PS I-enriched membrane samples contain CA [8]. There is an evidence that PS II-associated CA generates bicarbonate ions at a rate sufficient to bound protons released in the inner space of thylakoid in the process of photosynthetic oxidation of water [9]. Hence, CA prevents acidic denaturation of manganese cluster of PS II, as critical increase in local H^+ concentration may damage the active centers of the complex and result in their destruction with manganese release in liquid environment [10, 11].

The existence of multiple forms of CA in spinach chloroplasts has been confirmed [12]. Nevertheless, the detailed study of localization of CA-active proteins has not been conducted yet.

The aim of the present work is to modify native gel charge shift electrophoresis and two-dimensional electrophoresis methods for separation and identification of native pigment-protein and protein complexes of spinach and study of their carbonic anhydrase activity.

To this end, we developed a method for solubilization of functional multiprotein complexes of thylakoid membranes with nonionic detergent digitonin. The complexes were identified through 2-D electrophoresis (by their molecular mass and peptide composition), and their carbonic anhydrase and ATPase activity was assayed by enzyme-specific reactions.

Materials and Methods

The thylakoids were extracted from fresh spinach leaves homogenized on ice in buffer (40 mM tris-HCl (pH 7.8), 400 mM sorbitol, 5 mM EDTA, 1 mM $MgCl_2$, 10 mM NaCl, 5 mM sodium ascorbate, 0.05% BSA). The suspension was filtered through double layer of tissue and centrifuged at 2500 g at 4 °C for 4 min. The sediment was resuspended in hypotonic buffer (20 mM tris-HCl (pH 7.5), 5 mM sorbitol and 5 mM $MgCl_2$), followed by centrifugation at 2500 g at 4 °C for 4 min. The sediment of isolated thylakoids was washed with storage buffer (200 mM sorbitol, 10 mM NaCl, 10 mM KCl, 2.5 mM $MgCl_2$, 10 mM tris-HCl, pH 7.8). The procedure was repeated twice, then the sediment was resuspended in small volume of storage buffer to chlorophyll concentration of 4 mg/ml. Chlorophyll

concentration in thylakoid membrane samples was assayed after Arnon [13].

In order to solubilize protein complexes the thylakoid membranes were incubated for 30 min at 4 °C in 10% solution of digitonin (at detergent:protein ratio of 1:1; 2:1; 4:1; 8:1) in the presence of protease inhibitors 0.75 M aminocaproic acid and 1 mM phenylmethylsulfonyl fluoride (PMSF). The soluble proteins of thylakoid membranes were then separated by centrifugation at 140,000 g at 4 °C for 45 min. Protein content in supernatant was assayed after Lowry et al. [14].

Native gel charge shift electrophoresis of membrane protein complexes was done after Andersson et al. [15] with modifications, Kolesnichenko et al. [16] in PAA gel slabs (70×80×1.5 mm) in acrylamide gradient (4-11%) in 0.38 M tris-HCl buffer (pH 8.8). Stacking gel contained 3.75% of acrylamide in 0.06 M tris-HCl buffer (pH 6.8). Cathode and anode buffers were glycine and tris solutions (25 mM tris-HCl, 192 mM glycine, pH 8.3). To create charge shift, SDS was added to cathode buffer to 0.005% concentration. The electrophoresis was conducted at 4 °C in order to maintain the native conditions of multiprotein functional complex of thylakoids. The protein bands were visualized with the help of coomassie G-250 dye.

The gel strips containing protein bands were excised and used for SDS denaturing electrophoresis in second direction in modified Laemmli system [17] in PAA gel slabs (70×80×1.5 mm) in order to analyze the peptide composition of complexes. The electrophoretic separation of proteins was conducted in acrylamide concentration gradient from 10% to 20% in 0.38 M tris-HCl buffer (pH 8.8), 0.1% SDS.

The stacking gel contained 4% acrylamide in 0.06 M tris-HCl buffer (pH 6.8), 0.1% SDS. Cathode and anode buffers contained 25mM tris-HCl, 192 mM glycine, 0.1% SDS, pH 8.3. Protein bands were visualized with coomassie R-250.

ATPase activity was assayed after Allen and Hyncik [18] and Gomori [19]. The gels after native electrophoresis were incubated for 10-12 hours in solution containing 10 mM ATP, 10 mM $CaCl_2$ in 100 mM tris-HCl (pH 9.5). The gels were then washed with water and incubated for 20 min in 3 mM solution of $Pb(NO_3)_2$ in 80 mM malate buffer (pH 7.0). After washing the gels were placed in 0.2% solution of Na_2S . ATP synthase localization was visualized as dark red bands of insoluble PbS .

CA activity was visualized according to Edwards and Patton [20]. The gels were incubated for 30 min in 0.2% of bromothymol blue in 50 mM veronal buffer (pH 8.1). The gel was then placed in water saturated with CO₂ at 0 °C. CA spots were visualized where indicator color changed from blue to yellow.

CF₁ coupling factor was extracted from cation-free thylakoids with 1 mM EDTA solution and purified after [21] with some modifications. ATPase activity of isolated CF₁ was assayed by phosphate generation measured after Lowry and Lopes [22]. Rate of CA reaction catalyzed by isolated CF₁ was assayed by CO₂ generation from dehydration of bicarbonate. CO₂ content was determined with infrared gas analyzer (S151, Qubit Systems Inc., Canada).

Results and Discussion

The thylakoid membranes are distinct in their heterogeneous localization and their association with numerous and diverse functional protein complexes. In accordance with their protein composition, there exist terminal membranes, lateral (marginal) membranes of stromal (lamellar) thylakoids and intragrana (stacked) thylakoids, which are in tight junctions with each other. PS II is localized mostly within grana in the regions of tight contacts of thylakoids. PS I is generally found in stromal thylakoids and in marginal and terminal regions of grana. This system is absent in stacked regions of grana. On the other hand, cytochrome complexes are distributed more or less evenly throughout the thylakoid membranes, with somewhat higher density within regions of tight thylakoid contacts in grana. ATP synthase complex is predominantly localized of stromal thylakoids, and in marginal and terminal regions of grana [3, 4].

Thylakoid membrane disintegration and native functional complexes separation are achieved with application of neutral (nonionic) detergents. The most efficient detergent or a mix of detergents and protein/detergent ratio in incubation medium has to be selected experimentally for solubilization of complexes from each particular membrane type. The native pigment-protein complexes of thylakoids may be extracted with dodecylmaltoside (DM), tridecylmaltoside (TM), digitonin or triton X-100 [23], which disrupt bonds between polypeptide complexes and lipid matrix of membranes and preserve the native composition and functional activity of photosynthetic complexes. The full extraction of all the multiprotein complexes from various thylakoid parts

under mild solubilization is unlikely to be achievable due to heterogeneous nature of protein and lipid thylakoid components and due to irregularities in localization of membrane complexes. So, the solubilization of particular pigment-protein complex is dependent on the nature of detergent used as well as on the ratio of detergent to protein in incubation medium. According to studies [23], native protein complexes of grana are extracted with DM or TM, and polypeptide complexes of thylakoid stroma may be extracted successfully by digitonin application to target membranes.

In the present work thylakoids washed from soluble components were then solubilized with DM or digitonin. The solubilized membrane material was analyzed by native gel charge shift electrophoresis. Fig. 1 shows the results of electrophoretic separation of isolated spinach thylakoid membranes in PAA gel after solubilization in 10% digitonin (detergent:protein ratio of 1:1; 2:1; 4:1; 8:1) and 5% dodecylmaltoside (detergent:protein ratio of 1:1; 2:1; 4:1; 8:1) in the presence of protease inhibitors 0.75 M aminocaproic acid and 1 mM PMSF.

The number of protein bands in an electrophoretic line is dependent on detergent:protein ratio, and the complexes with high molecular mass appear at higher concentrations of detergent. It is apparent that lower concentrations of detergent cannot provide for separation of these complexes from lipid matrix. As can be seen on Fig. 1, the most effective solubilization of native thylakoid protein complexes is achieved at digitonin:protein ratio 8:1. This solubilization procedure allows for detection of protein structures with molecular mass over 900 kDa. According to published data, the mass of the main functional protein complexes of photosynthetic membranes are approx. 400 kDa (cytochrome b6f), 600 kDa (ATP synthase), and 700 kDa (PS I) [23]. These complexes are organized as dimers within membranes (cytochrome b6f complex, PS II) [1, 4] or trimers (light-harvesting complex, LHC). The identification of high molecular mass components (over 900 kDa) on electrophoretic plates affirms that this method is suitable for solubilization of oligomeric membrane components with digitonin.

Therefore, the chosen concentrations of digitonin allowed for efficient separation of primary polypeptide complexes of spinach thylakoids. Previously, dodecylmaltoside application allowed for a fairly clear resolution of thylakoid membrane complexes from *Arabidopsis* [23] and rice [24]. In con-

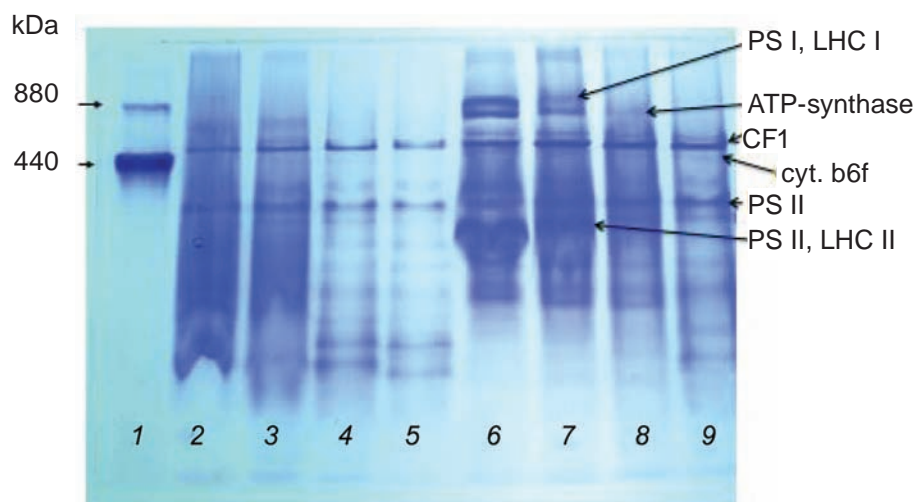


Fig. 1. Electrophoretic analysis of solubilized native protein complexes of spinach thylakoid membranes in PAA gel: 1 – marker proteins (monomeric and dimeric ferritin); 2 – DM:protein ratio was 8:1; 3 – DM:protein ratio was 4:1; 4 – DM:protein ratio was 2:1; 5 – DM:protein ratio was 1:1; 6 – digitonin:protein ratio was 8:1; 7 – digitonin:protein ratio was 4:1; 8 – digitonin:protein ratio was 2:1; 9 – digitonin:protein ratio was 1:1

trast to this, the electrophoretic separation of integral protein complexes from spinach thylakoids after DM solubilization is inferior to the results obtained with application of digitonin.

On unstained electrophoretic plates of digitonin-solubilized thylakoid complexes there is a clear red band within the region corresponding to the marker of 440 kDa molecular mass. This band is conceivably related to cytochrome b6f complex, which has a natural red color due to the presence of cytochromes.

We analyzed the specific enzymatic activity in PAA gels in order to identify protein bands with ATPase activity that may be part of native ATP-synthase complex. As can be seen on Fig. 2, the ATPase activity in gels incubated in buffer system with ATP and $\text{Pb}(\text{NO}_3)_2$ is detected within a zone that corresponds to 700 kDa of molecular mass, which is consensually viewed as the full mass of the ATP-synthase complex. Moreover, the ATPase activity was detected in zones corresponding to molecular masses of over 900 kDa, which probably signifies oligomeric enzyme forms, and 400 kDa, which is approximately the molecular mass of coupling factor CF_1 . The latter is the catalytic hydrophilic part of the ATP-synthase complex containing no hydrophobic subunits responsible for anchoring the complex in membrane. CF_1 retains the ability to catalyze ATP hydrolysis, which permits its easy detection after electrophoresis. The second dimension electrophoresis in denaturing conditions in the presence of SDS

(Laemmli system) may also be used to identify protein zones associated with ATP synthase.

The two-dimensional protein electrophoresis in PAA gel, which combines the potentials of native gel and denaturing gel electrophoresis (in the presence of SDS), is widely used for analysis of protein and peptide composition of biological objects [23]. As the native proteins and protein complexes have been separated (the first dimension), the gel strip is cut out, incubated in the presence of SDS and mercaptoethanol and then separated in denaturing SDS system (Laemmli system) in the second dimension. This approach makes possible the analysis of peptide composition of protein complexes that exhibit certain specific enzymatic activity.

As the number, stoichiometry, and molecular mass of all the ATP-synthase subunits are known, it is possible to identify certain protein zones of ATPase activity with full ATP-synthase complex or with its catalytic part CF_1 , in accordance with their polypeptide composition. The electrophoretic plate of polypeptide zones with ATPase enzymatic activity (Fig. 3) demonstrates that protein content associated with the polypeptide zone of molecular mass approximating 700 kDa corresponds the polypeptide composition of the full ATP-synthase complex – CF_0CF_1 (of nine polypeptide component: CF_0 subunits I (19 kDa), II (16.5 kDa), III (8 kDa), IV (25 kDa); and CF_1 subunits α (60 kDa), β (56 kDa), γ (39 kDa), δ (20.5 kDa), ϵ (14.7 kDa)) [25]. Thus, the zone of native gel that corresponds to molecular

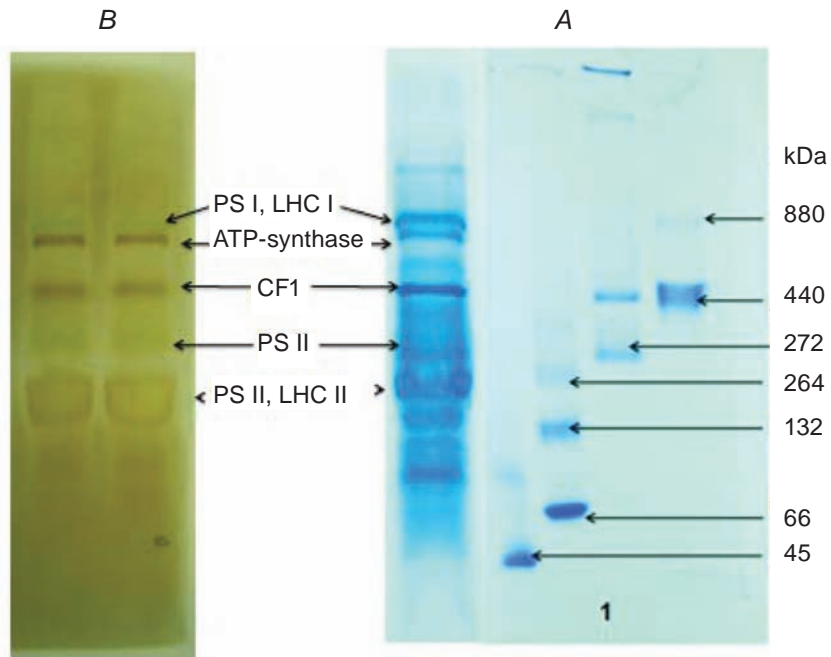


Fig. 2. Electrophoretic analysis of solubilized native protein complexes of spinach thylakoid membranes in PAA gel: A – the gel was stained with coomassie G-250 (1 – marker proteins); B – the gel was incubated in ATPase activity detection medium

mass of 700 kDa may be identified (according to its protein composition) as the full ATP-synthase complex, and the zone corresponding to molecular mass of 400 kDa (Fig. 2 and 3) as the coupling factor CF₁.

The most pronounced CA activity out of those detected in native gel polypeptides was associated with PS II protein complex (Fig. 4). As mentioned above, there are two proteins with CA activity in PS

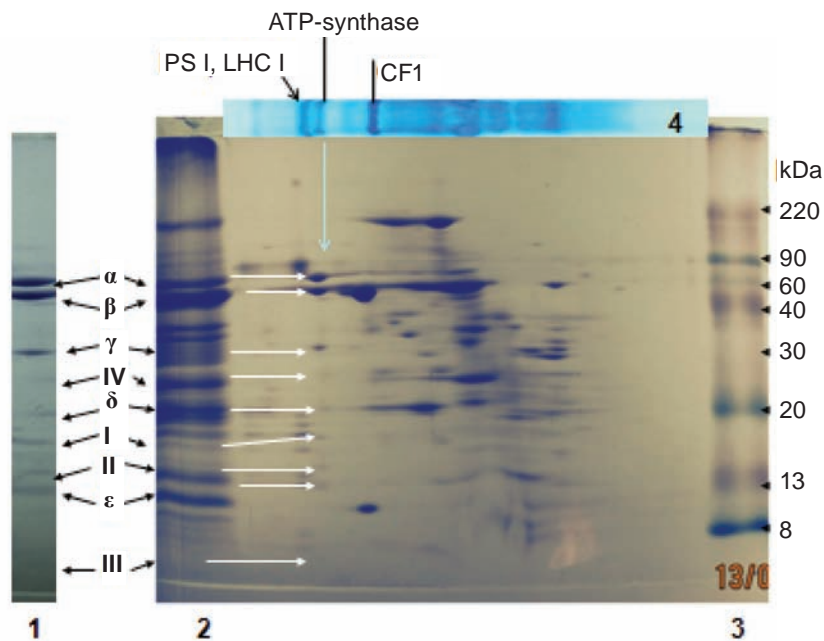


Fig. 3. Electrophoretic analysis of peptide composition of protein complexes from spinach thylakoid membranes (second dimension): 1 – peptide composition of ATP-synthase complex; 2 – peptide composition of functional complexes; 3 – marker proteins; 4 – PAA gel strip after native electrophoresis

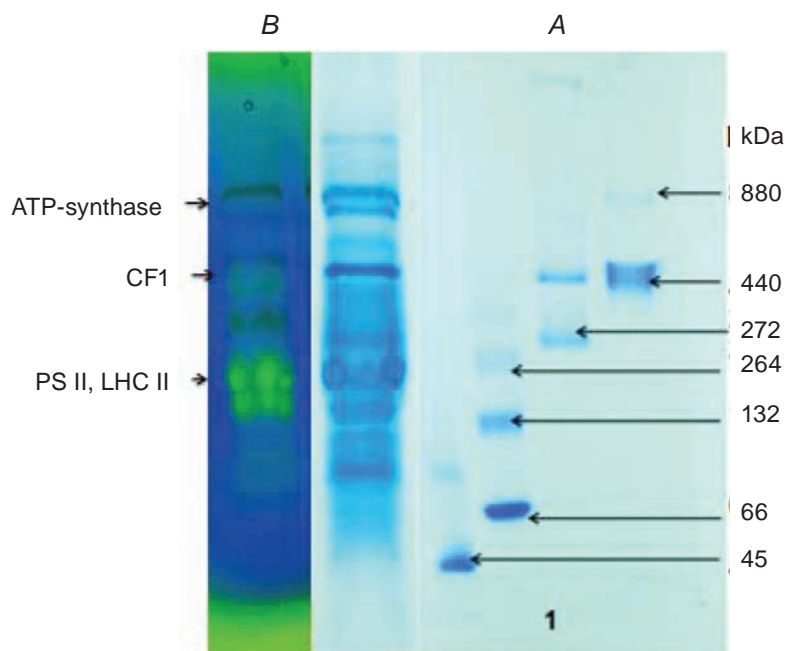


Fig. 4. Electrophoretic analysis of solubilized native protein complexes of spinach thylakoid membranes in PAA gel: A – the gel was stained with coomassie G-250 (1 – marker proteins); B – the gel was incubated in carbonic anhydrase activity detection medium

II complex [7, 8], which may explain the more intensive coloration of PS II protein zone.

The protein zones of native gel identified as ATP synthase complex and coupling factor CF_1 as per their ATPase activity and subunit composition were catalytically active in CA reaction under our experimental conditions (Fig. 4).

We extracted and purified coupling factor CF_1 from thylakoid membrane components in order to define the specific localization of CA activity. The results of electrophoretic analysis of isolated CF_1 demonstrate its peptide composition (Fig. 5). Apparently, under native conditions the sample contains single polypeptide with approximate molecular mass of 400 kDa, which corresponds to molecular mass of CF_1 as described elsewhere. The purified CF_1 polypeptide is degraded under the effect of SDS and mercaptoethanol during second dimension electrophoresis into five protein zones with molecular masses close to those of coupling factor's subunits [25]. The sample was catalytically active both in ATPase reaction and in CA reaction (Fig. 5 and 6; Table).

As can be seen, the CA activity of isolated CF_1 is inhibited by water-soluble acetazolamide (AZA), and is not significantly inhibited by lipophilic ethoxzolamide (EZA). This is in contrast to the results of the studies of effects by these inhibitors on CA activity of PS II, where EZA had been more efficient in inhibition than AZA [7, 8].

Therefore, our results prove that main native protein and pigment-protein complexes from spinach thylakoids that take part in proton transfer are capable of catalyzing CA reaction. Although CA activity of PS II and PS I and heterogeneous nature of CA activity of thylakoids has been previously demonstrated, this study has a priority in the detection of CA activity of multiprotein functional complex of ATP-synthase and of its catalytic part – the coupling factor CF_1 . The significance of this enzymatic activity for light-dependent ATP-synthesis and hydrolysis in chloroplasts requires more extensive studying. Another prospective research subject will be the identification of a specific carrier with CA activity within coupling factor CF_1 .

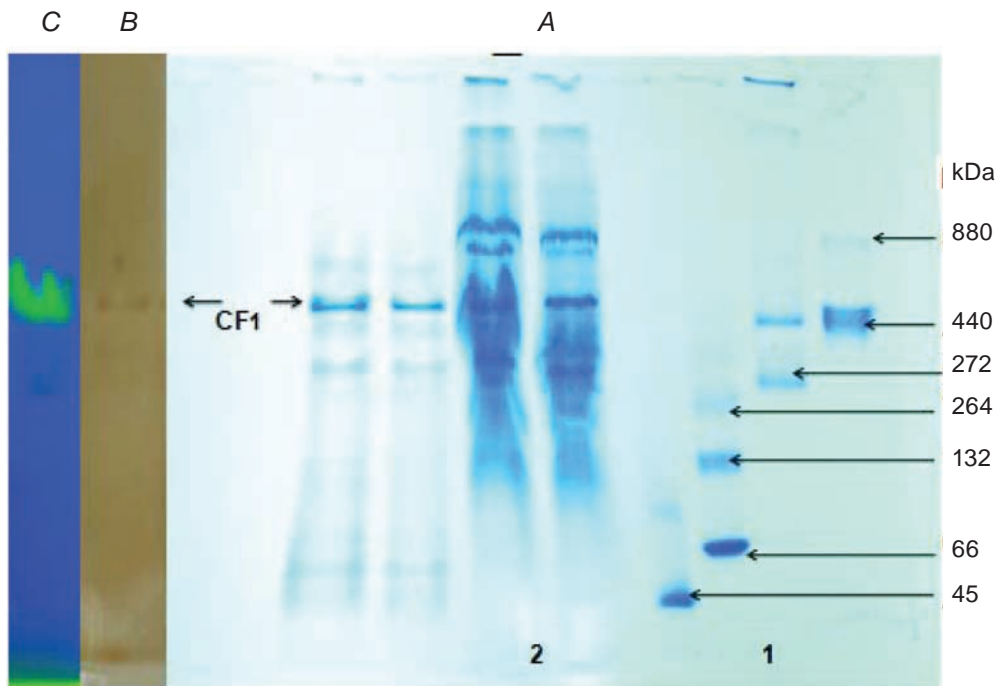


Fig. 5. Electrophoretic analysis of purified native coupling factor CF_1 in PAA gel: A – The gel was stained with coomassie G-250 (1 – marker proteins, 2 – native protein complexes of thylakoid membranes); B – the gel was incubated in ATPase activity detection medium; C – the gel was incubated in carbonic anhydrase activity detection medium

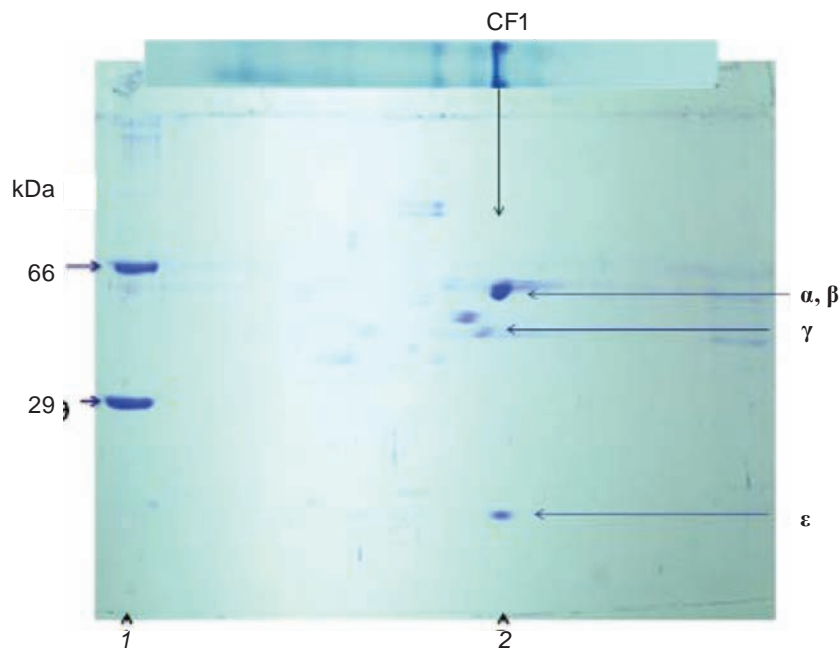


Fig. 6. Electrophoretic analysis of peptide composition of coupling factor CF_1 in PAA gel (second dimension): 1 – marker proteins; 2 – peptide subunits of coupling factor CF_1

ATP hydrolysis by isolated coupling factor CF₁ and effects of carbonic anhydrase inhibitors on reaction rate of bicarbonate dehydration by coupling factor CF₁ (M ± m, n = 6)

	HCO ₃ ⁻ dehydration rate, μmol CO ₂ ×(min×mg of protein) ⁻¹	ATPase reaction rate, μmol P _i ×(min×mg of protein) ⁻¹
Control	920 ± 85	18 ± 2
+ 1 mM AZA	549 ± 60	
+ 5 mM AZA	0	
+5 mM EZA	340 ± 40	

AZA – acetazolamide; EZA – ethoxyzolamide.

КАРБОУАНГІДРАЗНА АКТИВНІСТЬ ІНТЕГРАЛЬНО-ФУНКЦІОНАЛЬНИХ КОМПЛЕКСІВ ТИЛАКОЇДНИХ МЕМБРАН ХЛОРОПЛАСТІВ ШПІНАТУ

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Ізольовані тилакоїдні мембрани обробляли неіонними детергентами дигітоніном або додецилмальтозидом, та солюбілізовані поліпептидні комплекси розділяли методом нативного електрофорезу зі зміщенням заряду. Розташування зон гелю, які містили АТР-синтазний комплекс і його відокремлену каталітичну частину (CF₁), визначали за допомогою кольорової реакції на АТРазну активність. Цитохромному b6f-комплексу тилакоїдів відповідала зона, яка завдяки наявності цитохромів мала червоний колір у нефарбованому гелі. Локалізацію АТР-синтази і CF₁ підтверджено аналізом субодичного складу відповідних протеїнових зон після SDS-електрофорезу в другому напрямку. За змінами кольору індикатору бромтимолового синього в гелях після нативного електрофорезу виявлено карбоангідразну активність у поліпептидних смужках фотосистеми II, цитохромного b6f-комплексу, АТР-синтази і CF₁. Активність

карбоангідрازی (КА) в ізольованому чиннику CF₁ визначали методом інфрачервоного газового аналізу за швидкістю дегідратації бікарбонату. Водорозчинний інгібітор КА ацетазоламід, на відміну від ліпофільного етоксизоламиду, пригнічував карбоангідразну активність CF₁. Вперше показано, що в складі АТР-синтази існує компонент, здатний каталізувати інтерконверсію форм вугільної кислоти, пов'язану із протонним обміном. Одержані дані свідчать про наявність у тилакоїдних мембранах хлоропластів шпинату множинних форм карбоангідрازی і підтверджують припущення про їх участь у перенесенні протонів до АТР-синтази.

Ключові слова: тилакоїдні мембрани, АТР-синтаза, чинник спряження CF₁, карбоангідраз, протонний транспорт.

КАРБОУАНГІДРАЗНАЯ АКТИВНОСТЬ ИНТЕГРАЛЬНО-ФУНКЦИОНАЛЬНЫХ КОМПЛЕКСОВ ТИЛАКОИДНЫХ МЕМБРАН ХЛОРОПЛАСТОВ ШПИНАТА

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Изолированные тилакоидные мембраны разрушали при обработке неионными детергентами дигитонином или додецилмальтозидом, и солюбилизированные полипептидные комплексы разделяли методом нативного электрофореза со смещением заряда. Положение зон геля, содержащих АТР-синтазный комплекс и его каталитическую часть (CF₁), определяли с помощью цветной реакция на АТРазную активность. Цитохромному b6f-комплексу тилакоидов отвечала зона, имевшая из-за присутствия цитохромов красную окраску в неокрашенном геле. Локализация цитохромного b6f-комплекса, АТР-синтазы и CF₁ подтверждена анализом субъединичного состава соответствующих протеиновых зон после SDS-электрофореза. С помощью индикатора бромтимолового синего в полипептидных зонах фотосистемы II, цитохромного b6f-комплекса, АТР-синтазы и CF₁ в неденатурирующих условиях была выявлена

карбоангидразная активность. В растворе активность карбоангидразы (КА) изолированно-го CF_1 определяли по скорости дегидратации бикарбоната методом инфракрасного газового анализа. Водорастворимый ингибитор КА ацетазоламид, в отличие от липофильного этоксизоламида, ингибировал карбоангидразную активность CF_1 . Впервые показано, что в состав АТФ-синтазы входит компонент, способный катализировать интерконверсию форм угольной кислоты, связанную с протонным обменом. Полученные результаты свидетельствуют о наличии в тилакоидных мембранах хлоропластов шпината множественных форм карбоангидразы и подтверждают предположение об их участии в переносе протонов к АТФ-синтазе.

Ключевые слова: тилакоидные мембраны, АТФ-синтаза, сопрягающий фактор CF_1 , карбоангидраза, протонный транспорт.

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