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Heterogeneous Origin of Carbonic Anhydrase Activity of Thylakoid Membranes

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Abstract—Carbonic anhydrase activities of pea thylakoids as well as thylakoid fragments enriched either in Photosystem 1 (PS1-membranes) or Photosystem 2 (PS2-membranes) were studied. The activity of PS1-membranes if calculated on chlorophyll basis was much higher than the activity of PS2-membranes. Acetazolamide, a non-permeable inhibitor of carbonic anhydrases, increased carbonic anhydrase activity of PS2-membranes at concentrations lower than 10^{-6} M and suppressed this activity only at higher concentrations. A lipophilic inhibitor of carbonic anhydrases, ethoxyzolamide, effectively suppressed the carbonic anhydrase activity of PS2-membranes ($I_{50} = 10^{-9}$ M). Carbonic anhydrase activity of PS1-membranes was suppressed alike by both inhibitors ($I_{50} = 10^{-6}$ M). In the course of the electrophoresis of PS2-membranes treated with *n*-dodecyl-β-maltoside "high-molecular-mass" carbonic anhydrase activity was revealed in the region corresponding to core-complex of this photosystem. Besides, carbonic anhydrase activity in the region of low-molecular-mass proteins was discovered in the course of such an electrophoresis of both PS2- and PS1-membranes. These low-molecular-mass carbonic anhydrases eluted from corresponding gels differed in sensitivity to specific carbonic anhydrase inhibitors just the same as PS1-membranes versus PS2-membranes. The results are considered as evidence for the presence in the thylakoid membranes of three carriers of carbonic anhydrase activity.

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Carbonic anhydrase (CA, carbonate hydrolyase; EC 4.2.1.1), an enzyme catalyzing the reversible hydration of CO_2 ($CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$), is widely distributed in all organisms from procaryotes to animals. This is a consequence of the participation of all components of the reaction in many metabolic processes including CO_2 fixation, respiration, energy conservation, and so on. At the present time, CA isoforms are divided into three classes: α, β, and γ [1]. All animal CAs belong to the α-class, and up to quite recent times all CAs of higher plants were relegated to the β-class. In recent years, the conception was formed that even within one organism many forms of CA are present. For example, more than 10 genes encoding CAs belonging to all three classes [2] (including three genes of γ-class CA; the only well-studied representative of this class being CA from archaebacteria *Methanosarcina thermophila* [3]) were detected in the *Arabidopsis thaliana* genome. Representatives of different classes differ in sensitivity to sulfamides, specific inhibitors of CA. CAs of the α-class have the greatest sensitivity to them, with 50% inhibition of the reaction rate at 2-200 nM, while such inhibition for CAs of the β-class occurs at 5-500 μM [4].

In plants, there are soluble forms of the enzyme found in mitochondria [5], cytoplasm, and stroma of chloroplasts [6], and membrane-associated forms found in the plasma membrane [7, 8] and thylakoids [9]. The evidence for CA activity of thylakoids appeared first in 1982 [10, 11], but the existence of specific thylakoid CA was accepted relatively recently [9]. A number of facts indicate the connection of the thylakoid CA with Photosystem 2 (PS2) [12-16]. Moreover, the presence of two carriers of CA activity in PS2-membranes isolated from mesophyll chloroplasts of maize was proposed. The first carrier readily passes into solution at high salt con-

Abbreviations: AA) acetazolamide; BTB) bromthymol blue; CA) carbonic anhydrase; Chl) chlorophyll; DM) *n*-dodecyl-β-maltoside; EZ) ethoxyzolamide; PS1, PS2) Photosystems 1 and 2; PS1- and PS2-membranes) fragments of thylakoid membranes enriched with pigment–protein complexes of corresponding photosystems; PMSF) phenylmethylsulfonyl fluoride.

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centration and has molecular mass of 33 kD, while the second one is more firmly bound to the membrane [13]. In our previous work, it was shown that core-complex of PS2 from wheat and pea had CA activity [14, 15]. CA activity differing from CA activity of PS2 was recently found in membranes enriched with Photosystem 1 (PS1) [16].

In this study the evidence in favor of the existence of several, possibly three, carriers of CA activity in thylakoid membrane are presented.

MATERIALS AND METHODS

Isolation of thylakoids. Thylakoids were isolated from 2-3-week-old pea plants according a method developed earlier [17] and which provides washing from highly active stromal soluble CA. The method was modified as follows:

– a low-speed centrifugation (120*g*) for 1 min for precipitation of cell fragments was added;

– the chloroplasts were broken for 15 min at 0°C in medium containing 50 mM Mes-KOH, pH 6.5, 8 mM $MgCl_2$, 5 mM dithiothreitol;

– the thylakoids were resuspended in medium containing 5 mM EDTA, 1 mM benzamidine, 1 mM α-aminocaproic acid, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Control experiments showed that thylakoids had CA activity while the supernatant after the last washing of thylakoids did not have the activity.

Isolation of fragments of thylakoid membranes enriched with either PS1 or PS2 (further, PS1- and PS2-membranes). Thylakoids were incubated with Triton X-100 for 30 min with stirring in an ice bath at Triton/chlorophyll (Chl) ratio (mg/mg) of 1 in medium containing 50 mM Mes-KOH, pH 6.5, 8 mM $MgCl_2$, 300 mM sucrose, and 1 mM PMSF. The thylakoids were centrifuged at 13,000*g* for 30 min, then the supernatant was thrown away, and the precipitate was incubated under the same conditions for 30 min in the same medium except that Triton X-100 was added up to Triton/Chl ratio (mg/mg) of 20. This was centrifuged at 32,000*g* for 30 min, and a precipitate containing PS2-membranes was resuspended in the same medium and centrifuged in the same regime to wash out the Triton. To get the PS1-membranes, the supernatant after precipitation of PS2-membranes was centrifuged at 144,000*g* for 1 h. (This supernatant was indicated as S20.) The precipitate containing PS1-membranes was washed free from Triton by suspending in the same medium and by precipitating under the same regime. All procedures of isolation of thylakoids and their fragments were carried out at 4°C. The membranes obtained were frozen in liquid nitrogen with addition of 10% glycerol and stored at –80°C.

Analysis of the fractions. The pigment composition of the fractions was analyzed using the spectra of low-temperature fluorescence (excited with light of 435 nm) [18] measured with a Hitachi-850 spectrofluorimeter (Hitachi, Japan). Spectra were corrected according to the spectral sensitivity of the apparatus. The purity of photosystem preparations was tested by measuring the content of reaction center components (P680, P700, and pheophytin) [19]. The functional activities of the preparations were determined from the measurements of the rates of either oxygen consumption or oxygen evolution using a pO_2-electrode in medium containing 30 mM Hepes-KOH, pH 7.6-7.8, 10 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 0.1 M sucrose, membrane preparations with chlorophyll concentration 10 μg/ml, and the additions indicated in Table 1.

Electrophoresis. The thylakoids were treated with dodecyl maltoside (DM) at DM/Chl ratio (mg/mg) of 10 for 30 sec in a shaker (Vortex, Latvia), and then they were precipitated at 12,000*g* for 30 min. PS1- and PS2-membranes were treated with DM at DM/Chl ratio of 15 for 20 sec in a shaker. PS2-membranes were precipitated

Table 1. Characteristics of thylakoid membrane fragments enriched with either PS1 or PS2. Photochemical activity of PS1 was measured with methyl viologen as acceptor in the presence of 10 μM 2,4-dichlorophenyl 1,1-dimethyl urea, 5 mM Na-ascorbate, 0.2 mM 2,6-dichlorophenolindophenol, 0.1 mM methyl viologen; photochemical activity of PS2 was measured in the presence of 1 mM potassium ferricyanide and 0.1 mM 2,6-dichloro-*p*-benzoquinone

Experiment number	Membranes enriched with:	Oxygen uptake or evolution, μmol O_2/h per mg Chl	Chl/P680	Chl/P700	Chl *a*/Chl *b*
1	PS1	–225 ± 5			2.9
	PS2	127 ± 10			1.6
2	PS1	–218 ± 3	abs.	470	4.8
	PS2	112 ± 9	169		1.7
3	PS1	–55 ± 3	abs.	349	7.7
	PS2	125 ± 4	376		1.9

Note: The minus sign indicates oxygen uptake; "abs." means that P680 was not detected.

under the same regime as thylakoids. The supernatants, after precipitation of thylakoids and PS2-membranes, containing 50-100 μg Chl, and PS1-membranes, treated as described above, containing ~20 μg Chl, were applied to gels after addition of glycerol up to 10%. Electrophoresis was carried out according to Peter and Thornber [20] with 0.2% derifate-160 (sodium *n*-lauryl-β-iminodipropionate) in the upper electrode buffer in 7% cylindrical gels containing 20% glycerol at 0.8-1 mA per tube and voltage 50-80 V for 17-19 h in the dark at 4°C. The preparative procedures and the conditions of electrophoresis produced native pigment–proteins complexes.

Visualization of CA activity in polyacrylamide gel. The gels were stained with bromthymol blue (BTB) according to Edwards and Patton [21] and were placed into water saturated with CO_2 at 0°C. Change in indicator color from blue to yellow was observed at the CA position. For further studies, the corresponding pieces of gels were cut out and stored at –20°C.

Determination of the protein in polyacrylamide gel. Protein was determined by staining of gels with Coomassie G-250. Urease (mixture of trimer and hexamer with molecular masses 272 and 545 kD, respectively) was used for determination of molecular masses of pigment–protein complexes. There were six protein bands in the gel from monomer (91 kD) to hexamer due to the action of the derifate present in the upper electrode buffer.

To analyze, using the low-temperature fluorescence, the pigment composition of green gel bands, they were cut out, put into a glass tube placed in liquid nitrogen in a Dewar vessel inside the apparatus chamber.

The CA activity was assayed with a glass electrode at 2°C in 14 mM veronal buffer (pH 8.4); the reaction was initiated by addition of water saturated with CO_2 at 0°C, and the time of pH change from 8.3 to 7.8 was measured. This time was corrected for the time of spontaneous hydration under the same conditions after addition of a corresponding volume of medium instead of the sample. CA activity was expressed in μmol H^+/min per mg Chl after correction for both medium and sample buffer capacities measured by titration with 0.1 N HCl. To analyze inhibitor actions, the samples were incubated with the inhibitors in the reaction vessel for 2 min before starting the reaction. Sulfamide inhibitors of CA, ethoxyzolamide (EZ) and acetazolamide (AA) (both from Sigma, USA), were dissolved in dimethylsulfoxide.

To analyze the CA activity of the pieces of gels, they were ground with a pestle in a cooled mortar in the presence of broken glass in 2 ml of the veronal buffer; the mix was centrifuged at 12,000*g* for 10 min, and CA activity of the supernatant was measured. The same eluate of a gel piece without sample was used as the control.

Chlorophyll in the membrane samples was determined in ethanol extracts [22]. To determine the chlorophyll content in gel pieces, they were ground with broken glass and $MgCO_3$, and after ethanol addition were centrifuged at 5500*g* for 5 min. The chlorophyll extraction from gel pieces was incomplete, but its distribution expressed as a percentage of overall chlorophyll in all the bands was constant, and this allowed correcting the data for incomplete extraction.

RESULTS

Carbonic anhydrase activity of thylakoids. The distribution of CA activity in the thylakoids was analyzed after their treatment with DM. In order to promote visualization of the activity, the electrophoresis at low buffer capacity of a separating gel (12.4 mM Tris-glycine, pH 8.5) was used [20]. The concentration of DM (keeping DM/Chl ratio of 10) and the time of treatment (30 sec) provided emergence of six green bands in PAGE (Fig. 1a, gel I). The spectra of low-temperature fluorescence showed that the start region of the gel (band *1*) contained large fragments of thylakoids, band *2* included membrane fragments containing PS1, as low-temperature fluorescence maximum at 735 nm indicated. The bands *3-6* contained mainly light-harvesting complex, PS2, and small amount of PS1 (Fig. 1b).

When visualizing CA activity in gel, the most distinguishable change of color of BTB (appearance of yellow on blue background) was observed in the region of proteins with apparent molecular mass near 50 kD as well as in the start region of the gel (Fig. 1, gel II). Chlorophyll content in the region of low-molecular-mass proteins was low, and CA activities of the corresponding bands, especially of band *6*, were high if calculated on chlorophyll basis (Fig. 1b, right column). The change in color of BTB was observed also in other bands but the registration was difficult owing to intensive green color of the bands. The arrows in Fig. 1a show the bands with CA activity. To evaluate the overall CA activity of the sample, the entire gel was cut as shown in Fig. 1a for gel I, and the band contents were eluted into veronal buffer, and then CA activity of each eluate was measured. CA activity was detected in all bands, and the greatest overall CA activity was in band *2* containing PS1-membranes (Fig. 1b, left column). The thylakoids isolated from plants grown in December and January had in this band CA activity comparable with CA activity of bands *3-5* containing fragments of PS2 and light-harvesting complex.

To establish the position of CA activity in thylakoids, further study was carried out with fragments of thylakoid membrane enriched with either PS1 or PS2.

Characterizations of PS1- and PS2-membranes. To obtain these preparations, thylakoids preliminarily treated with Triton X-100 were used, and the CA activity carriers, which could be either in the lumen or weakly bound to membranes, passed into the supernatant. Thus, further, only CA activity tightly bound to thylakoid membrane was analyzed. Determinations of both PS1 content

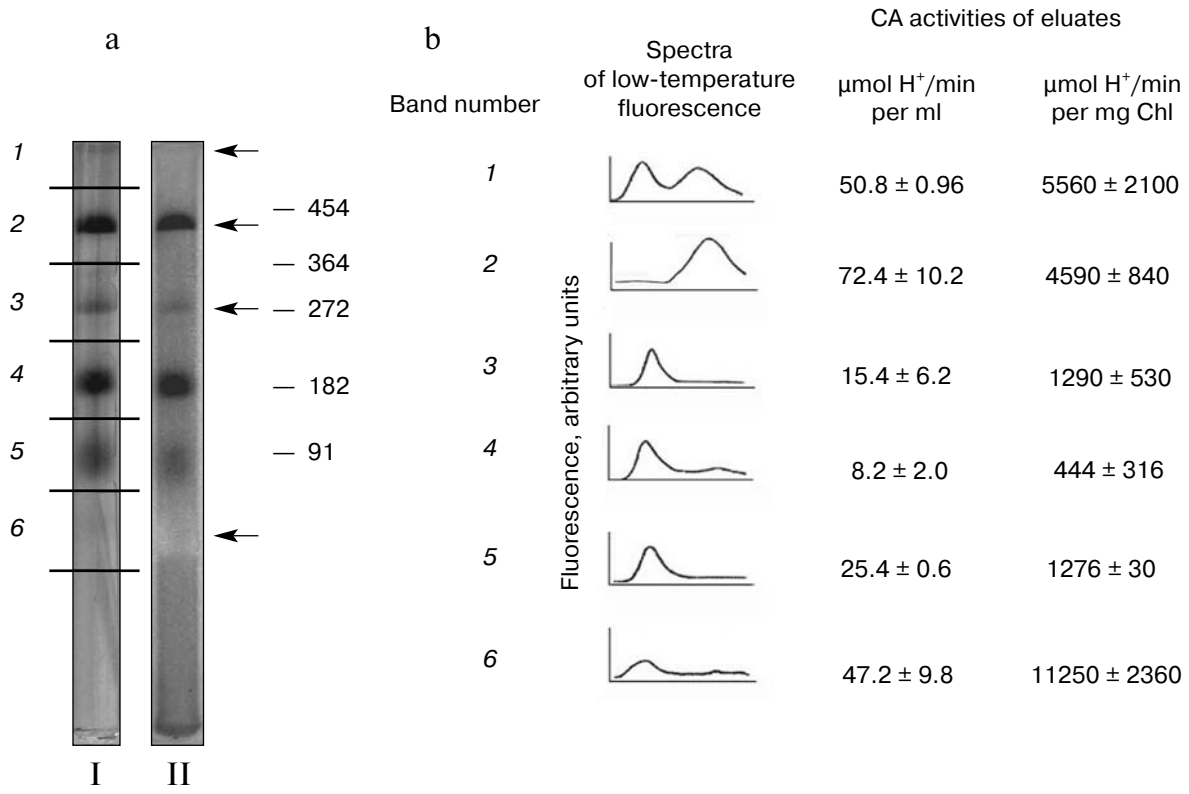

Fig. 1. Carbonic anhydrase activity of dodecyl maltoside extract from pea thylakoids. a) Electrophoregrams of the control "green" gel (I, the lines of cutting are shown) and the gel stained with bromthymol blue (II, the arrows show the positions of CA activity manifestation). The numbers on the right represent the molecular masses of marker proteins in kD. b) Spectra of low-temperature fluorescence of cut out green bands and CA activities of eluates of these bands. Left column, CA activity of 1 ml of eluate; right column, CA activity calculated on chlorophyll basis.

and PS2 content in the preparations obtained showed that PS2-membranes had small PS1 impurity (up to 10%) while PS1-membranes did not contain P680, the reaction center of PS2 (Table 1). The light-induced O_2 evolution rates in PS2-membranes reached 125 μmol O_2/h per mg Chl (that corresponded to 500 μ-eq electrons per h per mg Chl), and the light-induced O_2 uptake rates in PS1-membranes reached 200 μmol O_2/h per mg Chl (that corresponded to 400 μ-eq electrons per h per mg Chl) (Table 1). These data indicated that the complexes of photosystems were not significantly destroyed during the isolation of the corresponding membrane fragments.

Table 2. Carbonic anhydrase activity of thylakoid membrane fragments enriched with either PS1 or PS2 washed free from Triton X-100 as well as supernatant S20 obtained after sedimentation of the photosystems

Fraction	CA activity, μmol H^+/min per mg Chl
PS1-membranes	460.5 ± 68.0
PS1-membranes + Triton	112.0 ± 5.5
PS2-membranes	58.3 ± 3.5
PS2-membranes + Triton	265.2 ± 38.5
Supernatant S20	109.6 ± 36.6

Note: The measurements in the presence of Triton X-100 were at Triton/Chl ratio of 1. Supernatant S20 contained Triton at Triton/Chl ratio of 20. The data of typical experiment are shown.

The PS1- and PS2-membranes possessed CA activity (Table 2). CA activity of PS1-membranes decreased due to their incubation with Triton at Triton/Chl ratio of 1 while CA activity of PS2-membranes under such conditions reached maximal values (previously we analyzed the stimulation of CA activity of PS2-membranes in the presence of Triton [15]). Supernatant S20 obtained after precipitation of the photosystems (see "Materials and Methods") also had high CA activity (Table 2). The spectrum of low-temperature fluorescence showed the presence of light-harvesting complex and PS1 in this supernatant (data not shown).

CA activity of PS2-membranes. The electrophoresis of PS2-membranes treated with DM at DM/Chl ratio of 15 was carried out in the same way as for thylakoids, and as in the thylakoids the change in BTB color was easily

observed in the region of proteins with low-molecular-masses (Fig. 2a). The change in BTB color was observed also in the bands containing high-molecular-mass pigment–protein complexes, which were very likely undamaged complexes of PS2 as well as the monomers and dimers of core complexes (Fig. 2a, arrows near upper part of gel). The green color of these bands masked the yellow color of BTB, but we previously showed [15] the presence of considerable CA activity in these components of PS2.

The effects of specific sulfamide inhibitors of CA activity, the liposoluble ethoxyzolamide (EZ) and weakly penetrating into membrane acetazolamide (AA), were studied in the presence of Triton to provide the manifestation of maximal CA activity of PS2-membrane. AA functioned in a paradoxical manner, namely, it stimulated CA activity at submicromolar concentrations and inhibited this activity only at concentrations more than 10^{-6} M (Fig. 3a, curve *1*). Previously we found such effects of this inhibitor when we measured dehydrase CA activity of both undamaged thylakoids [17] and PS2-membranes isolated by another procedure [14]. It is necessary to note the high sensitivity of CA activity of PS2-membrane to the liposoluble EZ. The activity was inhibited completely at its concentration 10^{-8} M (Fig. 3a, curve *2*), and I_{50} was 10^{-9} M.

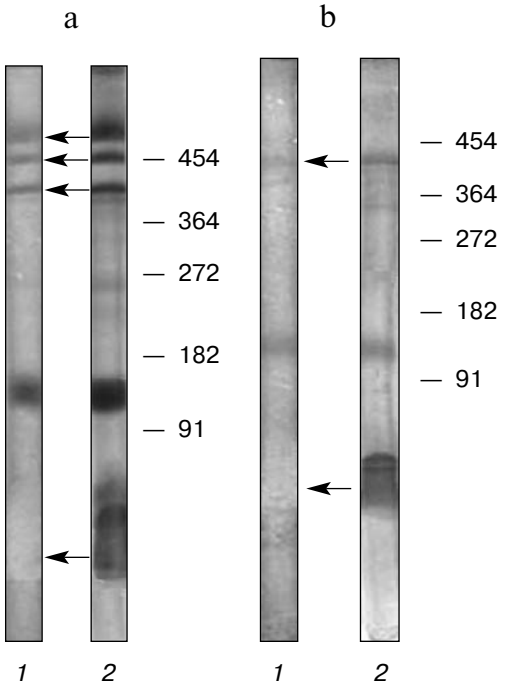

Fig. 2. Electrophoresis of PS2- (a) and PS1-membranes (b) after treatment with dodecyl maltoside. PS2- and PS1-membranes contained 50 and 20 μg Chl, respectively. Lanes: *1*) gels stained with bromthymol blue; *2*) gels stained with Coomassie G-250. The arrows show the bands manifesting CA activity. The numbers on the right represent the molecular masses of marker proteins in kD.

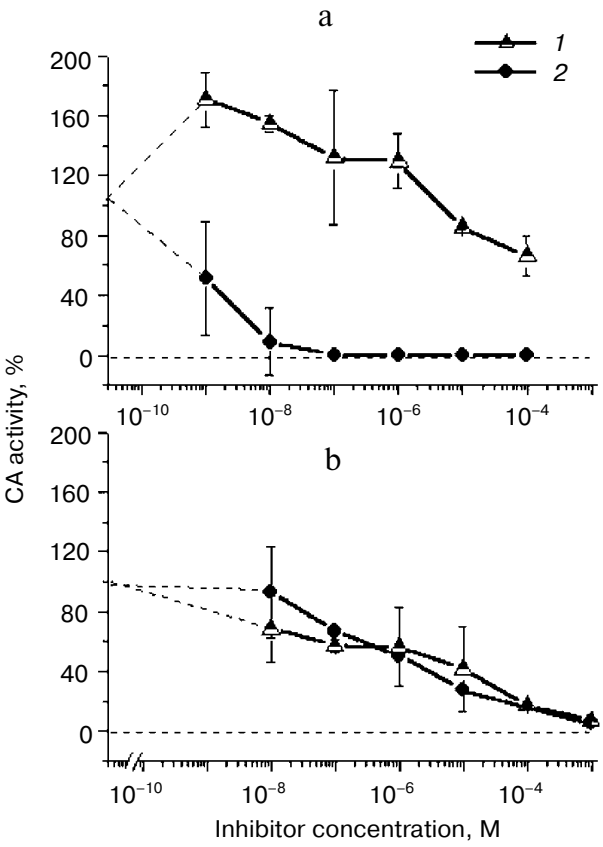

Fig. 3. Effects of sulfamide inhibitors acetazolamide (*1*) and ethoxyzolamide (*2*) on carbonic anhydrase activities of PS2- (a) and PS1-membranes (b). Activity of PS2-membrane was measured in the presence of Triton X-100 at Triton/Chl ratio of 1. In the experiments with acetazolamide 100% activities were 45 (a) and 540 μmol H^+/min per mg Chl (b); in experiments with ethoxyzolamide 100% activities were 60 (a) and 256 μmol H^+/min per mg Chl (b).

CA activity of PS1-membranes. CA activity of PS1-membranes if calculated on chlorophyll basis was 5-10 times higher than the CA activity of PS2-membranes (Table 2) even after the treatment of PS2-membranes with Triton X-100. Only CA activity of the PS1-membranes isolated from thylakoids of winter plants was comparable with CA activity of PS2-membranes.

To exclude that the presence of CA activity in PS1-membranes is the result of an adsorption, in the course of isolation, of protein possessing CA activity, PS1-membranes were incubated in medium with NaCl at high concentrations (0.1 and 0.5 M) followed by centrifugation. It was established that neither passing of CA activity into solution, nor decrease in CA activity of these membranes occurred (data not shown).

The electrophoresis of PS1-membranes treated with DM in the same way as PS2-membranes revealed four pigment–protein complexes (Fig. 2b, gel *1*). A readable change in BTB color occurred in the region of proteins

Table 3. Effects of carbonic anhydrase inhibitors on low-molecular-mass CA revealed on PAGE of dodecyl maltoside-treated PS1- and PS2-membranes

Eluate of	CA activity, μmol H^+/min per mg Chl		
	without inhibitor	+ ethoxyzolamide	+ acetazolamide
PS1-membranes	8833 ± 1239	4570 ± 1453	5125 ± 685
PS2-membranes	5296 ± 1631	0	14,444 ± 605

Note: Bands with CA activity in the region of low-molecular-mass proteins were cut out, and their content was eluted by veronal buffer. The concentration of both inhibitors was $1.5 \cdot 10^{-7}$ M.

with low molecular masses. Moreover, the yellow color appeared also at the start region containing heavy fragments of membranes.

In distinction from the above described effects on CA activity of PS2-membranes, EZ and AA affected CA activity of PS1-membranes similarly (Fig. 3b) (I_{50} was close to 10^{-6} M for both of them). It can be noted that in the experiments with PS1-membranes the stimulation of CA activity by submicromolar concentrations of AA was never observed. Also, the inhibition of CA activity of PS1-membranes by AA in the presence of Triton was somewhat higher than without detergent (not shown).

Effects of inhibitors on CA activity of low-molecular-mass proteins of PS1- and PS2-membranes solubilized with dodecyl maltoside. As noted above, after DM treatment of both PS2- and PS1-membranes the presence of CA activity in polyacrylamide gel in the region of low-molecular-mass proteins was observed. However, the electrophoresis conditions favorable for visualization of CA activity of high-molecular-mass pigment–protein complexes did not provide good resolving power for low-molecular-mass proteins. In order to establish the similarity or distinction of low-molecular-mass CA activity carriers solubilized with DM from PS1- and PS2-membranes, the effects of inhibitors on CA activities of eluates of the corresponding gel bands were studied. AA ($1.5 \cdot 10^{-7}$ M) stimulated CA activity of eluate of PS2-membranes and EZ suppressed it, while the both inhibitors equally suppressed CA activity of low-molecular-mass proteins eluted from PS1-membranes (Table 3). These effects of inhibitors were similar to their effects on undamaged PS1- and PS2-membranes (Fig. 3), and they suggest the presence of different proteins possessing CA activity in these membranes.

DISCUSSION

Carbonic anhydrase activity is linked up with the high-molecular-mass components of the core complex of PS2 (Fig. 2a). Previously, using affinity chromatography [15] we showed that both monomers and dimers of this complex possessed CA activity. Now we have found that PS2-membranes also have a low-molecular-mass carrier of CA activity. The paradoxical effect of AA originates from peculiarities of this carrier since CA activity of eluate of gel region with low-molecular-mass proteins of PS2 increased significantly in the presence of this inhibitor (Table 3). Both the high- and low-molecular-mass carriers of CA activity associated with PS2-membranes exhibited high sensitivity to the lipophilic inhibitor of CA, ethoxyzolamide, with I_{50} close to 10^{-9} M, and such high sensitivity was observed in the presence of Triton X-100, i.e. under conditions of the manifestation of maximal activity of these membranes. It is known that high sensitivity to sulfamides is distinctive of CA belonging to α-class [4]. In fact, the proteins of PS2-membranes isolated from mesophyll thylakoids of both corn and pea showed cross-reaction with antibodies to α-CA of *Chlamydomonas reinhardtii* [13, 16].

The low-molecular-mass protein possessing CA activity was found not only in DM extract from PS2-membranes (Fig. 2a) but also in PS1-membranes treated with DM (Fig. 2b). It might be suggested that a certain low-molecular-mass CA is evenly distributed along the thylakoid membrane, and, consequently, could be observed in the preparations of both photosystems. However, a number of facts indicate the difference between the low-molecular-mass carriers of CA activity in the preparations of the two photosystems. First, a carrier in PS1-membranes was less sensitive to sulfamides. Second, both inhibitors, lipophilic and hydrophilic, inhibited CA activity of PS1-membranes equally, and this indicated the accessibility of the carrier to the action of the both inhibitors. Third, AA did not stimulate CA activity of PS1-membranes at any concentration. These distinctions, which were observed in large membrane structures, were preserved after solubilization of the carriers of CA activity with dodecyl maltoside. Lastly, CA activity of PS1-membranes was suppressed by Triton under conditions when it stimulated the activity of PS2-membranes (Table 2).

Some literature data also evidence the presence in PS1-membranes of protein possessing CA activity, different from the carriers of the activity in PS2-membranes. Membranes enriched with PS1 possessing CA activity did not show a cross-reaction with antibodies to the connected with PS2 α-CA from *C. reinhardtii* [16]. Taking into account both these facts and that the carrier of CA activity in PS1-membranes is less sensitive to sulfamides, as it is shown in the present paper, it is possible to propose that this carrier belongs to the β-class of CA. Two groups of scientists registered colloidal gold particles bound to antibodies against soluble β-CA of pea leaves in the thylakoid membranes from pea leaves [23, 24]. In potato leaves, the binding of colloidal gold with thylakoid membranes was

observed when an antiserum to synthetic oligopeptide of N-terminal end of the chloroplasts CA was used [6]. We however earlier showed the absence of cross-reaction of proteins solubilized with SDS from pea thylakoids with antibodies against soluble β-CA of spinach [14].

What could be the functions of the thylakoid membrane CAs? It is known that bicarbonate ions are required for normal electron transport at the acceptor side of PS2 in the segment Q_A (primary quinone acceptor) → ferrous iron → Q_B (secondary quinone acceptor) [25], and a number of facts indicates their participation in processes at the donor side of this photosystem, probably in reactions of water oxidation [26]. The presence of CA regulating supply of bicarbonate to the required site in pigment–proteins complex of PS2 could provide the effective proceeding of these reactions. The assumption about participation of CA in the protection of PS2 from photoinhibition under conditions of high illumination, specifically, in binding the excessive protons with formation of uncharged CO_2 molecule well soluble in lipid membrane phase, was made earlier [26]. The function of CA found in PS1-membranes could be "delivery" of CO_2 to ribulose-bis-phosphate carboxylase/oxygenase (Rubisco) connected with the stromal membranes where PS1 is disposed. The presence of CA in a multienzyme complex executing CO_2 fixation and connecting Rubisco with thylakoid membrane was shown [23, 27]. A hypothesis proposing the participation of the membrane-associated CA in bicarbonate dehydration accompanied by production of CO_2 was made by Pronina and Semenenko [28]. We recently showed that protons accumulated in thylakoids in the light are used in the course of dehydration of bicarbonate added into a suspension of isolated thylakoids, and we suggested that this reaction could occur at the stromal membrane surface if the CA provides a channel for protons leaving the lumen [29].

It was found that addition of zinc suppressed CA activity of thylakoids [9] and PS2-membranes [14], and apparently not all thylakoid CAs constitute the typical CAs containing zinc coordinated by three histidine molecules in the active center [30]. Possibly, substitution of zinc for another metal, for example, for manganese on the donor side of PS2 and for ferrous iron on its acceptor side occurred in the process of evolution. The substitution of zinc for cobalt in animal α-CAII did not cause the loss of its activity [31], and substitution of zinc for ferrous iron in γ-CA of *M. thermophila* even caused an increase in CA-activity under anaerobic conditions [32].

Thus, our data suggest the presence in thylakoid membranes of three CAs, two connected with PS2 and one connected with PS1.

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