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Two Types of Ammonium Uncoupling in Pea Chloroplasts

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Abstract—The effect of ammonium on ATP synthesis, electron transfer, and light-induced uptake of hydrogen ions in pea chloroplasts was studied. It is shown that the dependence of these reactions on ammonium concentration could be due to effects of two different uncoupling processes. The first process is induced by low ammonium concentrations (<0.2 mM); the second one is observed in the NH_4Cl concentration interval of 0.5-5.0 mM. The first type of uncoupling is stimulated by palmitic acid or by *N*,*N*′-dicyclohexylcarbodiimide, while the second is stimulated by chloroplast thylakoid swelling caused by energy-dependent osmotic gradients. In the presence of the fluorescent dye sulforhodamine B, which does not penetrate through the cell membrane, this swelling causes the dye to enter the lumens. It is supposed that ammonium activates two different routes of cation leakage from the lumen. The first route involves channel proteins, while the second is a mechanosensitive pore that opens in response to osmotic gradients.

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To understand how the light energy transformation in plant chloroplasts is regulated, it is necessary to study the effect on this process of low molecular weight substances—substrates and cell metabolism regulators. One such substance is ammonium, which serves as substrate for glutamine synthase (GS), a key enzyme of nitrogen metabolism. Pea chloroplasts contain up to 60% of the glutamine synthase in leaves (GS2). In GS2 the mean K_m value for NH_4^+ is in the range 0.1-0.6 mM [1-3]. This means that ammonium concentration in chloroplast stroma should be in the range of 0.2-0.5 mM to maintain active amino acid synthesis. However, it was believed for a long time that it did not exceed 10 μM [4]. The development of analytical methods resulted in a change of this concept. It was shown that ammonium concentration in the aqueous phase in tomato leaf apoplast and in rape leaf tissue is from 0.1 [2] to 1.0 mM [3, 5].

Ammonium in the form of ammonia easily penetrates through the membrane and it is easily lost during the isolation of organelles. Since ammonium is not detected in chloroplasts *in vitro*, it was not previously considered as an endogenous regulator of chloroplast energetics.

During stationary illumination of chloroplasts, ammonium is accumulated in the thylakoid lumen in accordance with the pH gradient. Accumulated ammonium cations are compensated by chlorine anions delivered into the lumen from the stroma. It is assumed that ammonium and chlorine ions accumulated inside illuminated thylakoid can cause osmotic swelling that induces the leakage of hydrogen or ammonium ions from the lumen [6, 7].

Isolated chloroplasts are characterized by two functional effects caused by ammonium. At low ammonium concentrations it stimulates ATP synthesis if photophosphorylation is catalyzed by linear electron transfer from water to photosystem 1 (PS1) acceptors [8, 9]. On the other hand, at 5-10 mM concentration ammonium eliminates the transmembrane pH gradient, i.e. it is an uncoupler of electron transport and ATP synthesis. Both effects can be the result of change in the membrane cationic

Abbreviations: Chl, chlorophyll; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; ΔH, light-induced uptake of hydrogen ions; ETC, electron transport chain; GS, glutamine synthase; MV, methyl viologen; PS1, photosystem 1; V_e, basal rate of electron transfer from water to acceptors of PS1.

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conductivity, but still there are no data concerning the existence of any ammonium or ammonia channels in chloroplasts. The complete decoding of the *Arabidopsis* genome made it possible to show that cytoplasmic and mitochondrial membranes of plants contain proteins that structurally and functionally resemble channel proteins of bacterial and animal cells [10, 11]. However, none of the known channel proteins was found in thylakoid membrane. The membrane ion conductivity was investigated only using electrophysiological methods. It was shown that thylakoid membranes contain potential-dependent potassium channels that might also conduct ammonium. Potassium channels with conductivity of 14 and 20 pS were observed in lipid bilayer with incorporated vesicles of thylakoid membranes [12]. In the thylakoid vesicle membranes obtained under conditions of osmotic shock, channels with higher conductivity of 60 and 40/90 pS were registered [13, 14]. A non-selective channel (or pore of 620 pS), through which molecules of the fluorescent dye Lucifer Yellow could penetrate into the lumen, was also found [14].

It can be supposed that in thylakoids the dependence of energy transformation on ammonium concentration should be at least biphasic, at low ammonium concentrations functional effects are determined by leakages via potassium channels, and in the case of high concentrations they are determined by the leakage via pores that open due to energy-dependent swelling of thylakoids.

In this work, ammonium uncoupling was studied under conditions close to those in stroma of the native chloroplasts in cells. The data show that ammonium really induces two types (pathways) of hydrogen ion leakage from the lumen. The first one is activated at low ammonium concentrations (<0.2 mM); it is stimulated by palmitate or *N*,*N*′-dicyclohexylcarbodiimide (DCCD). The second type of leakage occurs at high ammonium concentrations. It is a membrane pore with unspecific conductivity, permeable for the fluorescent dye sulforhodamine B. The pore opens upon swelling of illuminated thylakoids in response to osmotic gradients.

It is supposed that ammonium and palmitate at low concentrations are able to provide for reversible endogenous uncoupling of energy transformation processes in chloroplasts of plant cells.

MATERIALS AND METHODS

Chloroplasts were isolated from 2-week-old pea seedlings as described earlier [15], washed in medium containing 0.2 M sucrose, 10 mM KCl, 5 mM $MgCl_2$, and 10 mM Tricine-NaOH, pH 7.8, resuspended in the same medium, and stored in the dark at 0°C. Chlorophyll concentration was determined according to Arnon.

Chloroplasts were treated with DCCD by the incubation of the organelles (0.1 mg chlorophyll (Chl) in 1 ml) for 5 min at 20°C in storage medium with 0.2 mM DCCD. The suspension was centrifuged for 10 min at 0°C, and the pellet was resuspended in fresh storage medium.

In experiments chloroplasts (40 μg Chl in 1 ml) were illuminated by red light ($\lambda > 600$ nm) for 1-2 min at 20°C. Ammonium was added in the dark before illumination. Electron transport was measured in reaction medium containing 0.2 M sucrose, 10 mM KCl, 5 mM $MgCl_2$, 3 mM Hepes, 3 mM Tricine, pH 7.8, 0.1 mM methyl viologen (MV) or 0.4 mM ferricyanide, and chloroplasts, or using a Clark electrode by oxygen uptake/release, or using a pH-electrode by H^+ release in reactions with ferricyanide. The reversible light-induced H^+ uptake (ΔH) was measured by the pH-electrode in media with low buffer concentrations (0.1 mM Hepes + 0.1 mM Tricine) and with MV or 50 μM PMS (phenazine methosulfate) + 2 μM diuron. ATP synthesis was calculated from H^+ uptake [16] in medium containing 2 mM KH_2PO_4, 0.25 mM ADP, 2 mM Tricine, pH 7.8, and MV or PMS + diuron. The sucrose, KCl, and $MgCl_2$ concentrations were as those in measurements of electron transport. The pH scales were calibrated by addition of known amounts of HCl to reaction media with chloroplasts. Data scattering for all measurements on chloroplasts of the same isolation did not exceed 5%.

To study sulforhodamine and chlorophyll fluorescence distribution in chloroplasts, a Leica TCS SPE confocal microscope was used. Chloroplasts were extruded from thin sections of young *Peperomia caperata* leaves directly into the reaction medium that was also used to register basal electron transfer. The medium contained MV, 50 μM fluorescent dye sulforhodamine B, and ammonium or palmitate additions according to the legend on Fig. 5. Specimens were placed under the microscope and distribution of the dye and chlorophyll fluorescence, excited by two lasers with wavelengths of 488 and 635 nm, was registered. The fluorescence intensity was registered in the range 538-590 nm for sulforhodamine B and 640-750 nm for chlorophyll. Sulforhodamine B at 50 μM did not influence chloroplast functions.

ADP, DCCD, sulforhodamine B, and methyl viologen from Sigma (USA) were used in this work.

RESULTS

The problem of studying the effect of ammonium on the processes of energy transformation in thylakoids is that it is impossible to measure the transmembrane pH gradient directly using suitable pH-indicators (such as ^{14}C-methylamine, 9-aminoacridine, or neutral red). These probes, like ammonium, are permeable amines, and therefore ammonium will compete with their cations for binding to anionic centers in the lumen [17]. Such competition is able not only to distort measured ΔpH val-

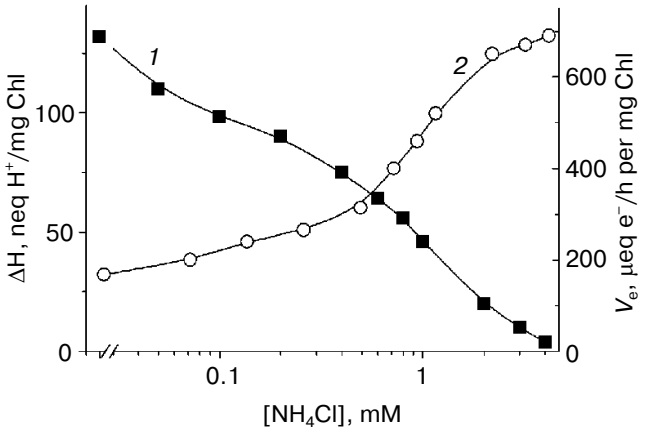

Fig. 1. Effect of ammonium on ΔH (*1*) and on the rate of basal electron transfer from water to MV (*2*). Measurement conditions are described in "Materials and Methods".

ues, but it can change the effect of ammonium on chloroplast functions. Besides, variations in ammonium and sucrose concentration in the reaction medium, which is necessary in this work, prevent determination of lumen volumes in each concrete case. Therefore, only qualitative estimation of pH gradient, convenient for comparison of various data, is possible. We measured parameters dependent on ΔpH but not on lumen volumes, such as the level of the light-induced H^+ uptake, rates of basal electron transport, and ATP synthesis. The ΔH value corresponds to the number of ionogenic groups binding H^+ upon pH gradient formation; the rate of electron transfer depends on pH in the lumen; ATP synthesis is defined by the pH gradient level and by the possibility of lateral transfer of H^+ to ATP synthases via domains not equilibrated with the aqueous phase of the lumen [9, 18, 19].

The effect of ammonium on ΔH and on the rate of basal electron transfer is shown in Fig. 1. Ammonium at low concentrations (<0.2 mM) caused noticeable inhibition of light-induced uptake of hydrogen ions (Fig. 1, curve *1*) accompanied by 1.5-2-fold stimulation of basal electron transfer (Fig. 1, curve *2*). Further uncoupling up to complete dissipation of the pH gradient was observed at high amine concentrations (0.5-5 mM). In all experiments changes of these two functions—ΔH formation and stimulation of basal electron transfer—corresponded to each other. In this case the two-phase character of all curves was observed, which is indicative of the existence of two partially overlapping concentration ranges of increased proton conductivity in membranes: below 200 μM and above 0.5 mM NH_4Cl.

In Fig. 2 the effects of ammonium on ATP synthesis and ΔH for reactions with MV (solid lines) and PMS in the presence of diuron (dotted lines) are compared. It is seen that in reactions with PMS the curves of ATP synthesis and ΔH correlate with each other (Fig. 2, curves *2* and *4*), whereas in reactions with MV no such correlation was observed (Fig. 2, curves *1* and *3*). Ammonium at low concentrations stimulated ATP synthesis with MV despite induction of H^+ leakage. This means that in the reaction with MV at low ammonium concentrations overlapping of two processes took place, the inhibition and stimulation of ATP synthesis. The possibility of stimulation was determined by a peculiarity of coupling of reactions under conditions of functioning of the whole electron transport chain (ETC).

When we supposed that the biphasic character of uncoupling curves could be caused by induction of two independent leakages of hydrogen ions or ammonium from the lumen, we searched for stimulators and inhibitors of ammonium uncoupling. It appeared that the first phase of uncoupling, ion leakage at low ammonium concentrations, is stimulated by palmitic acid (Fig. 3, curve *1*) that in the absence of ammonium does not cause a decrease in pH gradient, does not inhibit ΔH, and does not accelerate electron transfer [20]. A similar effect was also caused by treatment of chloroplasts with the protein carboxyl modifier DCCD (Fig. 3, curve *2*). Unlike control (Fig. 3, curve *3*), ΔH dependence on ammonium concentration in the presence of these stimulators was always monophasic (Fig. 3, curves *1* and *2*), and in this case curves of the basal electron transfer acceleration also became monophasic [20, 21].

To clarify the contribution of osmotic gradients to ammonium uncoupling, we used sucrose-free reaction mixtures. This provided for the possibility of light-induced chloroplast swelling in response to ammonium concentrations lower than those in sucrose-containing media.

Data obtained on chloroplasts of the same isolation and placed in the sucrose-free medium (solid lines) and

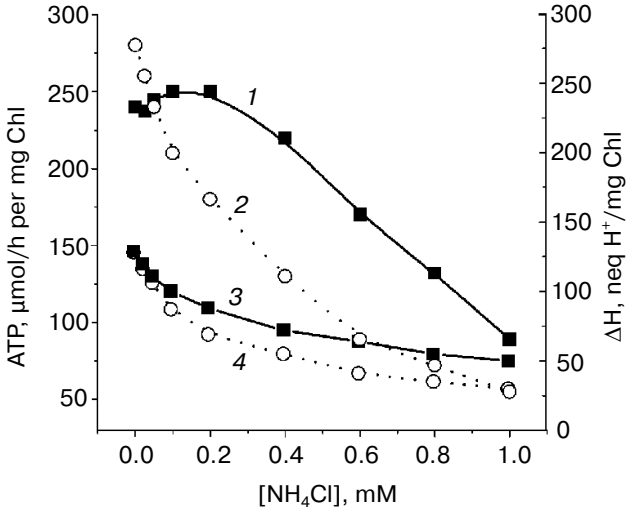

Fig. 2. Effect of ammonium on ATP synthesis (*1*, *2*) and ΔH (*3*, *4*) in reactions with MV (solid lines) and PMS in the presence of 2 μM diuron (dotted lines). Chloroplasts of the same isolation were used.

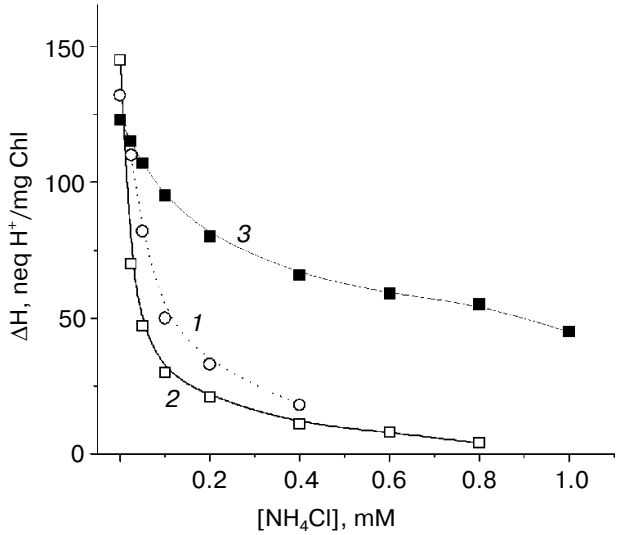

Fig. 3. Inhibition of ΔH by ammonium in the presence of 40 μM palmitate (*1*), in samples treated by DCCD (*2*), and in control chloroplasts (*3*).

in medium with sucrose (dotted lines) are shown in Fig. 4. In sucrose-free media, ammonium at low concentrations always inhibited ATP synthesis in the reaction with MV (Fig. 4a, curve *1*), and in this cases both phases of inhibition are well seen. In the absence of sucrose ΔH level (Fig. 4b, curve *1*) was always higher that in sucrose-containing media (Fig. 4b, curve *2*), which is indicative of emergence in the lumen of additional groups binding H^+ in the light. As seen in Fig. 4, in sucrose-free media the second phase of uncoupling was induced by amine at lower concentrations compared to those in the presence of sucrose. Thus, it could be supposed that this uncoupling phase is activated by chloroplast swelling.

To test this hypothesis and to study the question whether pores permeable for fluorescent dye are formed in the membrane, we used confocal microscopy. Chloroplasts of *Peperomia caperata* served as the model system because their size is twice larger than that of the pea chloroplasts. Figure 5 shows paired photographs of sulforhodamine B (upper row) and chlorophyll (lower row) fluorescence. Without additions, the size of chloroplasts was 10-15 μm. As seen in the top photograph of Fig. 5a, without additions the chloroplast grana are dark, i.e. they contain no dye. In the presence of 0.2 mM ammonium and 40 μM palmitic acid (Fig. 5b) the chloroplast size did not increase and the dye did not penetrate into grana. In the presence of 2 mM ammonium (Fig. 5c, top photograph) the dye fluorescence intensity inside the chloroplast sharply increased and became close to the level of its fluorescence in the reaction medium. In this case the chloroplast was not destroyed, because the distribution of chlorophyll fluorescence (Fig. 5c, bottom photograph) shows the 2-4-fold enlargement of chloroplast volume. Thus, as shown in Fig. 5, sulforhodamine B penetrated into thylakoids upon illumination of chloroplasts in the presence of ammonium at high concentrations.

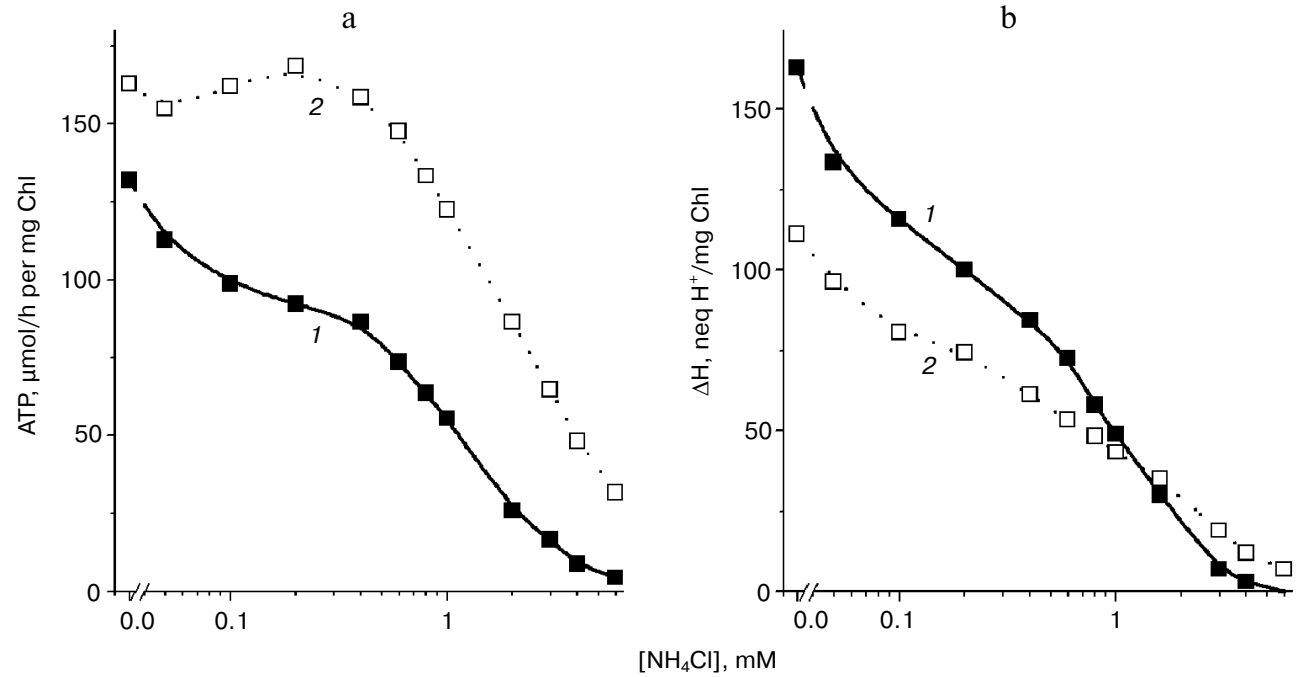

Fig. 4. Effect of ammonium on ATP synthesis (a) and ΔH level (b) in sucrose-free media (solid lines) and under standard conditions, i.e. in the presence of 0.2 M sucrose (dotted lines). Chloroplasts of the same isolation and the electron acceptor MV were used.

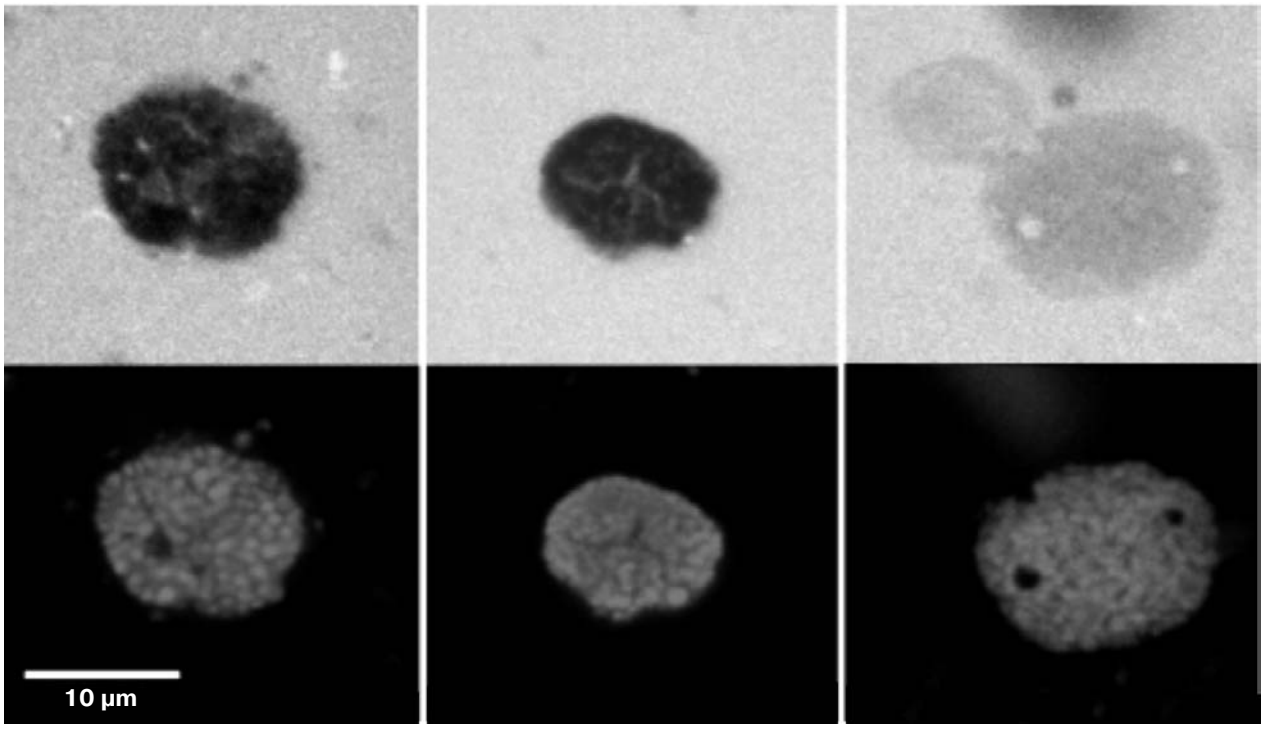

Fig. 5. Distribution of sulforhodamine B (top) and chlorophyll (bottom) fluorescence in *Peperomia caperata* chloroplasts 1 min after illumination with red light in standard medium (a), in the presence of 0.2 mM NH_4Cl and 40 μM palmitate (b), and in the presence of 2 mM NH_4Cl (c). MV was the electron acceptor.

DISCUSSION

Our results demonstrate the existence of biphasic uncoupling by ammonium. On curves of ΔH, V_e with MV, and cyclic reactions with PMS these phases were detected in identical ranges of ammonium concentrations—0-0.2 and 0.5-5 mM (Figs. 1, 2, and 4).

In sucrose-containing media the dependence of ATP synthesis on ammonium concentration in the reaction with MV was more complicated: the photophosphorylation was stimulated rather than inhibited by ammonium at low concentrations. It is supposed that in this reaction ATP synthesis involves two pools of ATP-synthase complexes, the first of which receives H^+ from the lumen, while the other one does it from the membrane-adjacent domains [9, 18, 19, 22]. The rate of ATP synthesis by the first pool of complexes depends only on pH gradient, while the rate of synthesis by the second pool depends on the rate of H^+ transfer via the ways localized in domains, which is proportional to the rate of electron transfer. Superposition of two processes takes place in the case of pH gradient lowering in response to low ammonium concentrations. ATP synthesis at the second complexes is stimulated due to acceleration of V_e, whereas the photophosphorylation at the lumen-associated complexes is inhibited. The H^+ transfer along domains is distorted by exclusion of sucrose due to which inhibition of ATP synthesis with MV becomes biphasic (Fig. 4a). The domains are not involved in the reaction of cyclic photophosphorylation with PMS, and therefore this reaction is always inhibited by ammonium at low concentrations (Fig. 2, curve *2*).

Curves of ammonium uncoupling can be represented as the sum of action of two different transport systems activated by ammonium. The first one is characterized by higher affinity to NH_4^+ ions, but it provides for rather low maximal rate of ion leakage, because in the absence of stimulators no more than twofold acceleration of basal electron transfer is observed. The second system exhibits low affinity to ammonium but is able to provide for high efficiency of ion leakage, i.e. the rate of ammonium-coupled electron transfer reaches the level characteristic of uncoupling by 1 μM gramicidin D. Each V_e curve (Fig. 6a) can be represented as the sum of a constant value and two Hill functions:

$$f(NH_4^+) = M_0 + M_1 \frac{K_1^{n1}}{K_1^{n1} + [NH_4^+]^{n1}} + M_2 \frac{K_2^{n2}}{K_2^{n2} + [NH_4^+]^{n2}}$$

$$M_0 + M_1 + M_2 = M_{max},$$

where K_1 and K_2 are apparent constants of dissociation of ammonium complexes with membrane components; $n1$

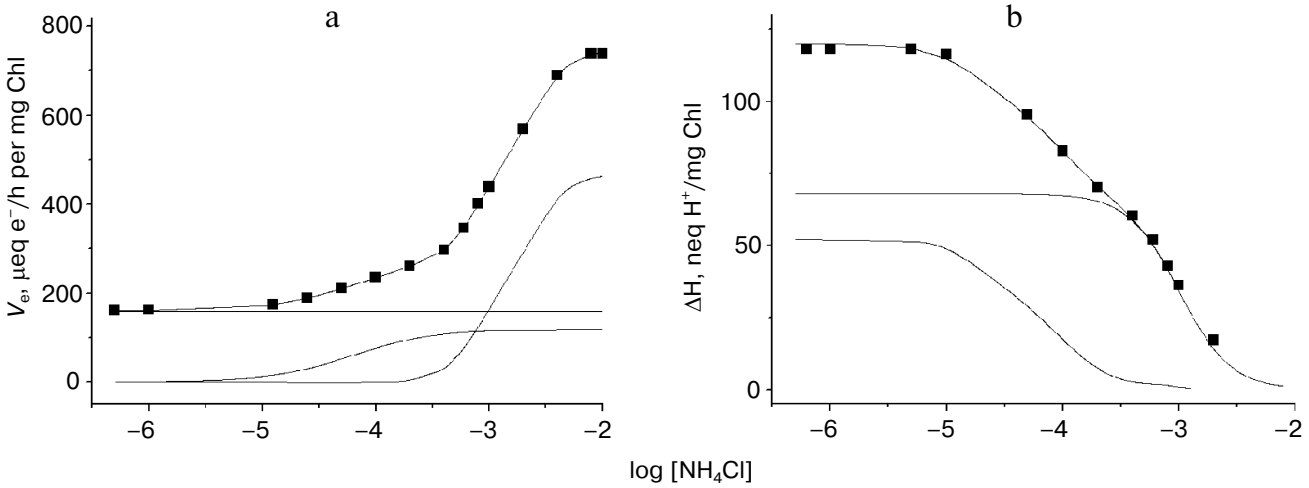

Fig. 6. Decomposition of experimental curves (symbols without lines) on the basis of Hill functions (solid lines without symbols): a) V_e with ferricyanide; b) ΔH with MV.

and *n*2 are Hill coefficients; M_0 is the rate of basal electron transport in the absence of ammonium; M_{max} is maximal value of the rate of electron transport.

There is no stable component in the ΔH dependence on ammonium concentration ($M_0 = 0$), but like V_e curves, it is well approximated by the sum of two Hill functions (Fig. 6b), and values of the sum of decomposition functions are within experimental error. Attempts to represent the experimental curves by a single function result in 10-100-fold increase in the error of approximation. The use of two expansion functions gives good results and approximate values of K_1 and K_2 can be estimated using experimental curves; therefore, there is still no grounds to introduce additional decomposition functions for ΔH and V_e data. However, curves of ATP synthesis with MV in the sucrose-containing medium require introduction of a third Hill function corresponding to the stimulation of ATP synthesis by ammonium at low concentrations.

We have carried out decompositions of over 50 V_e and ΔH curves using Hill functions similar to those shown in Fig. 6 as examples. Hill functions and their sums are shown by lines, and the experimental data are designated by symbols. The first uncoupling phase has K_1 values of 60 ± 20 μM, and K_2 values are in the range of 1-2 mM in the presence of sucrose and 0.5-1 mM in the sucrose-free media.

In the presence of palmitate or DCCD, the contribution to total uncoupling sharply increases and the curves become practically monophasic (Fig. 3). Mechanisms of action of these stimulators are still unclear. It should be noted that in the reaction with palmitate K_1 remains unchanged. This means that palmitate increases conductivity of the first system but does not change its affinity to ammonium. It is possible that the effect of palmitate is mediated by the ADP/ATP antiporter recently found in thylakoids [23, 24], which can be activated by ammonium. The second stimulator, DCCD, is usually used as a modifier of protein carboxyls [25] and a blocker of ATP synthase H^+ channel [26, 27]. In our case it decreases the K_1 value, i.e. it increases affinity to ammonium of the first uncoupling system (Fig. 3). DCCD is known to inhibit the light-induced uptake of ammonium [21] and accelerate the ΔΨ decrease after a light flash [28, 29]. It is also known that at DCCD/Chl = 2, DCCD is able to bind covalently to carboxyls of the CF_o channel and to polypeptides of the LHC family of photosystem 2 [28, 30-35]. It has been shown that the isotope is bound to groups localized close to the membrane luminal surface [33, 34]. However, there are no data showing that just these groups are involved in ion transfer, since irreversible modification of carboxyls in amphiphilic domains is also possible without covalent binding of isotope [25].

It is known that DCCD displaces calcium bound by amphiphilic loops of polypeptides CP29 and psbS [34, 35]. Calcium, in turn, can serve as a channel blocker for monovalent cations potassium and ammonium. It is also possible that in our experiments DCCD increases conductivity of the first uncoupling system for monovalent cations by the removal of calcium that closes the channel gate.

It became possible to clarify the mechanism of induction of the second uncoupling phase using variations of the osmotic strength of the medium and using the confocal microscopy. In the absence of sucrose K_2 values decreased in all reactions (Fig. 4), i.e. induction of the second phase of uncoupling as a rule required only half as high ammonium concentration.

In the state of photosynthetic control at reaction medium pH 7.8, pH value inside the lumens of illumi-

nated chloroplasts is in the range of 5.4-5.7 [36]. The transmembrane proton gradient is 2.2 ± 0.1 pH units, so ammonium concentration inside the lumen should be 100-200-fold higher than in the medium. It is quite probable that ammonium binding to membrane components, which induces uncoupling, takes place on the lumen surface of the membrane. In this case K_1 and K_2 should be at least 100-fold higher, i.e. K_1 will be 8-10 mM, and K_2 of the order of 100 mM. The electric charge of ammonium ions in lumens are compensated by chlorine anions. This means that at ammonium concentrations in the medium above 1 mM osmotic pressure in the lumens of illuminated chloroplasts will exceed that in the medium containing 0.2 M sucrose. Swelling of thylakoids becomes possible, which is confirmed by data shown in Fig. 5c. This means that the second leakage system can be mechanosensitive.

A pore with high but not selective conductivity was found in thylakoids swollen after osmotic shock [14]. We supposed that thylakoid swelling, caused by the establishment of osmotic gradients in the light, will promote opening of membrane pores at sufficiently high ammonium concentrations. In this case dissipation of all concentration gradients should take place due to exchange between lumen content and environment. Therefore, it could be expected that the fluorescent dye sulforhodamine B, added to the medium and not penetrating through membranes, would be able to get into thylakoids. As seen in Fig. 5a, in the absence of ammonium chloroplast grana remained dark because the dye did not get into them. Ammonium at low concentrations caused neither granum lightening nor chloroplast swelling even in the presence of 40 μM palmitate, when electron transport reached the level characteristic of complete uncoupling by gramicidin (Fig. 5b). Only ammonium at high concentrations caused the increase in chloroplast volume and in the sulforhodamine B fluorescence inside chloroplasts, which is indicative of penetration of the dye into granum lumens, i.e. of emergence of open pores in thylakoid membranes.

Since osmotic pressure in lumens falls along with pore opening, pores might close. In the case of continued chloroplast illumination, it is possible that ion gradients on thylakoid membranes are formed again, which might stimulate pore re-opening and the penetration of additional sulforhodamine amount into lumens. The frequency of such cycles should be increased along with the increase in ammonium concentration in the reaction medium, which will also provide for accompanying acceleration of electron transfer.

It is possible that just these pores were detected in [14]. The authors supposed that pores in native chloroplasts are closed for ions and serve for protein transport through thylakoid membranes. Systems that transport small proteins through thylakoid membrane are well known [38, 39].

In this work the existence of two types of ammonium uncoupling of photosynthetic reactions on chloroplast thylakoids was shown for the first time.

Uncoupling of the first type might be due to an ion-selective channel or channels. These channels are activated by ammonium at low concentrations characteristic of stroma of a native cell. The channel activation causes a decrease in ion gradient, which, in turn, results in the stimulation of electron transport and ATP synthesis catalyzed by electron transfer via the complete ETC. The channel conductivity sharply increases in response to palmitate at low concentrations, which can be involved *in vivo* in efficient regulation of energy transformation processes in a vegetative cell, thus optimizing coupling and preventing excessive lumen acidification upon decrease in ADP level.

Uncoupling of the second type is induced by ammonium at high concentrations and corresponds to the classic type of ammonium uncoupling. Our data show that this uncoupling is activated by energy-dependent chloroplast swelling caused by osmotic ion gradients. The appearance in thylakoids of open non-selective pores allows passage through the membrane of sulforhodamine B molecules and smaller ions, including different amine cations.

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