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Synergism of Ammonium and Palmitic Acid in Uncoupling of Electron Transfer and ATP Synthesis in Chloroplasts

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Abstract—Uncoupling by ammonium of electron transfer and ATP synthesis during linear transfer of electrons from water to photosystem 1 acceptors was studied in pea chloroplasts. It was shown that 40 μM palmitic acid decreased several-fold the ammonium concentrations necessary for 50% inhibition of ATP synthesis. The protonophore carbonyl cyanide *m*-chlorophenylhydrazone has no such property. The enhancement by palmitate of ammonium-induced uncoupling is accompanied by acceleration of basal electron transfer and decrease in the photoinduced uptake of hydrogen ions (H^+). In the absence of ammonium, palmitate has no effect on basal transport and stimulates uptake of hydrogen ions. This means that in the case of combined action of palmitate and ammonium an additional leakage of H^+ takes place, resulting in dissipation of the pH gradient. Synergic action of two metabolites, free fatty acid and ammonium, is supposed to provide for functioning of a system of mild regulation of energy coupling processes in native plant cell chloroplasts. Possible mechanisms of synergism are discussed.

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Key words: ammonium uncoupling, palmitic acid, electron transfer, ATP synthesis, chloroplasts

The effects of substrates and cell metabolism products on chloroplast energetics are usually studied individually, whereas these substances circulate simultaneously in the living cell cytoplasm.

Among these metabolites there are, in particular, ammonium and free fatty acids. The content of these substances in a native cell depends on many factors. Ammonium is absorbed from the environment and is formed of nitrates; it is used for syntheses of amino acid and nitrogenous bases. Fatty acids are produced and catabolized during intracellular lipid synthesis and hydrolysis, their amount and composition depending on growth conditions and plant age.

The effects of ammonium and free fatty acids on electron transfer and ATP synthesis have been rather comprehensively studied on chloroplasts of higher plants, but combined action of these metabolites was not investigated. It is known that ammonium at low concentrations (0.2-0.4 mM) decreases electrochemical pH gradient by 0.1-0.2 unit, but in this case it stimulates ATP synthesis [1, 2], and at high concentrations (5-10 mM) it is a classical uncoupler. It was shown on lettuce chloroplasts that saturated long-chain free fatty acids (FFA) have no effect on electrochemical gradient of hydrogen ions, but they inhibit ATP synthesis and stimulate basal electron transfer to the level of coupled transfer. They are not uncouplers but rather decouplers of the energy transformation processes. The inhibition of photophosphorylation by these acids is due to changes in lateral pathways of H^+ transfer to ATP synthases along the surface layer of the lumen [3, 4]. According to different data, palmitate causes mitochondrial "decoupling" [4] or uncoupling mediated by ATP/ADP antiporter or by specific uncoupling proteins able to transfer fatty acid anions through the membrane [5-8].

It is shown in this work that palmitic acid in pea chloroplasts is an endogenous stimulator of ammonium uncoupling. Palmitate and ammonium at low concentrations cause non-additive increase in transmembrane leakage of hydrogen ions resulting in dissipation of pH gradient, inhibition of ATP synthesis, stimulation of basal electron transfer, and lowering the photoinduced H^+ uptake. It is supposed that the synergistic action of

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Chl, chlorophyll; FFA, free fatty acids; ΔH, photoinduced uptake of hydrogen ions; PA, palmitic acid.

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ammonium and palmitate may provide for mild regulation of the energy transformation coupling in thylakoids of native plant cell chloroplasts.

MATERIALS AND METHODS

Class C chloroplasts were isolated from the two-week-old pea leaves as described earlier [9], but chloroplasts after isolation were washed at 4°C in solution containing 0.5 mg/ml BSA, 0.2 M sucrose, 10 mM Tricine-KOH, pH 7.8, 10 mM NaCl, and 5 mM $MgCl_2$. The chloroplasts were resuspended and kept in the same medium at chlorophyll (Chl) concentration 2-3 mg/ml. Chlorophyll concentration was determined following Arnon.

Albumin-free reaction media thermostatted at 20°C and containing salts and sucrose in above-mentioned concentrations, chloroplasts (40 μg Chl/ml), electron acceptors (0.1 mM methyl viologen or 0.4 mM ferricyanide), and buffers in different concentrations were used. Electron transport was measured using 2 mM Hepes + 2 mM Tricine, pH 7.8; photoinduced uptake of hydrogen ions (ΔH) was determined in the presence of 0.1 mM Hepes + 0.1 mM Tricine, pH 7.8; ATP synthesis was measured with 0.25 mM ADP, 2 mM K_2HPO_4 + 2 mM Tricine, pH 7.8. Palmitic acid (PA) dissolved in ethanol was added to reaction medium immediately after chloroplasts, and ammonium was added in 20-30 sec. The chloroplasts were devoid of catalase activity, and for this reason catalase inhibitors were not used in the experiments. Chloroplasts were exposed to red light (λ > 600 nm, 200 W/m^2) for 1-1.5 min.

The speed of electron transport with methyl viologen was measured by oxygen uptake using a Clark electrode, and in the case of ferricyanide as the acceptor it was determined by oxygen release or using a pH electrode following medium acidification.

Photoinduced uptake of hydrogen ions (ΔH) and ATP synthesis during electron transfer from water to methyl viologen were registered by alkalization of the reaction medium. The amount of absorbed H^+ was determined by titration of the medium with known doses of HCl. The rate of ATP synthesis was calculated using a published technique [10]. Data scattering for all measurements on chloroplasts of the same isolation did not exceed 5%.

ADP, ATP, BSA (fatty acids free), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and methyl viologen were from Sigma (USA).

RESULTS

Chloroplasts for investigation of the combined action of ammonium and palmitate were washed and kept in the presence of BSA, whereas no albumin was added to reaction media.

The effect of PA on thylakoid functions in ammonium-free media and in the presence of 0.2 mM NH_4Cl is shown in Fig. 1. Figure 1a shows that in the absence of ammonium, palmitate at PA/Chl = 1 inhibited ATP synthesis by 50% (curve *1*). The rate of basal electron transport did not increase in most experiments at PA concentrations below 40 μM (Fig. 1b, curve *1*). In approximately 10% of experiments, it decreased or increased by 10-20% in the presence of PA. A noticeable rate increase was registered at PA/Chl > 1. Addition of 0.2 mM ammonium to the reaction medium enhanced the inhibition of photophosphorylation by palmitate (Fig. 1a, curve *2*) and caused additional stimulation of basal electron transfer in the PA range 10-40 μM (Fig. 1b).

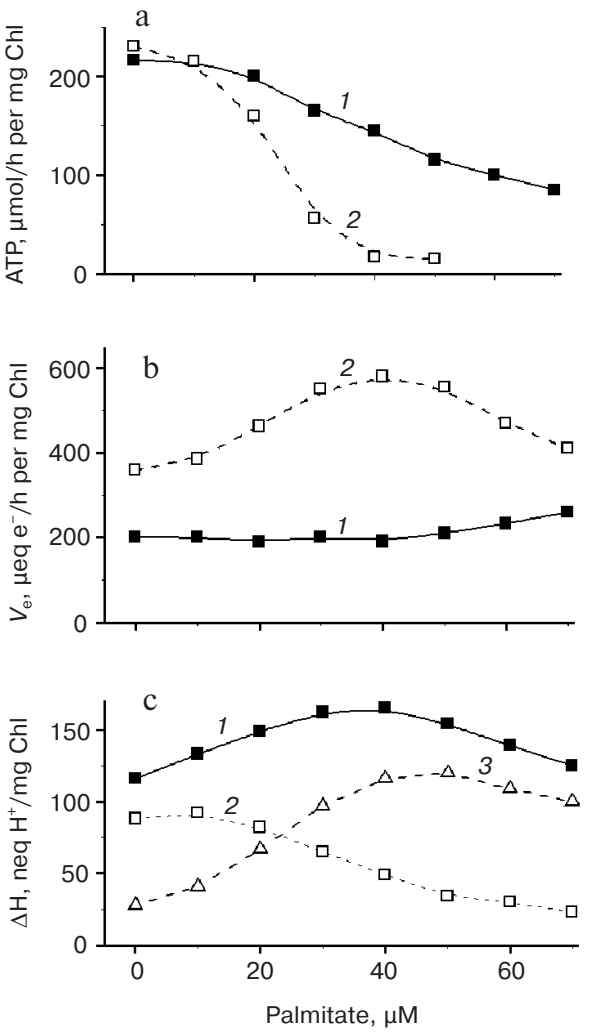

Fig. 1. Effect of palmitate on ATP synthesis (a), rate of basal electron transport (b), and ΔH level (c) in ammonium-free media (curves *1*) and in the presence of 0.2 mM NH_4Cl (curves *2*). Curve *3* in Fig. 1c is the difference between curves *1* and *2*. Measurement conditions are described in "Materials and Methods", and methyl viologen was used as the electron acceptor.

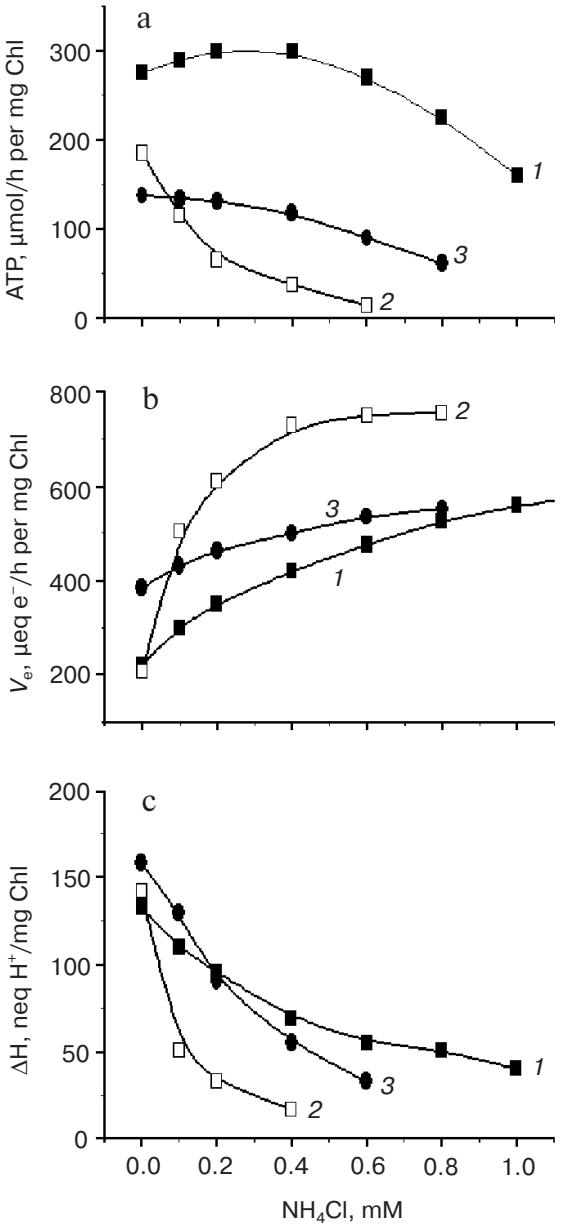

Fig. 2. Palmitic acid stimulates uncoupling by ammonium: ammonium concentrations necessary for (a) 50% inhibition of ATP synthesis, (b) stimulation of basal electron transfer, and (c) ΔH dissipation decrease several-fold. The protonophore CCCP causes practically no stimulation of uncoupling by ammonium. Curves: *1*) control without additions; *2*) + 40 μM PA; *3*) + 2 μM CCCP. Conditions as in Fig. 1 but with 0.4 mM ferricyanide as the electron acceptor in Fig. 2b.

Photoinduced uptake of hydrogen ions under conditions of basal electron transfer (ΔH) corresponds to the number of groups able to bind H^+ upon acidification of the thylakoid lumen. Palmitate (40 μM) in ammonium-free reaction medium stimulated ΔH, but basal electron transfer was not enhanced in this case. At PA concentrations above 40 μM, ΔH stimulation decreased (Fig. 1c, curve *1*). As shown in Fig. 1c, ammonium inhibits both ΔH and its stimulation by palmitate (Fig. 1c, curves *1* and *2*): the difference between curves *1* and *2* (curve *3*) grows as PA concentration increases. In the presence of 0.2 mM ammonium, curves of ΔH decrease are within the interval corresponding to the electron transfer acceleration and inhibition of ATP synthesis (Figs. 1a and 1b). It follows from these data that ammonium at low concentrations enhances the uncoupling effect of palmitate.

As shown in Fig. 2, palmitate, in turn, significantly increased the efficiency of ammonium uncoupling. Palmitate (40 μM) decreased ammonium concentrations necessary for 50% inhibition of ATP synthesis (Fig. 2a, curves *1* and *2*). The complete uncoupling of basal electron transfer in the presence of 40 μM palmitate was achieved at low ammonium concentrations (Fig. 2b, curves *1* and *2*). In ammonium-free media, the ΔH level was noticeably stimulated by PA, but in the presence of ammonium, the uptake of hydrogen ions sharply decreased after PA addition (Fig. 2c, curve *2*). The protonophore CCCP (1-2 μM) caused classical uncoupling in the absence of synergism with ammonium (Fig. 2, a-c, curves *3*).

Stimulation of ΔH by both weak acids, palmitate and CCCP, is not caused by the increase in pH gradient in the presence of these reagents, but rather by the increase in the lumen buffer capacity due to binding of the fatty acid anions and rupture of hydrogen bonds with involvement of protein carboxyl groups. This may damage lateral pathways of hydrogen ion transfer along the lumen surface layer and serve as a factor responsible for the uncoupling by FFA [3]. In the presence of ammonium, decoupling by palmitate is replaced by uncoupling, and ATP synthesis is inhibited by PA at low concentrations (Figs. 1a and 2a) causing stimulation of basal electron transport (Figs. 1b and 2b) and decrease in the ΔH level (Figs. 1c and 2c).

DISCUSSION

Protonated free fatty acids are able to quickly cross the lipid membrane by flip-flop, but transmembrane translocation of fatty acid anions is hampered by their retention at the surface of the membrane/water interface with carboxyl turned toward water and with "fatty tail" turned towards lipid [11, 12]. Due to this, on bilayer lipid membranes palmitate at low concentrations does not increase conductivity for hydrogen ions. However, investigations of mitochondria, lasting for many years, show that palmitic acid in micromolar concentrations increases the conductivity of the inner mitochondrial membrane [5-8]. To explain the uncoupling effect of FFA, Skulachev supposed that it should be mediated by special membrane proteins providing for the palmitic acid anion rather than CCCP transfer through the membrane [5, 8]. It was shown that in addition to the ATP/ADP carrier, able to perform such function, mitochondrial membranes contain a whole family of uncoupling proteins (UCP). Uncoupling pro-

teins have not yet been found in thylakoid membranes of chloroplasts, but the ATP/ADP carrier has been recently found there [13, 14]. It is possible that in thylakoids it may stimulate uncoupling by fatty acids, but our data show that activation of this process requires ammonium.

The pH gradient on chloroplasts under conditions of stationary illumination is 2-3 units. In the presence of weak acids (AH) and bases (BH^+) transmembrane gradients of A^- and BH^+ ions, equal in their value to the hydrogen ion gradient, are formed. The factor responsible for the ion gradient formation is free crossing of the membrane by neutral forms of these reagents (i.e. $AH_{in} = AH_{out}$ and $B_{in} = B_{out}$). At the same time, ionized molecules do not penetrate through bilayer, and as a result $A^-_{out}/A^-_{in} = H_{in}/H_{out} = BH^+_{in}/BH^+_{out}$.

It can be supposed that ammonium binding, which activates transmembrane transfer of palmitate anions by uncoupling protein, takes place at its lumen side, because in the case of chloroplast illumination ammonium concentration in the lumen increases more than 100 times in accordance with the level of the pH gradient.

In contrast, the concentration of PA anions in the stroma also increases more than 100-fold due to the pH gradient formation. Therefore, it might be that palmitate anions after binding on the stromal side of the membrane activate a cationic channel able to transport ammonium or potassium via their concentration gradient. Such channel has not yet been identified on thylakoids, but its existence is revealed using the patch-clamp technique [15] and microelectrodes [16]. It is known that the treatment of chloroplast with N,N′-dicyclohexyl carbodiimide, a modifier of carboxylic groups of proteins, results in a sharp increase in the thylakoid membrane conductivity for ammonium and potassium [17], which is indicative of the involvement of unknown proteins in transport of these cations.

Thus, it seems probable that the combined use of ammonium and palmitate can stimulate synergistic uncoupling by affecting membrane proteins, such as the ATP/ADP carrier or another uncoupling protein, and a K^+/NH_4^+ channel.

Ammonium is not efficient in uncoupling of mitochondria due to low pH gradient, and owing to this, its activity in these organelles has not been studied in detail. However, in the case of combined use of hydrophobic anions and amphiphilic penetrating amines or CCCP and lipophilic cations, non-additive increase in conductivity for hydrogen ions is registered on mitochondrial and model membranes, which is usually explained using a mechanism based on formation of neutral pairs between fatty acid anions and cations [18, 19]. Such a mechanism was demonstrated in experiments on liposomes [20], UCP1-containing proteoliposomes [19], and on an octane/water interface [21]. It was shown that the efficiency of H^+ transmembrane leakage due to formation of neutral pairs depends on ion lipophilicity, pK of weak acids and alkali, and constants of inter-ionic complex formation. It is supposed that neutral pairs can be incorporated into the membrane, thus forming a specific pathway for leakage of hydrogen ions [22].

Neutral pairs also freely cross a bilayer, i.e. their concentrations within and outside the lumen should be equal. It is quite possible that synergism in ammonium and palmitate action includes formation of $PA^- + NH_4^+$ pairs penetrating into the membrane both from the reaction medium and from the lumen. If in this case neutral pairs form a specific pathway for hydrogen ion leakage via any protein or lipid layer, cyclic transfer of PA through the membrane should not occur. Therefore, under uncoupling conditions palmitate may act not as a cyclic protonophore but rather as a participant of pore or channel formation, the emergence of which is stimulated by PA and is registered in mitochondrial membranes [23, 24].

Thus, analysis of the data obtained in this work and those from the literature shows that there are several probable variants for explanation of the mechanism of the palmitate and ammonium synergism, and each variant requires additional investigation.

This work shows for the first time the existence of synergism in uncoupling action of two metabolites, ammonium and palmitic acid, but not of CCCP. This synergism may be the basis for "mild" uncoupling in native chloroplasts. At low PA concentration the proton gradient is somewhat decreased by ammonium at low concentrations, which results both in stimulation of electron transfer and in increased rate of ATP synthesis catalyzed by the complete electron transport chain, when lateral pathways of hydrogen ion transfer to ATP synthases are preserved [25]. Increase in palmitate concentration causes mild reversible uncoupling, thus preventing chloroplast swelling and deformation in response to the pH gradient increase due to exhaustion of ADP or to the excess of ammonium.

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