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RESEARCH PAPERS

Involvement of Energy-Dissipating Systems in Modulating the Energetic Efficiency of Respiration in Mitochondria from Etiolated Winter Wheat Seedlings

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Abstract—Effects of cyanide-resistant alternative oxidase (AOX) and modifiers of plant uncoupling mitochondrial proteins (PUMP) on respiration rate and generation of transmembrane electric potential ($\Delta\psi$) were investigated during oxidation of various substrates by isolated mitochondria from etiolated coleoptiles of winter wheat (*Triticum aestivum* L.). Oxidative phosphorylation in wheat mitochondria during malate and succinate oxidation was quite effective (it was characterized by high respiratory control index as defined by Chance, high ADP/O ratio, and rapid ATP synthesis). Nevertheless, the effectiveness of oxidative phosphorylation was substantially modified by operation of energy-dissipating systems. The application of safranin dye revealed the partial dissipation of $\Delta\psi$ during inhibition of cytochrome-mediated malate oxidation by cyanide and antimycin A and demonstrated the operation of AOX-dependent compensatory mechanism for $\Delta\psi$ generation. The complex I of mitochondrial electron transport chain was shown to play the dominant role in $\Delta\psi$ generation and ATP synthesis during AOX functioning upon inhibition of electron transport through the cytochrome pathway. Effects of linoleic acid (PUMP activator) at physiologically low concentrations (4–10 μM) on respiration and $\Delta\psi$ generation in mitochondria were examined. The uncoupling effect of linoleic acid was manifested in activation of the State 4 respiration, as well as in $\Delta\psi$ dissipation; this effect was eliminated in the presence of BSA but was insensitive to purine nucleotides. The uncoupling effect of linoleic acid was accompanied by reversible inhibition of AOX activity. The results are discussed with regard to possible physiological role of mitochondrial energy-dissipating systems in regulation of energy transduction in plant cells under stress conditions.

Keywords: Triticum aestivum, mitochondria, membrane potential, oxidation substrates, respiratory inhibitors, linoleic acid.

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INTRODUCTION

The energy-dissipating systems of plant mitochondria attract growing interest, because they diminish the energy conversion efficiency in oxidative phosphorylation by dissipating to heat a part of energy generated by electron transport chain (ETC). These losses occur both before and after energy conversion to the form of transmembrane proton gradient ($\Delta\mu H^+$). Studies of the alternative electron-transport pathways deal primarily with the cyanide-resistant alternative oxidase (AOX) that short-circuits two out of three energy-coupling sites in ETC [1, 2]. In addition, a great deal of attention is being paid to thermogenin-like plant uncoupling mitochondrial proteins (PUMP) that are able to elevate the proton conductance of the mitochondrial inner membrane, thereby diminishing the membrane potential ($\Delta\psi$) [3, 4]. In view of ubiquitous occurrence of AOX and PUMP, a challenging task is to elucidate physiological significance of these systems in non-thermogenic plant tissues. The main postulate is that dissipative systems act as mild uncouplers and reduce $\Delta\psi$ in a controlled manner, thereby restricting $\Delta\psi$-dependent generation of reactive oxygen species (ROS), which are known to exert toxic action on membranes, proteins, and other polymers in the cells and mitochondria [5]. The supporting evidence to this view came from observations that the activity of dissipative systems is stimulated by exogenous ROS, as well as by abiotic stresses and associated oxidative stress [2, 3, 6–9]. Furthermore, it is envisioned that AOX optimizes the respiratory metab-

Abbreviations: $\Delta\psi$—membrane potential; AOX—alternative oxidase; CRR—cyanide-resistant respiration; DTT—dithiothreitol; ETC—electron transport chain; FCCP—carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; FFA—free fatty acids; PUMP—plant uncoupling mitochondrial proteins; RCI—respiratory control index; ROS—reactive oxygen species; SHAM—salicylhydroxamic acid.

olism during plant growth and development by integrating this metabolism with photosynthesis and other metabolic processes [2, 10, 11]. At the same time, it should be noted that the major part of hypotheses concerning physiological roles of AOX, PUMP, and other dissipative systems in plant mitochondria need further experimental verification.

The last few years were marked by impressive advances in molecular identification of mitochondrial dissipative systems, characterization of genes coding for the respective proteins, and elucidation of factors controlling their functional activity. Nevertheless, the functioning of dissipative system remains unresolved in many aspects [10, 12]. It is still an open question whether all endogenous factors involved in activation/inactivation of these systems have been already revealed and how these systems interact and coordinate during regulation of energy conversion efficiency in mitochondrial respiration.

Investigations of energy-dissipating systems in plant mitochondria are mainly performed with isolated organelles. In this case it is often difficult to identify the operation of such systems, because this task requires optimally adjusted conditions for mitochondria isolation and for analysis of their functional activity. In this study with isolated mitochondria from etiolated winter wheat seedlings, we carefully optimized experimental conditions, which allowed us to assess the influence of various dissipative systems on oxidative phosphorylation and membrane potential generation.

MATERIALS AND METHODS

We used 3-day-old etiolated seedlings of soft winter wheat (*Triticum aestivum* L., cv. Mironovskaya 808) grown by hydroponic method on tap water at room temperature.

Mitochondria were isolated according to Voinikov [13] with some modifications. Shoots (coleoptiles with embryonic leaves, 15–20 g) measuring 1.5–2.0 cm in length were cooled, sliced, and ground in a mortar with an isolation (homogenization) medium at a proportion of at least 1 : 4 (w/v). The homogenization medium contained 0.3 M sucrose, 18 mM KH_2PO_4 (pH 7.9), 1 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothreitol (DTT), and 0.1% fatty acid-free BSA. The homogenate was filtered through a cotton cloth and centrifuged at 8000 *g* for 5 min. The supernatant was used for sedimentation of mitochondria by centrifugation at 20000 *g* for 4 min. Isolated mitochondria were resuspended in a small volume of medium containing 0.3 M sucrose, 18 mM KH_2PO_4 (pH 7.2), and 0.1% fatty acid-free BSA. The suspension of mitochondria was stored on ice. All operations were carried out in a cold room at 4–5°C.

The generation of $\Delta\psi$ was monitored from safranin absorbance changes at 511 nm relative to 533 nm ($\Delta A_{511-533}$), as described by Moore and Bonner [14], using a Hitachi-557 spectrophotometer (Japan). The incubation medium (2 ml) contained 0.3 M sucrose, 18 mM KH_2PO_4 (pH 7.2), 1 mM $MgCl_2$, 5 mM EDTA, 5 μM safranin O, and 0.2 mg/ml of mitochondrial protein. In most cases, the incubation medium contained 0.1% BSA, except for experiments with PUMP activation. The activation of PUMP by linoleic acid was performed in the presence of 10 μM oligomycin and 25 μM atractyloside, provided that BSA was excluded from the reaction medium.

The rate of oxygen uptake by mitochondria was measured at 25°C by an amperometric method using an LP-7 polarograph (Czechia) and an oxygen electrode [15]. The incubation medium (1 ml) contained 0.3 M sucrose, 18 mM KH_2PO_4 (pH 7.2), 1 mM $MgCl_2$, 5 mM EDTA, 0.1% BSA, and an optimal amount (0.4–0.5 mg) of mitochondrial protein. The respiration rate in metabolic states 3 and 4, the ADP/O ratio, the respiratory control index (RCI), and phosphorylation rates were calculated according to Chance and Williams [16]. The main (cytochrome-dependent) respiratory pathway was inhibited by means of 0.5–2.0 mM KCN or 3 μM antimycin A; the alternative (cyanide-resistant) respiration was inhibited by means of 3 mM salicylhydroxamic acid (SHAM). The content of mitochondrial protein in the suspensions was determined according to Lowry, using BSA as a standard sample.

Each experiment was performed in 4–5 replicates with 2–3 assay per replicate. Figures display the results of representative experiments.

RESULTS

Characterization of Functional Activity of Isolated Organelles

Figure 1 displays typical polarographic curves of oxygen uptake by isolated mitochondria from etiolated winter wheat seedlings during oxidation of malate or succinate in the presence of glutamate. The respiration of mitochondria proceeded at a sufficiently high rate; it was readily stimulated in the presence of ADP (State 3) and was characterized by rather high respiratory control index (RCI) as defined by Chance. The RCI was particularly high during oxidation of NAD-linked substrates.

As can be seen from Fig. 1, isolated mitochondria from etiolated seedlings were capable of cyanide-resistant respiration (CRR), which was almost fully inhibited by SHAM, a specific inhibitor of AOX. According to our estimates, the maximal activity of AOX during malate and succinate oxidation was 40 and 70 nmol O_2/(mg protein min), respectively. It should be also noted that such a high AOX activity was only observed after inclusion of 5 mM DTT into the homogenization medium. However, the CRR rate remained almost unchanged when DTT was added to the wash medium

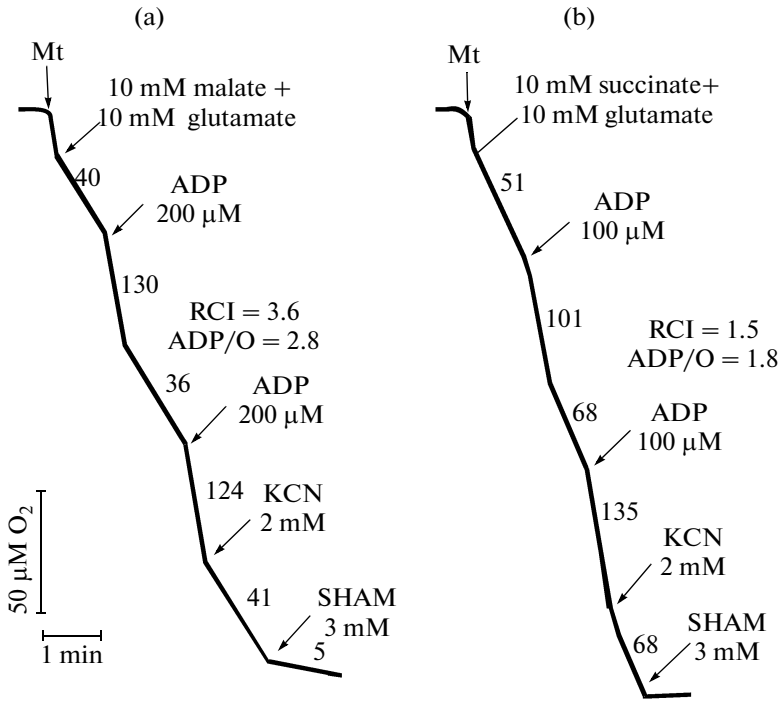

Fig. 1. Polarographic curves of oxygen uptake by isolated mitochondria from etiolated winter wheat seedlings during oxidation of (a) malate and (b) succinate.

Experimental conditions are described in Materials and Methods. Mitochondria (Mt) were added to the medium (pH 7.2) containing 0.3 M sucrose, 18 mM KH_2PO_4, 1 mM $MgCl_2$, 5 mM EDTA, and 0.1% BSA. Numbers near the curves designate the rate of oxygen uptake expressed in nmol O_2/(mg protein min).

for isolated organelles or included into the incubation mixture where the functional activity was assayed.

Action of Respiratory Inhibitors on Δψ Generation and ATP Synthesis in Mitochondria of Winter Wheat Seedlings

High functional activity of isolated organelles was also evident from their ability to rapidly generate Δψ on the mitochondrial inner membrane upon oxidation of respiratory substrates, malate in particular (Fig. 2). It is remarkable that Δψ generation was characterized by high rate and large amplitude, which was manifested in a substantial increase and sustained maintenance of the safranin absorbance signal. Figure 2 also shows that the addition of ADP to mitochondrial samples induced temporal decrease in Δψ. Afterwards, Δψ recovered to its initial level following the depletion of ADP during oxidative phosphorylation. It can be seen also that the extent and duration of ADP-triggered Δψ depolarization were directly related to ADP concentration. At the same time, the described effect of ADP disappeared in the presence of oligomycin, an inhibitor of mitochondrial ATP synthase (data not shown).

Next, we observed that the treatment of mitochondria with cyanide at a concentration sufficient for complete inhibition of cytochrome oxidase caused only a minor decrease in Δψ (Fig. 2). An important point is that mitochondria retained their capacity of oxidative phosphorylation, which was evident from the reversible decrease in Δψ upon the addition of ADP (Fig. 2). The phosphorylation rate estimated from the observed Δψ changes was about 70 nmol ATP/(mg protein min), which accounts for more than 20% of ATP synthesis rate in the absence of cyanide. The subsequent addition of rotenone to mitochondria substantially reduced Δψ, indicating the prevalent role of rotenone-sensitive NADH dehydrogenase (complex I) in Δψ generation (Fig. 2). A similar pattern of Δψ changes was also observed upon the treatment of wheat seedling mitochondria with antimycin A that inhibited the cytochrome pathway at the level of complex III (data not shown).

As noted above, the rate of CRR in wheat seedling mitochondria depended strongly on the nature of respiratory substrate. In view of these results, we had to elucidate whether Δψ generation in malate- or succinate-oxidizing mitochondria was sensitive to inhibitors of cytochrome-linked and alternative electron transport pathways. Figure 3 shows that inhibition of cytochrome pathway in succinate-oxidizing mitochondria upon the addition of antimycin A or cyanide resulted in almost complete suppression of Δψ generation. Nevertheless, the Δψ depression in mitochon-

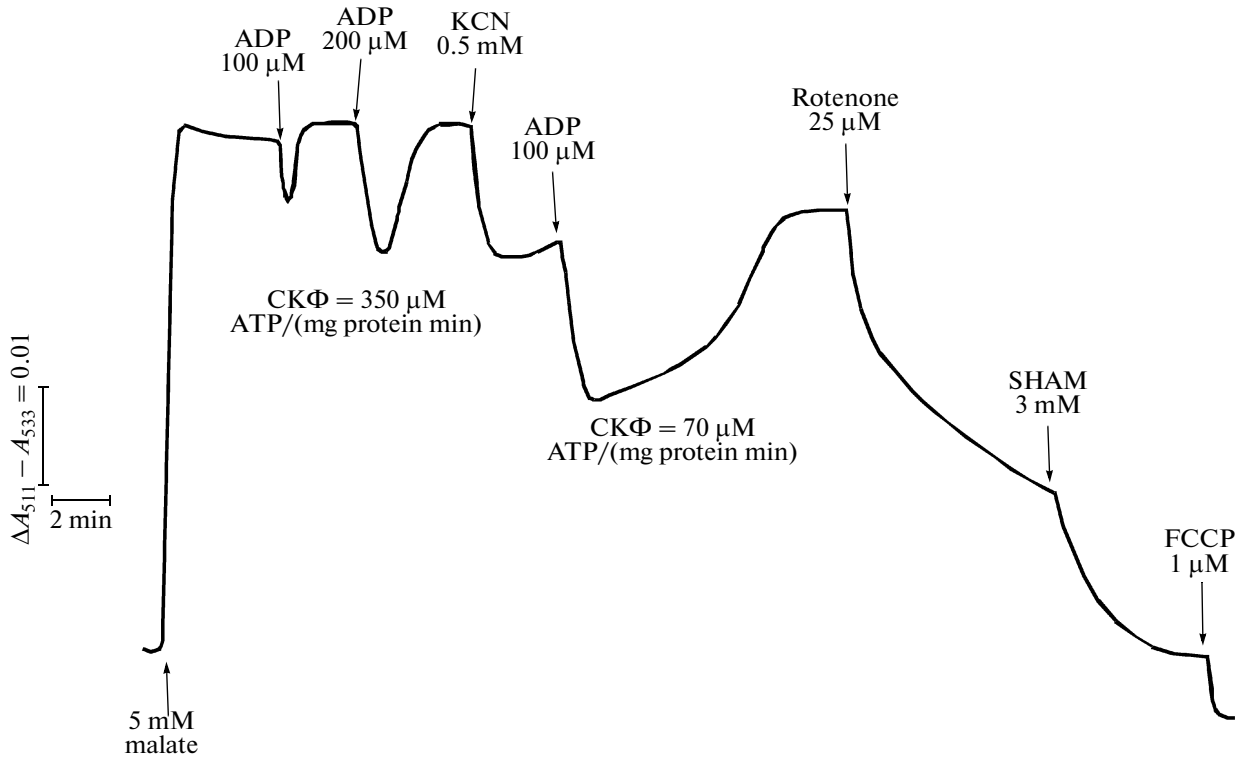

Fig. 2. Generation of Δψ on the inner membrane of wheat seedling mitochondria during malate oxidation and depolarization of Δψ upon the addition of ADP and ETC inhibitors.
Experimental conditions are described in Materials and Methods.

dria was reversed upon the addition of NAD-linked substrate (malate), but the restored Δψ was completely eliminated in the presence of SHAM. This results confirms the importance of complex I in Δψ maintenance when the cytochrome pathway of oxidation is inhibited and only AOX is operative. It is remarkable that the malate oxidation in State 4 (in the absence of ADP) occurred at comparatively low Δψ, which might be important for the avoidance of ROS formation in mitochondria (see Discussion).

Sensitivity of Δψ Generation in Mitochondria of Winter Wheat Seedling to Modulators of Uncoupling Proteins

Based on the above results, it was necessary to elucidate whether PUMP are involved in controlling the energy conversion efficiency of mitochondrial respiration. To this end, we analyzed the influence of linoleic acid, a potential enhancer of PUMP activity, on mitochondrial respiration rate and Δψ generation. These experiments were performed in the presence of oligomycin and atractyloside, the inhibitors of mitochondrial ATP-synthase and the ADP/ATP antiporter, respectively. These experimental conditions prevented possible involvement of ADP/ATP antiporter in the transmembrane transport of fatty acids [17, 18]. In addition, we removed BSA from the reaction medium, since BSA inhibits the uncoupling proteins by binding free fatty acids.

Figure 4 shows the effect of exogenous linoleic acid, taken at physiologically low concentration (4 μM) on Δψ in malate-oxidizing mitochondria. The decrease in Δψ in these experiments depended on the concentration of linoleic acid added and was reversible, unlike Δψ changes induced by carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP). The addition of purine nucleotides (ATP, ADP, and GTP) to mitochondria incubated in the presence of linoleic acid had no discernible effect on Δψ. By contrast, the addition of BSA to the organelles induced a clearly pronounced recoupling effect (Fig. 4).

Our polarographic data showed that the presence of linoleic acid or FCCP enhanced the mitochondrial respiration to comparable extents. Furthermore, the effect of linoleic acid was reversible and fully eliminated upon the subsequent addition of BSA (Fig. 5). Unlike BSA, the addition of ATP, another PUMP inhibitor, had no influence on the effect of linoleic acid. According to our estimates made as described in [7], the increment of mitochondrial respiration rate upon PUMP activation (the difference of respiration rates in the presence of linoleic acid as an activator and in the presence of BSA + purine nucleotides as inhibitors) was 20 nmol O_2/(mg protein min) during malate oxidation by mitochondria from etiolated wheat seed-

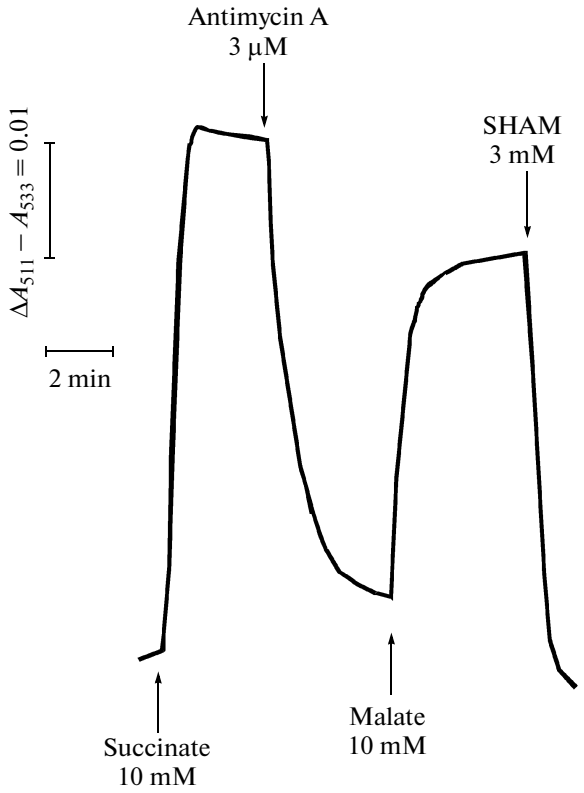

Fig. 3. Capacity of various respiratory substrates to support oxidation-induced Δψ generation under inhibition of the cytochrome pathway.

Experimental conditions are described in Materials and Methods.

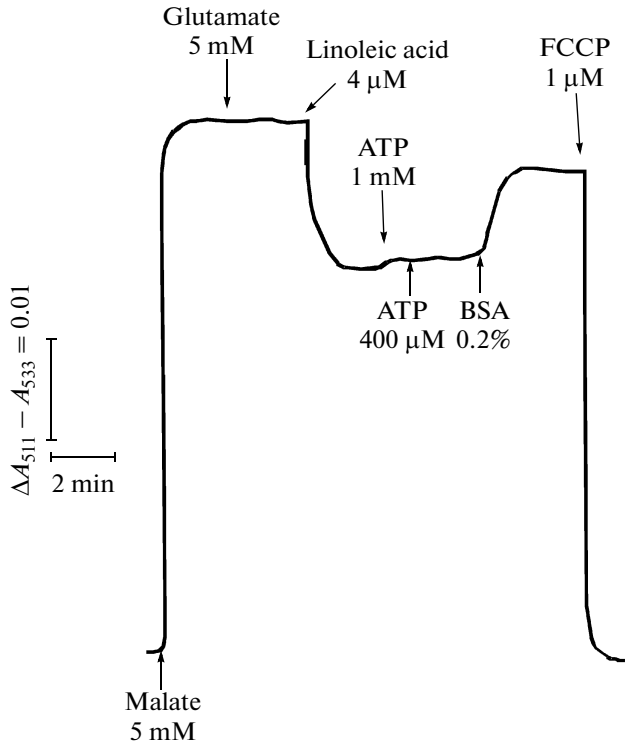

Fig. 4. Uncoupling action of exogenous linoleic acid on Δψ generation during malate oxidation by wheat seedling mitochondria and its reversal upon BSA addition.

The incubation medium (pH 7.2) contained 0.3 M sucrose, 18 mM KH_2PO_4, 1 mM $MgCl_2$, 5 mM EDTA, 5 μM safranin, 10 μM oligomycin, and 25 μM atractyloside; BSA was omitted from the medium.

lings (Fig. 5a). At the same time, the contribution of CRR to the total rate of O_2 consumption by mitochondria rose from 23% in the presence of linoleic acid to 36% after the inhibition of uncoupling proteins (Fig. 5b). After inhibiting the uncoupling proteins, the respiration rate approached the initial value observed in the absence of linoleic acid (Fig. 1a). This fact suggests that the uncoupling effect of linoleic acid is accompanied by reversible inhibition of AOX activity, which is in line with previous findings for isolated mitochondria from other plant materials [19].

DISCUSSION

The results of this study indicate that mitochondria of etiolated winter wheat seedlings are characterized by high activity of the alternative CN-resistant oxidase. We found that the AOX activity could be as high as 50% of the State 3 oxidation rate observed in the presence of various respiratory substrates (Fig. 1). In the previous study with mitochondria isolated from etiolated seedlings of the same winter wheat cultivar (Mironovskaya 808) by a similar isolation procedure, the CRR activity did not exceed 21–23% during oxidation of either malate or succinate [20]. Such a substantial difference in estimates of CRR capacity is apparently due to the choice of different reducing agents in the composition of isolation media. In the present study we used DTT, which is more active than the previously used cysteine [20].

It is known that the redox state of sulfhydryl–disulfide bond between the AOX monomers plays an important role in the post-translational regulation of AOX activity [1]. The presence of DTT in the reaction medium and all isolation solutions was shown to facilitate the maintenance of AOX dimer in the highly active "reduced" form [21]. In our experiments, the presence of 5 mM DTT in the homogenization medium was already sufficient for obtaining the maximum AOX activity, while the subsequent addition of 5 mM DTT to the media for resuspension and incubation of mitochondria had no additional influence on the extent of CRR. Thus, the choice of optimal conditions for mitochondria isolation and the presence of sufficiently high concentrations of reducing agents are essential requirements for obtaining the maximum AOX activity in mitochondria of wheat seedlings.

Despite significant advances in deciphering the AOX structure and molecular mechanisms of its activity regulation, elucidation of AOX physiological role in

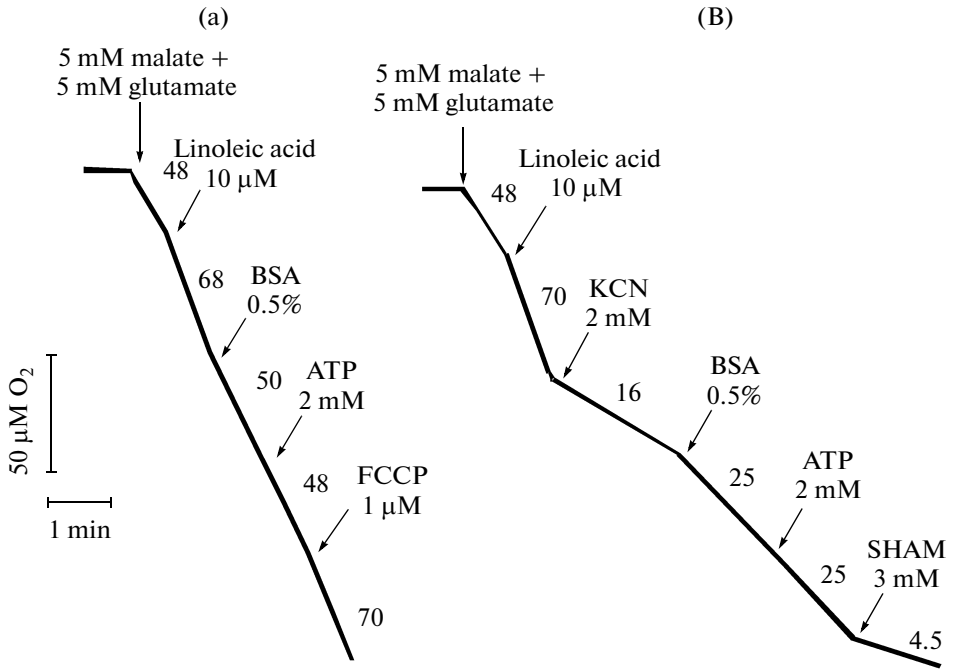

Fig. 5. Effect of linoleic acid on the rate of (a) total respiration and (b) cyanide-resistant respiration of mitochondria. Measurement conditions were the same as in Fig. 4. Numbers near the curves indicate oxygen uptake rates expressed in nmol O_2/(mg protein min).

non-thermogenic plant tissues is currently at the beginning stage. When the alternative respiratory pathway becomes operative, two of three energy-coupling sites (sites II and III) in the electron-transport chain stop functioning. For this reason, the alternative respiratory pathway is considered to be low effective for energy conservation in the forms of both ATP and $\Delta\psi$. Indeed, the addition of cyanide to isolated plant mitochondria, specifically to mitochondria of wheat seedlings, was usually accompanied by significant dissipation of $\Delta\psi$ [7, 9]. By contrast, the cyanide-induced changes of $\Delta\psi$ during malate oxidation in our experiments were not strongly pronounced and were comparable to those induced by the ADP addition. In this case, mitochondria retained the capacity of oxidative phosphorylation, though at a lowered level (Fig. 2). Rotenon (the inhibitor of complex I in the respiratory chain) caused almost complete dissipation of $\Delta\psi$ (Fig. 2). Hence, the operation of rotenone-sensitive NADH dehydrogenase and highly active AOX in isolated winter wheat mitochondria sufficed for generation of rather high $\Delta\psi$ during oxidation of NAD-linked substrates, despite the inhibition of the cytochrome pathway. This activity was sufficient to compensate for the lacking function of two energy-coupling sites. In accordance with expectations, such compensation was not observed during succinate oxidation, when the coupling site I was inactive (Fig. 3). In this case, the addition of malate to the reaction medium caused a compensatory increase in $\Delta\psi$ that was sensitive to SHAM (Fig. 3). Thus, our results provide direct evidence for the ability of CRR to support $\Delta\psi$ generation and ATP synthesis upon the blockade of the cytochrome pathway; this ability is implemented through operation of the first energy-coupling site (complex I) in the respiratory pathway. The above results are consistent with the assumed significance of alternative oxidation pathway for modulation of ATP synthesis in plant mitochondria [21].

It is remarkable that $\Delta\psi$, at which ATP was synthesized during malate oxidation mediated by complex I and AOX, was markedly lower than $\Delta\psi$ generated by the main cytochrome-mediated respiratory pathway (Fig. 2). Although the origin of this phenomenon remains unknown, its relation to the uncoupling action of KCN is unlikely, because a similar pattern of $\Delta\psi$ changes was observed in the presence of antimycin A (Fig. 3). Considering the dependence of ROS production by respiratory chain on the extent of $\Delta\psi$ [5], one may assume that functioning of the alternative mitochondrial oxidation pathway should be accompanied by the decreased rate of ROS production. This effect could be significant for the prevention of oxidative stress; e.g., in plants exposed to water deficit or low temperature, when the cytochrome pathway is suppressed while AOX is activated [6]. The analysis of our and published data led us to conclude that AOX, unlike other uncoupling systems (PUMP, Pmito-K_{ATP}, anion channels), does not act solely as energy-dissipative system but can pay an important role under certain conditions in $\Delta\psi$ generation and ATP synthe-

sis in plant mitochondria. However, the validity of this hypothesis should be verified by further studies.

As noted in the Introduction, the activation of PUMP under oxidative stress conditions in the presence of long-chain fatty acids is supposed to uncouple oxidation and phosphorylation, which would reduce ROS production in the respiratory chain by the feedback mechanism [4]. Our experiments with winter wheat mitochondria showed that the presence of linoleic acid at low concentration stimulated substantially the State 4 respiration in the presence of respiratory substrates (Fig. 5) and depressed $\Delta\psi$ significantly, while the addition of BSA restored $\Delta\psi$ level (Fig. 4). These results clearly demonstrate that free fatty acids exert the uncoupling effect, probably in relation to PUMP activity. The possible involvement of adenine nucleotide translocator in the uncoupling effect of free fatty acids [18] can be excluded in these experiments because this translocator was inhibited by atractyloside. Apart from the uncoupling action of linoleic acid, this substance inhibited also AOX activity (Fig. 5). It is not excluded that such inhibition may reflect the operation of a mechanism regulating the interactions of AOX and PUMP in plant mitochondria [19]. The inclusion into the reaction medium of ATP, ADP, and other nucleotides known to inhibit the uncoupling proteins in plant mitochondria, including hard wheat mitochondria [9, 17], had no appreciable influence on $\Delta\psi$ (Fig. 4) and on the rate of substrate oxidation during State 4 respiration (Fig. 5). Therefore, further studies based on Western blot analysis are needed to prove the presence of PUMP in wheat mitochondria.

It should be noted that identification of PUMP activity in isolated mitochondria from plants and animals still remains a rather complicated task. First, the PUMP activity can be only detected at low endogenous level of free fatty acids in mitochondria, which is accomplished by BSA addition. However, the application of nonoptimal (too high or too low) BSA concentration would lead to underestimates of PUMP activity. The PUMP detection is strongly hampered by possible nonspecific uncoupling action of free fatty acids on respiration and $\Delta\psi$ generation, without involvement of uncoupling proteins [18]. Finally, the PUMP sensitivity to purine nucleotides is highly labile depending on functional condition of mitochondria [18, 22] and on the source of plant materials [23], which introduces further complications to experimental data analysis. For example, the specific inhibitor of uncoupling proteins in animal mitochondria GTP had often no inhibitory action on PUMP, while ATP was quite effective in inhibiting the PUMP activity in mitochondria from hard wheat seedlings [9, 17]. Furthermore, purine nucleotides had no inhibitory action on PUMP activity in isolated mitochondria from thermogenic spadices of *Symplocarpus renifolius* [24].

In conclusion, it should be pointed out that regulatory mechanisms of energy transduction in animal mitochondria have received renewed attention of the researchers. The particular interest is centered around unveiling new mechanisms that regulate $\Delta\psi$ in different metabolic states and affect the key mitochondrial functions including ATP synthesis and ROS production [18, 25]. The regulation of mitochondrial respiration and energy transduction in plants is a particularly significant issue because plant mitochondria possess a highly branched and flexible system of bypass electron transport routes and diverse mechanisms of energy dissipation.

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