Volume 336, number 1, 83–86 FEBS 13377 December 1993

Catalytic and activating protons follow different pathways in the H^+-ATPase of potato tuber mitochondria

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Received 17 September 1993; revised version received 21 October 1993

The effect of some F_0F_1 inhibitors on the activation of the H^+-ATPase by the electrochemical proton gradient was investigated in mitochondria extracted from potato tubers. Transient activated state of the ATPase was revealed by addition of ATP and of the detergent lauryldimethylamine oxide (LDAO) to energized mitochondria. Venturicidin, tri-*n*-butyltin and aurovertin at high concentrations did not affect the process of $\Delta\tilde{\mu}_H{}^+$-activation, whereas oligomycin fully blocked it. The results support the idea of separate pathways or binding sites for catalytic and activating protons.

F_0F_1 H^+-ATPase; Electrochemical proton gradient; Enzyme activation; Inhibitor; Lauryldimethylamine oxide; Plant mitochondria

1. INTRODUCTION

In chloroplasts and mitochondria, the electrochemical proton gradient $\Delta\tilde{\mu}_H{}^+$ activates the F_0F_1-ATPase in addition to supplying it with energy (for reviews see [1–3]). In some situations, as in that of the chloroplast ATPase in its oxidized form [1,4], and probably also that of the ATPase of potato tuber mitochondria [5,6], the shortness of the activated state does not allow to detect ATP hydrolysis once $\Delta\tilde{\mu}_H{}^+$ has been fully dissipated. In the latter case however, the addition of lauryldimethylamine oxide (LDAO) instead of an uncoupler to energized mitochondria prevents the ATPase from deactivating [6].

In a previous report [7], we have got evidence for the existence of different routes for catalytic and regulatory protons within the ATPase. This came from the fact that thylakoids treated with venturicidin (VTCD), a reversible inhibitor of the F_0 sector, before or after $\Delta\tilde{\mu}_H{}^+$-activation, had the same final activity. However, due to the impossibility to rapidly remove the bound inhibitor, non-saturating concentrations of VTCD had to be used, assuming that all the F_0 channels were actually blocked but at different times [7].

In this report, we have used the same approach in potato tuber mitochondria, but with massive, largely over-saturating concentrations of F_0 inhibitors. The activity was revealed by LDAO, which presents the advantage to stabilize the $\Delta\tilde{\mu}_H{}^+$-activated state of the ATPase [6] and to fully reverse the effect of inhibitors.

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Abbreviations: F_0 and F_1, membranous and extrinsic parts of the proton ATPase; IF1, inhibitory peptide of the mitochondrial ATPase; $\Delta\tilde{\mu}_H{}^+$, transmembrane difference in electrochemical proton potential; $\Delta\varphi$, trans-membrane potential difference; TPP^+, tetraphenylphosphonium; LDAO, lauryldimethylamine oxide; VTCD, venturicidin; TNBT, tri-*n*-butyltin.

2. MATERIALS AND METHODS

Mitochondria were extracted from potato tubers as previously described [8,5] and assayed at 25°C in a medium containing 0.4 M mannitol, 5 mM $MgCl_2$, 30 mM KCl, 0.1% (m/v) bovine serum albumin, 4 mM K–K_2 phosphate buffer, 4 mM tricine, 7 μM TPP^+, pH 7.5. The protein concentration was 0.13 mg · ml^{-1}, determined using the Bradford method [9]. Other additions are indicated. Oxygen uptake was measured with a Clark-type electrode. Membrane potential was measured with a TPP^+-sensitive electrode [10]. ATPase activities were followed by pH changes [11] using a fast and sensitive glass electrode, the suspension being titrated by HCl at the end of all kinetics. The three signals were simultaneously monitored in a previously described setup [6].

3. RESULTS

In the first type of experiment we had to ensure that LDAO fully reversed the effect of inhibitors. The protocol used is shown in Fig. 1. With succinate 10 mM initially present, ADP (100 μM) was added to mitochondria and was 100% phosphorylated. This first 'state 3–state 4' [12] transition is necessary to obtain fully active mitochondria, as shown by the lag in ATP synthesis (Fig. 1). This conditioning process has already been described [13] and involves not only the ATPase, but also the enzymes of the respiratory chain. 150 s later, a second addition of ADP (50 μM) was made. ATP (1 mM) and LDAO (10 mM) were injected 150 s and 180 s after this second ADP addition, respectively (Fig. 1, 'a', full trace). When checked, inhibitors were

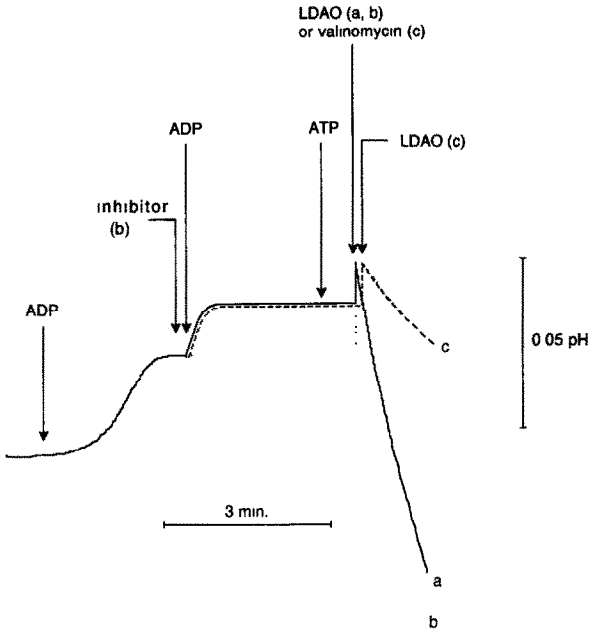

Fig. 1. pH variations induced by ATP synthesis and LDAO-triggered ATP hydrolysis. Conditions were as described in section 2. Different additions are indicated by vertical arrows, letters in parentheses mentioning in which cases these additions were made. pH increase (upwards) corresponds to ATP synthesis, pH decrease to ATP hydrolysis. Note the lag of ATP synthesis after the first ATP addition. Continuous trace, 'a': control. Dotted trace, 'b': inhibitor added; note the total absence of ATP synthesis after the second ATP addition. Dashed curve, 'c': no inhibitor, but activity revealed by LDAO only 10 s after a valinomycin addition. The beginning of the trace (before the second ADP addition) is common to the three conditions. The ATP hydrolase activities of Fig. 2 were calculated from the slopes of the traces after LDAO addition. The initial peak is an artifact due to the addition of LDAO and was discarded. See text for details.

added 10 s before the second ADP addition ('b', dotted trace). Lastly, to be sure that the measured activity required the presence of $\Delta\tilde{\mu}_H^+$ ($\Delta\varphi$ in the present case [8,5]), mitochondria were deenergized by valinomycin ($0.38\ \mu g \cdot mg^{-1}$ protein), and the activity was revealed by LDAO only 10 s later (Fig. 1, 'c', dashed trace).

Four ATPase inhibitors were checked. Three were directed against F_0: oligomycin [14], VTCD [15], and tri-*n*-butyltin (TNBT) [14], and one against F_1: aurovertin [14]. At the concentrations used, all the inhibitors fully abolished ATP synthesis (as in Fig.1, trace 'b') and ATP hydrolysis in pea leaf mitochondria deenergized by valinomycin (not shown; the latter control cannot be made in potato tuber mitochondria, due to the short lifetime of the activated state [5,6]; the limit of detection was about 2% of the control). Fig. 2 shows that the LDAO-revealed activity is insensitive to the previous addition of the F_0 inhibitors. By contrast, the effect of aurovertin was only partly reversed. It can also be seen that 10 s after the collapse of $\Delta\varphi$ by valinomycin, only a small fraction of the activity is still revealed by LDAO, as previously observed [6].

Data of Fig. 2 indicate that the effects of oligomycin, VTCD and TNBT are fully reversed by LDAO, which probably traps the inhibitors. But since these substances were added on already activated enzymes, this does not mean that they are without effect on the $\Delta\tilde{\mu}_H^+$-activation. To solve this question, other experimental conditions must be used. The main difficulty is that since mitochondria have to be conditioned by at least one state 3–state 4 transition, it is impossible to study the effect of inhibitors on ATPases which have never been activated. We have thus used a complex protocol detailed in Fig. 3. The ATPase were activated in the presence of succinate and ADP (mitochondria conditioning, steps 1–2), deactivated for a long time in the quasi-absence of $\Delta\varphi$ (addition of malonate and of a small amount of valinomycin, steps 3–4), then reactivated by $\Delta\varphi$ (NADH addition) in the presence of ADP (steps 6–7) with or without inhibitor addition (added in step 5), and finally revealed by ATP + LDAO (steps 8–9). ADP was present during the reactivation stage to prevent the enzymes from being in state 4, which could equilibrate protons within the ATPase and mask a possible effect of F_0 inhibitors (see [7]).

Fig. 4 shows the effects of the different inhibitors, added before the reactivation, on the final rate of ATP hydrolysis triggered by LDAO. As in Fig. 2, VTCD and TNBT had no effect. This shows that blocking the F_0 channel does not affect the $\Delta\tilde{\mu}_H^+$-activation. Aurovertin, which inhibits the F_1 moiety, did not modify the activity, in contrast to that was observed in Fig. 2. Lastly, the rate of ATP hydrolysis found on the oligomycin-treated sample was equal to the background activity of the non-reactivated sample.

4. DISCUSSION

When it is added to mitochondria, the most trivial effect of LDAO is to instantaneously disrupt the membranes. But we have previously shown [6] that LDAO

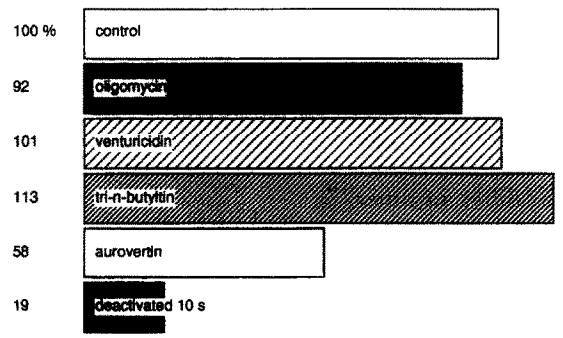

Fig. 2. Effect of inhibitors added on preactivated material on the rate of ATP hydrolysis triggered by LDAO. Conditions of Fig. 1. Top bar (control) corresponds to trace a in Fig. 1; bottom bar (deactivated 10 s) corresponds to trace c; other bars (inhibitors) correspond to trace b. The rates are expressed as a percentage of the control. Concentrations of inhibitors in μg (mg protein)$^{-1}$: oligomycin, 12.8; venturicidin, 6.1; tri-n-butyltin, 4.4; aurovertin, 190. Control activity: 10.0 μmol ATP (mg protein)$^{-1}$ min^{-1}.

also reveals transient activated states of the ATPase. This makes this detergent a powerful tool to check the activation of the ATPase by the $\Delta\tilde{\mu}_H^+$.

The results presented here clearly indicate that two universal F_0 inhibitors (VTCD and TNBT, acting on F_0 in *E. coli* [16,17], chloroplasts [18,19] and mitochondria [14,15]), used at concentrations which lower the proton flow below the limit of detection, do not prevent the $\Delta\tilde{\mu}_H^+$-activation of the ATPase in potato tuber mitochondria. This strengthens the previous suggestion [7] according to which catalytic and regulatory proton binding sites are different.

Aurovertin, which inhibits the F_1 moiety, seems to deactivate a fraction of the ATPases, but only when

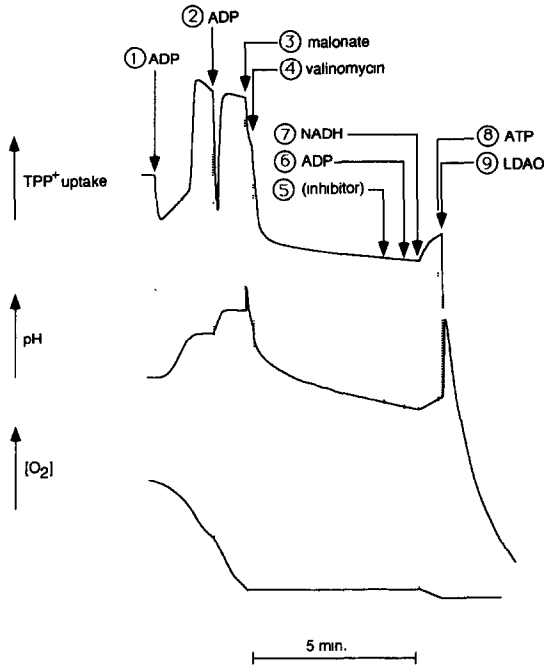

Fig. 3. Time-course of TPP^+, pH and oxygen signals during conditioning of mitochondria, deactivation and reactivation of the ATPase and LDAO-triggered ATP hydrolysis. Conditions are as indicated in section 2. TPP^+ uptake and increase of pH and O_2 concentration are directed upwards. The example displayed corresponds to the control (no inhibitor). Succinate 2 mM initially present. Timing (in s), additions and subsequence events: (1) $t = 0$, ADP 100 μM: conditioning, increase of the oxidation rate, starting of ATP synthesis (note the lag), fast decrease then slow increase of $\Delta\varphi$, then reaching of state 4, where ATP synthesis stops, the oxidation rate decreases and $\Delta\varphi$ rapidly increases; (2) $t = 105$ s, ADP 50 μM: new ATP synthesis, then reaching of state 4; (3) $t = 165$ s, malonate 50 mM: inhibition of the oxidation, $\Delta\varphi$ decrease, positive pH peak (artifact), then pH decrease (ATP hydrolysis); (4) $t = 175$ s, valinomycin 6.4 ng (mg protein)$^{-1}$: faster $\Delta\varphi$ decrease, acceleration of ATP hydrolysis, then slow ATPase deactivation, $\Delta\varphi$ being not fully collapsed; (5) $t = 420$ s, time of inhibitor addition; (6) $t = 460$ s, ADP 100 μM; (7) $t = 480$ s, NADH 0.5 mM: triggering of the respiratory chain, $\Delta\varphi$ increase, pH increase, a fraction of this pH increase being due to the scalar H^+ consumption accompanying the electron transfer from NADH to O_2; (8) $t = 520$ s, ATP 1 mM; (9) $t = 525$ s, LDAO 10 mM: oxidation stop, negative peak of the TPP^+ signal (artifact), positive peak of pH (artifact), then pH decrease (ATP hydrolysis). Since the electron transfer stops, the pH decrease at the end is only due to ATP hydrolysis (no scalar protons coming from the respiratory chain).

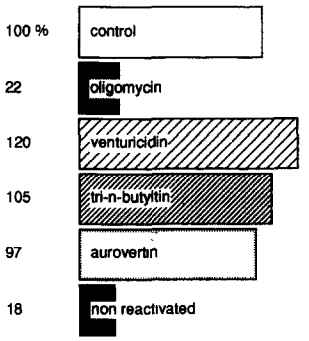

Fig. 4. Effect of inhibitors added before the $\Delta\tilde{\mu}_H^+$-reactivation on the rate of ATP hydrolysis triggered by LDAO. Conditions as in Fig. 2. Top bar (control): no inhibitor in step 5; bottom bar (non-reactivated): no NADH in step 7. The rates are expressed as a percentage of the control. Concentrations of inhibitors as in Fig. 2. Control activity: 4.4 μmol ATP · mg^{-1} protein · min^{-1}.

added during state 4, on already activated ATPase (Fig. 2). This effect can hardly be related to protons. It might depend on the proportion of the different microscopic states experienced by the enzyme during the catalytic cycle, or on the position of the inhibitory peptide IF1. All these parameters probably differ in the conditions encountered in Figs. 2–4.

Compared to other F_0 inhibitors, oligomycin has a special effect, in the sense that it blocks the reactivation of the ATPase (Fig. 4). It is possible that two different pathways exist in F_0 for catalytic and regulatory protons, the second being blocked only by oligomycin. But the specific effect of oligomycin might be not directly related to protons, as that of aurovertin in Fig. 2. Interestingly, these two inhibitors were reported to change in an opposite way the energy-sensitive IF1–F_1 interactions in submitochondrial particles [20]. It should also be pointed that contrary to VTCD and TNBT, oligomycin is not an universal F_0 inhibitor (it has no significant effect on *E. coli* and chloroplasts [17]); in mitochondria, it requires F_0 subunits specific to this organelles. So it is not a simple inhibitor and its action probably involve complex conformational changes (including IF1–F_1 interactions [20]).

The most important fact remains that VTCD and TNBT only inhibit the catalytic H^+-flow without interfering with the process of H^+-activation. The distinction between catalytic and regulatory proton binding sites thus seems to be a common feature of chloroplast and mitochondrial F_0F_1-ATPases. However, it cannot yet be decided whether distinct parallel channels are involved, or if the sites of action of VTCD and TNBT are located downstream the H^+-regulatory site, in the same channel.

Acknowledgements: Thanks are due to Dr. Philippe Diolez for helpful discussions and his expertise in plant mitochondria. The excellent technical assistance of Marie-Thérèse Crosnier was greatly appreciated. Catalase-treated LDAO was a gift of Dr. Françoise Reiss-Husson.

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