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ATP synthase: activating versus catalytic proton transfer

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Abstract ATP synthase (F-ATPase) of chloroplasts, CF_0CF_1, is both activated and driven by transmembrane protonmotive force. We dichotomized between activating and driving proton transfer by specific inhibitors, tentoxin and venturicidin. Thylakoids membranes were submitted to voltage steps (by flashing light) superimposed to a steady pH-difference. Transient proton intake, transfer and release by CF_0CF_1 was monitored by spectroscopic probes. Both activities, activation and catalysis, required all three partial reactions of the proton, however, activating proton transfer rose first (monophasically, $\tau_{1/2}$~15 ms) followed by another phase of equal magnitude with a time lag of about 15 ms. Both types of consecutive proton transfer reactions contribute free energy for ATP synthesis.

Key words: F-ATPase; ATP synthase; Phosphorylation; Activation; Protonmotive force; Proton transfer

1. Introduction

F-ATPases of chloroplasts, mitochondria and eubacteria synthesize ATP at the expense of protonmotive force. Protonmotive force not only drives ATP synthesis but also activates these enzymes [1–5]. In the chloroplast enzyme, CF_0CF_1, the activation threshold is low (about 2 pH-units) if a disulfide bridge on the γ-subunit is open (reduced) and high if it is closed (3 units) [6]. The proportion of active enzyme molecules has been assayed indirectly by the rate of ATP synthesis/hydrolysis [6], and by supposed indicators of the active state as the release of tightly bound nucleotides [7–10], the accessibility to SH-reagents of Cys^{85} on the γ-subunit [11,12] and the accessibility of the covalently linked probe eosin-isothiocyanate to the triplet quencher dioxygen [13]. Attempts to dichotomize between protolytic reactions that drive ATP synthesis and activate the enzyme have led to the proposal that proton binding and release at opposite sides of the enzyme may be responsible for activation [5,14] whereas ATP synthesis requires proton transfer across the membrane. It is an important question whether the activating reactions involving protons are separated from or integrated in the translocation of four protons [15] for each molecule of ATP which is formed.

Based on the release of tightly bound nucleotides [16,17] and on the thiol modification of the γ-subunit [12] it has been concluded that tentoxin inhibits only catalytic turnover but not activation. Tentoxin, a phytotoxin from the fungus *Alternaria alternata* binds to subunit β of the CF_1-portion [18,19] of certain plant species. Contrastingly, inhibitors of proton transport through the F_0-portion like venturicidin (DCCD, and organotins) inhibit both reactions.

We followed the strategy by Valerio et al. [12] who compared the differential behaviour observed with/without tentoxin and with/without venturicidin to dichotomize activating and catalyzing protolytic reactions. Aiming at kinetic and spatial resolution of protolytic reactions, thylakoids were excited by groups of three short flashes of light. They induced voltage jumps across the thylakoid membrane on top of a steady pH-difference which was supported by continuous background illumination. Transient proton intake by, proton transfer across and proton release from CF_0CF_1 were monitored by different spectroscopic probes. This approach (previously coined as 'complete tracking of proton flow') has been applied to study protolytic reactions of CF_0CF_1 under ATP synthesis [20], proton slip through CF_0CF_1 in the absence of added nucleotides [21], and proton leakage through the exposed channel portion CF_0 [22].

2. Materials and methods

Flash spectrophotometric experiments were carried out as described elsewere [23]. Stacked thylakoids were prepared from laboratory grown pea seedlings according to [24]. Aliquods from concentrated stock were suspended at an average chlorophyll concentration of 10 μM in a reaction medium containing 3 mM $MgCl_2$, 10 μM methylviologen as electron acceptor, bovine serum albumin (2.6 mg/ml), as a selective (i.e. not membrane permeable) buffer and 0.1 mM ADP and 0.1 mM Na_2HPO_4. The sample was filled into an optical cell with 2 cm path length and the pH was adjusted to 8.0 units. The continuous measuring light (band filter Schott DT-Grün, 495–595 nm, 1.5 mW/cm^2) induced a transmembrane pH-difference across the thylakoid membrane of about 1.5 units, as monitored by fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine [25]. Three turnovers of photosystem II and one of Photosystem I were induced by a flash group consisting of 3 closely spaced Xenon-flashes (2 ms interval) which together generated a voltage jump of about 100 mV [26]. The repetition period of flash groups was 10 s.

The threshold of ATP-synthesis [6] was only surpassed after induction of the flash-induced transmembrane voltage (see [20]), which added about 100 mV [26] or 1.7 pH-units to the steady pH-difference of 1.5 units. Flash-induced charge transfer across the thylakoid membrane was detected by electrochromic absorption changes of intrinsic pigments at a wavelength of 522 nm [26,27], proton release into the suspending medium by the dye Cresol red [24], and proton intake from the thylakoid lumen by Neutral red [28]. The pH-transients were calculated as the difference of two original recordings obtained with and without the respective indicator dye. Those components which were attributable to CF_0CF_1 were separated from background events by their sensitivity to CF_0CF_1-specific inhibitors, here venturicidin and tentoxin. Broadly speaking, the difference between two transients obtained with and without added inhibitor represents the transient protolytic event related to a given reaction in CF_0CF_1. This simple procedure was justified for the pH-transients, but for the charge transfer a finer analysis was required which took into account the leak conductance of thylakoid membranes for other ions, as detailed in a previous study on proton slip [21]. A calibration of the extents in terms of protons per CF_0CF_1 (total) was

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based on the assumption that three closely spaced flashes of red light caused the stroma directed and transmembrane displacement of 4 electrons per electron transport chain, three by photosystem II and one by photosystem I. This caused the uptake of four protons from the stroma and the release of four protons into the lumen [26]. Based on typical figures of the number of chlorophyll molecules per electron transport chain in our material (550, M. Haumann et al., in press) and per CF_0CF_1 molecule (about 1,000, S. Engelbrecht, pers. communication) we assumed that about 8 protons per CF_0CF_1-complex were pumped into the lumen by each flash group.

3. Results

Fig. 1A,C shows transients of proton intake from the lumen (filled circles), charge transfer across the thylakoid membrane (lines) and proton release into the suspending medium (open circles) as calculated from the differences of original transients obtained plus/minus inhibitors. The traces in Fig. 1A were obtained as the difference plus/minus venturicidin. Venturicidin, which binds to the proteolipid of the F_0-portion [29], blocks any proton transfer through CF_0CF_1 and CF_0. Therefore, the traces in Fig. 1A represent transients of the sum of activating and catalytic protons. Small differences between the extents of the three partial reactions were within error and noise limits. Fig. 1B shows transients of the same three partial protolytic reactions, now recorded as the difference plus/minus tentoxin, which eliminates only catalytic turnover but not activation. These traces are hence attributed to catalytic turnover. By the same rationale the difference between the traces in Fig. 1A,B, as shown in Fig. 1C, is attributable to activating proton transsfer. We note that the calibrated extents of proton intake, transfer and release were about equal, among each other and between both types of reactions (catalytic and activating).

Fig. 2B,C is a close up on data points from Fig. 1B,C at greater time resolution. The points and lines denote the same observables as in Fig. 1 and the horizontal bars the time interval where three flashes were fired. In each case (activation and catalysis) proton intake, transfer and release occured with the same kinetic behaviour. Even the activating step necessitated all three reactions, including the *electrogenic transfer* of protons. The kinetics, however, differed between these two types of reactions. For activation there was an approximately exponential rise ($\tau_{1/2} \approx 15$ ms), whereas the proton transfer attributable to catalytic turnover rose more slowly with a pronounced time lag ($\tau_{lag} \approx 15$ ms). The extent of proton transfer, about 4 mol/mol CF_0CF_1(total), was equally shared between activation and catalysis. The kinetic disparity implied that all CF_0CF_1-molecules were active under conditions of flashing light. If only a small fraction of total CF_0CF_1 was activated, say ten percent, each activated molecule had to perform ten turnovers to account for the observed translocation of four protons per CF_0CF_1 total. No kinetic disparity between activating and catalytic protons was expected under these conditions.

4. Discussion

We relied on the differential effect of two inhibitors, tentoxin and venturicidin, on the catalytic turnover and on the activation of the ATP synthase, which is amply documented in the literature. Under pulsed energization of the thylakoid membrane by three closely spaced light flashes we observed two consecutive proton transfer reactions, a prompt phase ($\tau_{1/2} \approx 15$ ms) attributed to the activation and, following with a time lag

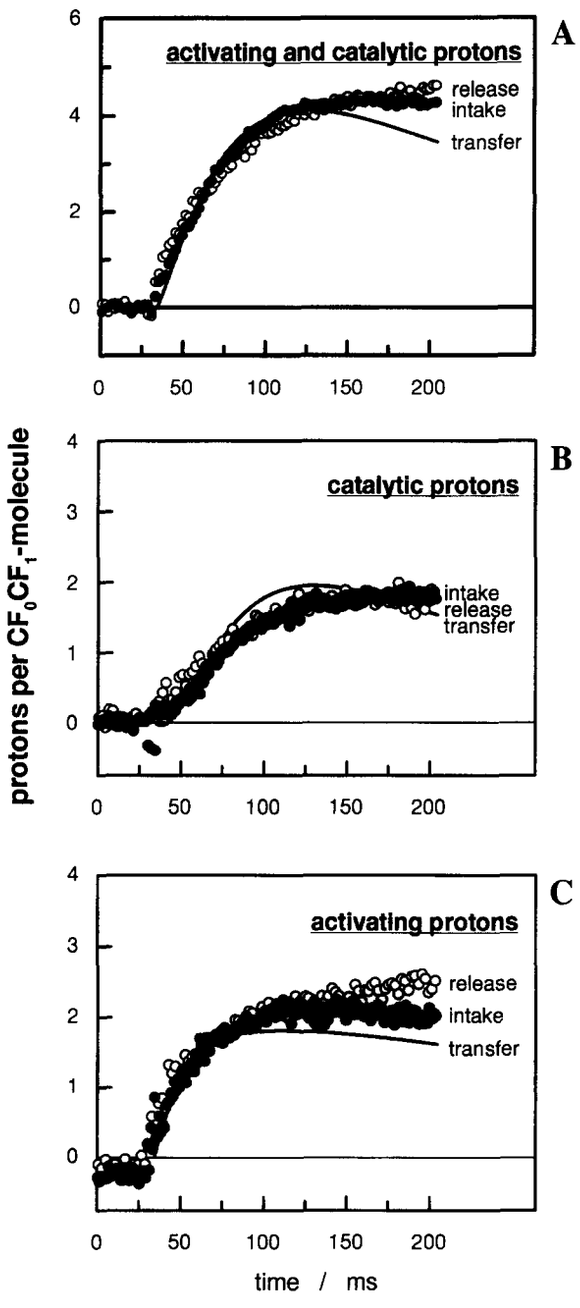

Fig. 1. Proton intake (●), proton transfer (−) and proton release (○) by CF_0CF_1 in response to a group of three Xenon flashes (2 ms interval) as applied to thylakoids. Each set of data points was calculated from original transients of the pH (in the lumen and at the stroma side of thylakoids) and of electrochromism as detailed in the text (see references therein). A: reactions obtained in the presence of ADP and P_i which are sensitive to venturicidin. These are attributable to 'activating' and 'catalytic' reactions. B: reactions which are sensitive to tentoxin, attributable to 'catalytic' events. C: the difference between traces in A and B. It represents 'activating' protons.

of about 15 ms, another phase of the same magnitude which was attributed to the catalytic turnover. The total transfer across CF_0CF_1 was 4 mol H^+/mol CF_0CF_1(total).

A ratio of 3 translocated H^+/ATP has been determined in the past [1,30,31] but more recently a figure of 4 was reported [15]. It is generally agreed on that the translocation of only two

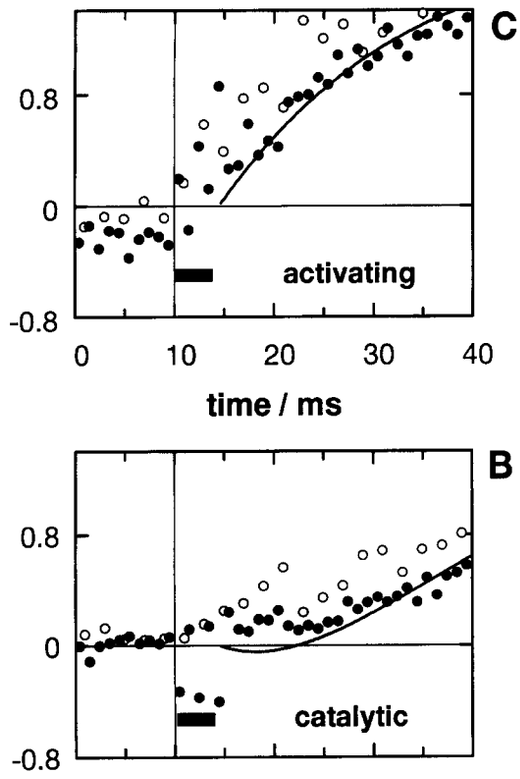

Fig. 2. Close up of the data from Fig. 1B,C at higher time resolution. The bars denote the time interval of the group of three flashes.

protons per ATP, using the protonmotive force which thylakoids sustain under continuous illumination, is insufficient to supply the free energy necessary for the observed ATP/ADP ratios under these conditions. In the light of a H^+/ATP ratio of three if not four our observations have two consequences:

(1) The consecutive nature of two partial translocation steps, each with about 2 H^+/CF_0CF_1 (total), is incompatible with the notion that only a small fraction of total CF_0CF_1 is activated. If this was the case every molecule in this subset had to perform several turnovers with 4 H^+. This would blur the consecutive reactions to the observer. Instead, our observation implies the activation of all CF_0CF_1 molecules even at a moderate peak energization slightly exceeding 3 pH-units or 180 mV.

(2) As there is no room to 'discard' the energy gained from the translocation of, say 2 activating protons, we conclude that the activating transfer of protons has to provide free energy for the synthesis of ATP. The observed consecutive nature of 'activating' (monophasic rise) and 'catalytic' proton transfer (rise only after atime lag) implies that the primary proton transfer step creates a metastable conformational state of the enzyme, which stores free energy. The secondary transfer step provides further free energy to finally promote the release of preformed ATP. This transient storage of free energy is in line with the currently discussed concepts on an 'energy linked binding change mechanism' which states that mainly product release requires energy input rather than phosphoester bond formation (reviewed in [32]). It is also in line with a mechanochemical or conformational coupling mechanism which, under discussion for long [33], has recently gained enormous thrust by the disclosure of a structure at 2.8 Å resolution of mitochondrial F_1 which is highly suggestive of the transmission of conformational changes through subunit γ into the catalytic triade of $(\alpha\beta)_3$ [34].

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