An Attempt To Discriminate Catalytic and Regulatory Proton Binding Sites in Membrane-Bound, Thiol-Reduced Chloroplast ATPase

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ABSTRACT: The question of the possible identity of catalytic and regulatory proton pathways in the chloroplast F_oF_1 ATPase has been studied using different energy-transfer inhibitors. Venturicidin, a reversible inhibitor of F_o, affects neither the $\Delta\tilde{\mu}_{H^+}$-dependent thiol reduction of the membrane-bound chloroplast ATPase nor its ability to be activated by the proton gradient. It seems therefore to block only the proton flow required by the catalytic function of the enzymes. Venturicidin, however, also slows down the deactivation of the thiol-reduced ATPases during uncoupled ATP hydrolysis, following a $\Delta\tilde{\mu}_{H^+}$ activation, but phloridzin, a reversible F_1 inhibitor, has the same effect. Tentoxin, an irreversible F_1 inhibitor, decreases the rate of ATP hydrolysis but does not affect the rate of deactivation. These findings suggest that catalytic and regulatory H^+-binding sites are different. No distinction can be made, if any, between protons involved in unmasking the thiol-sensitive groups of F_1 and in activating the enzyme. The effect of venturicidin and phloridzin on the deactivation is consistent with an inhibitory effect of newly formed—by ATP hydrolysis—ADP molecules, which might affect the enzyme without passing through the medium. Phosphate at millimolar concentration has an effect similar to low concentrations of phloridzin and venturicidin, probably by a simple back-reaction effect.

In chloroplasts, the electrochemical proton gradient $\Delta\tilde{\mu}_{H^+}$,[1] generated by the electron-transfer chain, is necessary to activate the membrane-bound F_oF_1 ATPase in addition to supplying it with energy for the ATP synthesis (Junge et al., 1970; Carmeli & Lifshitz, 1972; Bakker-Grunwald & Van Dam, 1974). The ability of this enzyme to be activated can be enhanced by thiol reduction (Bakker-Grunwald & Van Dam, 1974) of a specific disulfide bridge located on the γ subunit of F_1 (Nalin & McCarty, 1984). This strongly diminishes the magnitude of the $\Delta\tilde{\mu}_{H^+}$ required to activate the ATPase (Mills & Mitchell, 1984; Junesch & Gräber, 1987). On the other hand, the lifetime of the activated state is also considerably increased in the case of the thiol-reduced enzyme: some tens of seconds (Rumberg & Becher, 1984; Biaudet et al., 1988) instead of some milliseconds (Schlodder & Witt, 1981; Schlodder et al., 1982). Magnesium would favor the deactivation of the thiol-reduced form (Bakker-Grunwald & Van Dam, 1974; Shahak, 1986), whereas divalent cations would favor the activation of the oxidized form (Shahak, 1986).

ATPase reduction by dithiothreitol requires hours in non-energized conditions but only minutes in energized membranes, which indicates that $\Delta\tilde{\mu}_{H^+}$ induces unmasking of the disulfide bond (Bakker-Grunwald & Van Dam, 1974; Ketcham & al., 1984). It has also been proposed that deenergization of the membrane facilitates the reoxidation of the dithiol (Shahak, 1985). In vivo, the γ subunit may be reduced, via a thioredoxin, by electrons diverted from photosystem 1 (Mills & Hind, 1979; Mills & Mitchell, 1982; Shahak, 1982).

The activation of membrane-bound chloroplast ATPase by $\Delta\tilde{\mu}_{H^+}$ is accompanied by a conformational change of the inhibitory ϵ subunit (Richter & McCarty, 1987; Komatsu-Takaki, 1989) and by the release of tightly bound nucleotides (Harris & Slater, 1975; Strotmann et al., 1976; Magnusson & McCarty, 1976; Gräber et al., 1977; Strotmann et al., 1979). Whether these nucleotides are bound on catalytic or on regulatory sites in debated (Rosen et al., 1979; Bar-Zvi & Shavit, 1982; Aflalo & Shavit, 1982; Zhou et al., 1988). Although less pronounced than in chloroplasts, an activation by $\Delta\tilde{\mu}_{H^+}$ was also observed on mitochondrial ATPases, in relation to the dissociation of the inhibitory IF_1 subunit (Schwerzmann & Pedersen, 1981; Husain & Harris, 1983; Klein & Vignais, 1983; Lippe et al., 1988). However, all these phenomena are probably not the primary events: the basic processes of activation by $\Delta\tilde{\mu}_{H^+}$ should consist in proton-transfer reactions.

The first step of the $\Delta\tilde{\mu}_{H^+}$-induced activation of the chloroplast ATPase probably consists of the protonation of one or several groups on the internal pole of the enzyme and perhaps also of the deprotonation of external site(s) (Schlodder et al., 1982; Mills & Mitchell, 1984; Rumberg & Becher, 1984; Biaudet & Haraux, 1987; Biaudet et al., 1988). It seems also that ATPase activity depends on the pH difference across the thylakoid membrane rather than absolute pH values, internal or external (Gräber & Witt, 1976; Schönfeld & Schickler, 1984; Davenport & McCarty, 1986; Biaudet & Haraux, 1987; Biaudet et al., 1988; Diedrich-Glaubitz et al., 1988). This suggests a strong coupling between the protonation of one pole of the enzyme and the deprotonation of the other pole (Mills & Mitchell, 1984; Biaudet et al., 1988). These two processes would be equivalent to a net proton translocation (Schlodder et al., 1982). This could be related to the fact that electrical and chemical components of $\Delta\tilde{\mu}_{H^+}$ seem to be equivalent for the activation (Gräber et al., 1984; Turina et al., 1991).

Finally, three kinds of phenomena involve protons in the chloroplast F_oF_1 ATPase: one is the catalytic reaction, and two are regulatory processes, i.e., activation and unmasking

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[1] Abbreviations: F_o and F_1, membranous and extrinsic parts of the proton ATPase; PS1 and PS2, photosystems 1 and 2; $\Delta\tilde{\mu}_{H^+}$, transmembrane difference in electrochemical potential of protons; ΔpH, transmembrane pH difference; DTT, dithiothreitol; BSA, bovine serum albumin; MES, 2-(*N*-morpholino)ethanesulfonic acid; DCCD, dicyclohexylcarbodiimide; EDTA, ethylenediaminetetraacetic acid.

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of the reactive disulfide bond of γ. Until now, very few investigations have addressed the question of their possible discrimination. Most of the kinetic models encountered in the literature assume, more or less explicitly but without experimental evidence, that catalytic and activator proton binding sites are merged [see, for example, Quick and Mills (1988), Heinen and Strotmann (1989), and Lohse et al. (1989)]. This would be supported by one report (Reimer & Selman, 1978) where it was shown that, in thylakoids, two inhibitors of the F_o moiety, DCCD and triphenyltin chloride, prevent the light-induced exchange of ADP between F_1 and the medium, a process generally related to activation. However, other effects of these inhibitors cannot be fully excluded (see Discussion). On the other hand, it was recently shown (Khodjaev et al., 1990) that triphenyltin does not change the rate of acid–base activation of mitochondrial ATPase reconstituted into liposomes. This would indicate, in principle, that activator protons do not cross F_o, but the vectorial or scalar nature of the protonation events examined in this report is not clear. Thus, the question remains open.

In this work, we have investigated this problem by using venturicidin, another inhibitor of F_o (Walter et al., 1967), and with an approach different from that above. Venturicidin is known to block the H^+ flow through the proton channel by interacting with subunit III of F_o (Galanis et al., 1989). The inhibition is fast and partly abolished by washing (Bizouarn et al., 1990). We have examined the consequences of this rapid and reversible switching of F_o on (1) the catalytic turnover and the $\Delta\tilde{\mu}_{H^+}$ activation of thiol-reduced ATPase, (2) the rate of deactivation of the thiol-reduced ATPase during ATP hydrolysis, and (3) the $\Delta\tilde{\mu}_{H^+}$-induced thiol reduction of the ATPase. The principle is illustrated in Figure 1. If all protons bind to a unique site on the internal pole of F_1, one would expect that venturicidin, present during the activation stage of thiol-reduced enzymes, at a moderate concentration to maintain a measurable activity, diminishes both the number of active enzymes and their rate of catalytic turnover during ATP hydrolysis (Figure 1a). In this case, venturicidin should also diminish the rate of deactivation of the ATPases during uncoupled ATP hydrolysis by blocking protons continuously injected onto the catalytic/regulatory site (Figure 1b). Otherwise, these protons would be rapidly released into the lumen, due to the very high conductance of F_o (Lill & Junge, 1989). One also would expect that venturicidin, present in an earlier stage, that is, during the $\Delta\tilde{\mu}_{H^+}$-induced thiol reduction, prevents the unmasking of the γ disulfide bond. It appears from the present data that protons involved in activation and disulfide bond reduction would involve sites different from those implied in catalysis.

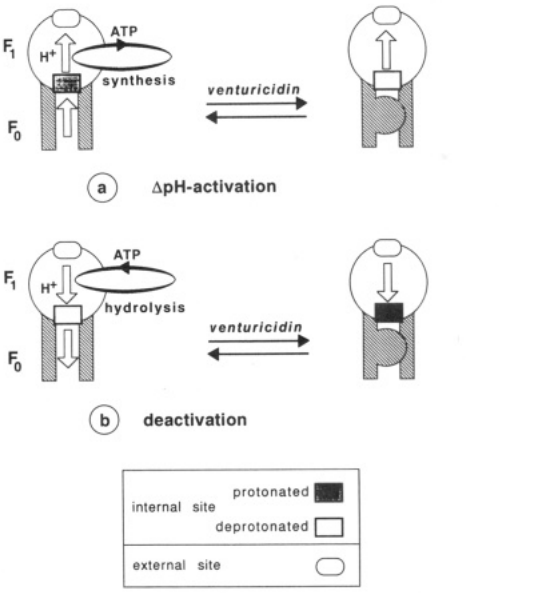

FIGURE 1: Expected effect of F_o closing on the F_1 activation and deactivation with an unique site for catalytic and activating protons. The phosphorylation reaction (a) is coupled to the translocation of protons from the internal (bottom rectangle) to the external (top rectangle) site. The hydrolysis reaction is coupled to proton translocation in the opposite direction (b). In (a, left) (energized condition), the internal site (grey) is protonated because the main $\Delta\tilde{\mu}_{H^+}$ drop is across F_1, due to its very high resistance compared to F_o. Periodical closing of F_o by venturicidin (a, right), by preventing proton binding on the internal site but not their escape in the external medium, helps to deprotonate this site (white), which leads to a decrease of the amount of activated enzyme at the end of the energized stage. In (b, left) (ATP hydrolysis in uncoupled condition), the internal site remains deprotonated, because protons freely escape in the internal medium, which is at the same pH as the external medium in uncoupled conditions. Closing of F_o (b, right) retains protons on the internal site, thus preventing the enzyme deactivation; statistically, the lifetime of the active form of the enzyme is increased.

Materials and Methods

Extraction of Thylakoids and Thiol Reduction. Green leaves of lettuce (*Lactuca sativa* L.) from local markets were chopped in a medium containing 0.4 mM sorbitol, 10 mM Tricine, 10 mM NaCl, 20 μM BSA, and 40 mM sodium ascorbate, pH 8.2. The crude extract was filtered through a nylon sieve (mesh diameter = 25 μm) and then centrifuged at 2000*g* for 10 min. The pellet was resuspended into 1–2 mL of the same medium and its chlorophyll concentration determined according to MacKinney (1941), with slight adjustments in the coefficients; a fraction was diluted at a final concentration of 80 μM chlorophyll into 40 mL of a hypotonic medium made with 10 mM Tricine and 10 mM NaCl, pH 8.3. After 2 min, a time sufficient to release thylakoids, the suspension was mixed with an equal volume of another buffer to get the final composition of 0.1 M sorbitol, 20 mM Tricine, 20 mM NaCl, 4 mM K_2HPO_4, and 0.5 mM $MgCl_2$, pH 8.3. All these steps were carried out at 5 °C and under dim light. Then the suspension, stirred and thermostated at 20 °C in a 100-mL glass vessel maintained in darkness, was supplemented with 50 μM phenazine methosulfate (to catalyze a light-induced cyclic electron transfer around photosystem 1) and 20 mM dithiothreitol (to reduce the ATPase). If necessary, the pH was rapidly readjusted to 8.3, optimal for thiol reduction (Biaudet & Haraux, 1986). After about 1-min incubation, the suspension was illuminated with a strong light devoid of infrared (wavelength between 550 and 700 nm, 1.5 kW m^{-2}) for 5 min unless otherwise stated. After the light was switched off, the 80-mL suspension was immediately mixed with an equal volume of a medium containing 0.1 M sorbitol, 30 mM NaCl, 6 mM K_2HPO_4, and 10 mM MES, pH 6.5, and then centrifuged at 2000*g* for 10 min. The pellet was diluted into this medium at pH 6.5 to obtain a final chlorophyll concentration of 1 mM. This suspension was stored on ice and in darkness before use.

This protocol of preparation of thiol-reduced membranes, adapted from Bakker-Grunwald and Van Dam (1974), was improved with respect to the previously described method (Biaudet et al., 1988) on two points: one centrifugation was omitted, which saved 30–40% of ATPase activity, and the pH of the storage medium was 6.5 instead of 7.8, which better preserved the thiol-reduced material.

EDTA-Treated Thylakoids. Crude extract of lettuce leaves was prepared and centrifuged as above. The pellet was resuspended into a medium containing 30 mM sorbitol, 2 mM Tricine, and 1 mM EDTA, pH 7.8, incubated for 3 min at

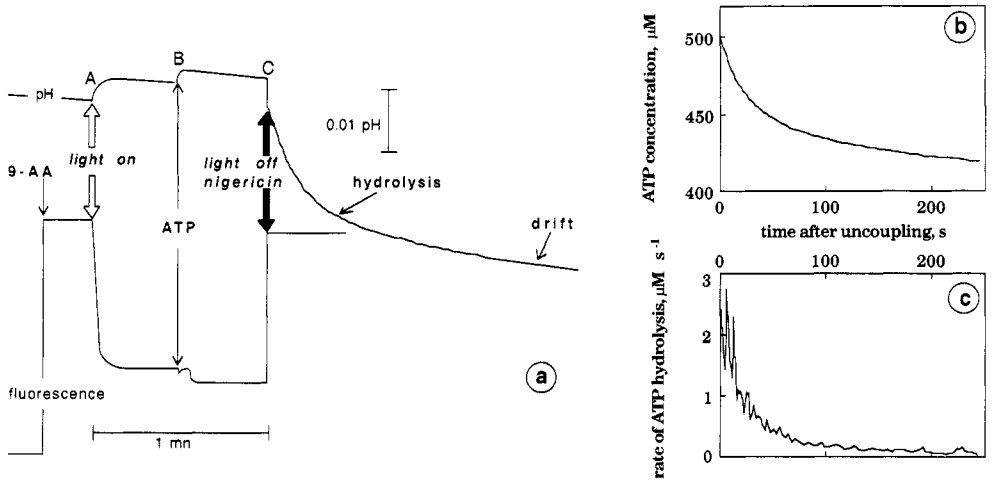

FIGURE 2: Monitoring of pH and 9-aminoacridine fluorescence (a) and time course of ATP hydrolysis after membrane uncoupling (b, c). Conditions were as described under Materials and Methods, with no inhibitor present. (a) Typical recording of pH (top) and 9-aminoacridine (9-AA) fluorescence (bottom). Turning on the light in (A) results in an external proton uptake (pH rise), hence, ΔpH generation (fluorescence quenching). ATP addition in (B) results in the phosphorylation of contaminating ADP (scalar proton production, then pH increase) and perhaps also to some additional proton uptake due to the ΔpH difference between "basal" and "static head" conditions. ATP addition in itself has also a slight quenching effect on 9-aminoacridine (instantaneous fluorescence decrease and uncomplete recovery of fluorescence after uncoupling). The slow increase and then decrease of fluorescence after ATP addition reflects the ΔpH decrease due to the phosphorylation of contaminating ADP and then its increase when the static head state is reached. Light extinction and nigericin addition in (C) result in an instantaneous proton release (pH decrease and fluorescence increase), followed by ATP hydrolysis (slow pH decrease) until all enzymes are deactivated; the very slow negative pH drift, insensitive to ATPase inhibitors, is instrumental. (b) ATP concentration vs time, directly computed from the pH-meter recording [phase C of pH recording in (a)]. The data are fitted with $ATP_t - ATP_\infty = (ATP_0 - ATP_\infty)e^{-kt} + V_\infty t$, where ATP_0 and ATP_t are the concentrations of ATP at times 0 and t (in s), respectively, ATP_∞ is the final ATP concentration, and V_∞ is an instrumental drift, not related to ATP hydrolysis. k is the constant of deactivation in s^{-1}. The initial change in ATP concentration is given by $V_0 = -k(ATP_0 - ATP_\infty)$. In this example, $V_0 = -2.1$ μM s^{-1}, $k = 0.036$ s^{-1}, $ATP_\infty = 442$ μM, and $V_\infty = -0.1$ μM s^{-1}. (c) Instantaneous rate of ATP hydrolysis vs time: first derivative of curve b (the sign of the ordinate axis was changed to give positive values). The asymptote (V_∞) is slightly above the 0 line due to the instrumental drift.

5 °C, and centrifuged at 2500*g* for 10 min. After determination of the chlorophyll concentration, the new pellet was diluted at a final concentration of 1 mM chlorophyll into 0.2 M sorbitol, 10 mM Tricine, 10 mM KCl, 6 mM $MgCl_2$, and 2 mM K_2HPO_4, pH 7.8, and kept on ice and in darkness [adapted from Strotmann et al. (1973)].

ΔpH Measurements. The energetic state of the membrane was controlled by the light-induced quenching of 9-aminoacridine (Schuldiner et al., 1972), ΔpH being calculated with previously published calibrations (Bizouarn et al., 1989).

Measurement of Ferricyanide Photoreduction. The absorbance of ferricyanide at 420 nm was monitored simultaneously with the fluorescence of 9-aminoacridine in a setup described elsewhere (de Kouchkovsky et al., 1982). EDTA-treated thylakoids were assayed at a chlorophyll concentration of 10 μM, at 20 °C, in the storage medium supplemented with 0.8 mM potassium ferricyanide and 4 μM 9-aminoacridine. The intensity of the red actinic light was 1.5 kW m^{-2}.

Hydrolysis Rate Measurements. ATP hydrolysis was followed by monitoring the "scalar" H^+ production due to acid–base equilibria (Nishimura et al., 1962) with a fast and sensitive glass electrode. Unless otherwise indicated, thiol-reduced thylakoids were suspended at a concentration of 30 μM chlorophyll in a medium containing 1 mM Tricine, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM K_2HPO_4, 50 μM pyocyanine (to ensure a cyclic electron flow around PS1), and 4 μM 9-aminoacridine (to measure ΔpH), adjusted at pH 8.2. Other additions, if any, are indicated in the Results section. The 1.5-mL sample, contained in a 1 × 1 cm spectroscopic cuvette, stirred and thermostated at 20 °C, was put into a setup described elsewhere (Biaudet & Haraux, 1986). Unless otherwise stated, the following procedure was applied (Figure 2a): after a few minutes of incubation, the suspension was illuminated for 1 min with strong red light (1.5 kW m^{-2}) to reactivate thiol-reduced enzymes; ATP (500 μM), preadjusted at pH 8.2, was added in the middle of the light stage and nigericin (1 μM) exactly at the end of the light stage. Complete discharging of the membrane, as controlled by the fluorescence of 9-aminoacridine, occurred in 1 s or less. ATP hydrolysis was followed immediately afterward, until complete deactivation of the enzymes, which occurred in 2–4 min. A slight instrumental negative drift, insensitive to ATPase inhibitors, was generally observed. The total pH decrease during ATP hydrolysis never exceeded 0.05. The buffering power of the suspension, determined in each condition by HCl titration, was constant in all this pH range.

Kinetic Analysis of ATP Hydrolysis. The pH-meter recording was digitized and fitted with an exponential decay superimposed on a linear drift (Biaudet et al., 1988), using an iterative method. The first 3–4 s of the recording, disturbed by the injection of nigericin and the output of vectorial protons, was disregarded. So the initial rate of ATP hydrolysis V_0, computed from the parameters of the exponential decay, was extrapolated at the time of deenergization). It is proportional to the amount of activated thiol-reduced ATPases at this time. The kinetic constant of deactivation k (in s^{-1}) was also easily obtained. Figure 2b shows an example of the kinetics redrawn from a pH recording, directly converted into ATP concentration, without subtraction of the linear drift (note that only a small fraction of the ATP is consumed during the experiment, so the substrate remains saturating). Figure 2c shows the first derivative of curve b, proportional to the instantaneous rate of ATP hydrolysis, superimposed on the slope of the drift (final baseline slightly different from zero, note the inversion of sign).

Since some of our conclusions are built on correlations between the initial rate of ATP hydrolysis, decreased by inhibitors, and the constant of deactivation, it was necessary to verify that no side effects appear when the initial rate becomes small, especially due to an increased weight of the instrumental

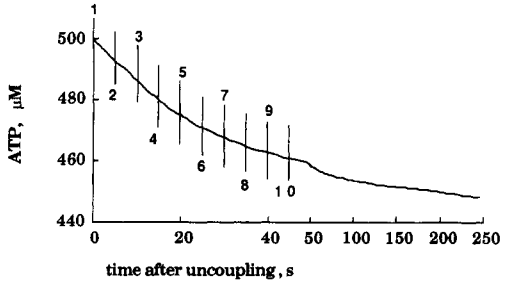

FIGURE 3: Determination of the first-order constant of decay at different times after deenergization. Conditions were as described under Materials and Methods, with no inhibitor present. See text for details. Four recordings were digitized and averaged to trace the curve shown. The time scale changes at $t = 50$ s. Successive fits were obtained with data comprised between the numbered vertical bars and the end of the kinetics. The equation used was that of Figure 2.

pH drift. We have thus checked that, for a given recording, the deactivation constant does not vary with time, i.e., when the activity itself decays. The principle is illustrated Figure 3. The ATP vs time curve, or its equivalent in pH, was fractioned into segments of about 5 s. First, the whole curve was fitted, which gives, as explained in Figure 2, the initial rate of ATP hydrolysis and the first-order constant of deactivation. Then the first segment was eliminated, and the remaining part of the curve, from $t = 5$ s to the end, was fitted in the same way. New values of the initial rate and of the deactivation constant were thus obtained. Of course, the pseudo initial rate decreased, as one moved in the direction of increased time of deactivation. Then the following segment ($t = 5$ s to $t = 10$ s) was removed in turn, the remaining curve (10 s to the end) was fitted again, and the operation was repeated until a very low pseudo initial rate, at least 10–20 times lower than the true initial rate, was obtained. In the example given in Figure 3, the pseudo initial rate of fit 10 was 90% less than the true initial rate (fit 1), but the deactivation constant was changed by only 10%, from 0.047 s^{-1} (fit 1) to 0.052 s^{-1} (fit 10). This negligible increase, compared to the large decrease of activity, indicates that no significant side effect is introduced by the computing method and therefore that the first-order kinetics is a quite good approximation. This test was made on each control of the experiments involving inhibitors; moreover, several kinetics were generally averaged in that purpose.

Stability of the Preparations. Controls made at different times showed that the preparations were stable for 3–5 h after thiol reduction. In some cases, however, a moderate decrease of the initial rate of ATP hydrolysis and increase of the deactivation constant were observed. These changes never exceeded 10–20% during the course of the experiment, and then corrections were made according to the order of taking the sample.

F_o and F_1 Inhibitors, Uncoupler. Venturicidin (F_o inhibitor), phloridzin (F_1 inhibitor), and tentoxin (F_1 inhibitor) were added to the chloroplast suspensions in small volumes of concentrated ethanolic solutions (no effect of ethanol alone was noticed). No incubation stage was found necessary. Venturicidin and phloridzin are reversible inhibitors, whereas tentoxin is irreversible (Bizouarn et al., 1990). Nigericin, as other uncouplers, has some inhibitory, poorly understood effect (Pick, 1988). In our hands, this effect did not occur for concentrations below 2 μM, which rules out any interference of this inhibition with the deactivation processes studied here.

Reagents. All chemicals were of analytical grade. Nigericin, venturicidin, phloridzin, tentoxin, and ATP (disodium salt, less than 1% ADP) were purchased from Sigma. Pyocyanine was prepared from phenazine methosulfate in this laboratory by Dr. C. Sigalat (McIlwain, 1937).

Table I: Lack of Effect on ATP Hydrolase Activity of the Presence of Venturicidin during Light-Induced Thiol Reduction[a]

venturicidin (μM)	addition: before light	addition: after light	DTT	light duration (s)	rate of hydrolysis [μmol of ATP (mg of chlorophyll)$^{-1}$ h^{-1}]
0			no	120	not measurable
0			yes	120	211 ± 13
0			yes	45	130 ± 13
0.3		+	yes	45	66 ● 4
0.3	+		yes	45	68 ± 7
0.3			yes	0 (dark)	not measurable

[a] Conditions were as described under Materials and Methods, with ADP plus arsenate to maintain a high proton flow across F_oF_1. Light refers here to the thiol-reduction stage, whereas the activity was always measured after a 1-min illumination period (reactivation stage). Each value is the average of three independent assays. For the control on the last line, venturicidin was added before the 140-s dark incubation with DTT which preceded the mixing with DTT-free medium.

RESULTS

Lack of Venturicidin Effect on the $\Delta\tilde{\mu}_{H^+}$-Induced Unmasking of the γ Disulfide Bond. We have checked the effect of venturicidin on the $\Delta\tilde{\mu}_{H^+}$-induced thiol reduction of the γ subunit. Thylakoids were illuminated in the presence of DTT as described under Materials and Methods, except that the light duration was limiting. In addition, ADP and arsenate[2] were present to maintain a continuous proton flow across the enzymes and then to make venturicidin-sensitive any acidobasic group located downstream of the site of action of this inhibitor. In the absence of the substrates of phosphorylation (the so-called "basal" condition), the net proton flow across F_o could indeed be so low that the local pH within the channel would not be affected by its closure.

After 45 or 120 s of illumination, the light was turned off, and NH_4Cl (0.5 mM) was added to accelerate the relaxation of the proton gradient. Venturicidin (0.3 μM) was added before illumination or 20 s after the light was switched off, when the proton gradient had fully decayed. After 140 s in darkness, the suspension was mixed with an equal volume of the DTT-free medium described under Materials and Methods, supplemented with BSA (2 g/L) to bind residual inhibitor. The membranes were then centrifuged, stored on ice, resuspended into the assay medium, reactivated with strong light for 1 min, and assayed for uncoupled ATP hydrolase activity. A typical experiment is described in Table I. The control, first line, shows that the activity represents only thiol-reduced ATPases, since it is zero for membranes illuminated without DTT. Comparison of lines 2 and 3 shows that 45 s is a limiting time for thiol reduction, which is a prerequisite to check the effect of venturicidin on the unmasking of the γ disulfide bond. Comparison of lines 3 and 4 shows that, even after membrane washing, the enzymes remain inhibited by around 50% (this will be examined in more detail below). The main point comes from the comparison of lines 4 and 5: the activity does not depend on the presence of venturicidin during the light plus DTT stage. Lastly, no significant activity was detected when

[2] The ATPases catalyze an energy-dependent condensation of ADP and arsenate, coupled to a proton flux through the enzymes (Gresser, 1981; Slooten & Nuyten, 1984). The complex formed is immediately cleaved in the medium, that is, without reverse proton translocation within the enzyme. Compared to phosphate, arsenate has the advantage of avoiding formation of ATP, thence, generation of a proton gradient during the dark stage, and related disturbing effects.

nonenergized thylakoids were incubated with DTT. These data show that venturicidin does not prevent the $\Delta\tilde{\mu}_{H^+}$-induced unmasking of the γ disulfide bond involved in the enzyme regulation.

We have also determined that in conditions similar to thiol modulation (same chlorophyll concentration, similar medium, saturating light intensity), but without subsequent washing, 0.3 μM venturicidin inhibits the ATPase activity by 80%. Thus the washing procedure reverses the inhibition by about 40%: (80% - 50%)/80% = 0.375. In other words, in the material washed after venturicidin treatment (condition of Table I), at least 60% of the rate of ATP hydrolysis is an activity which has been restored by washing: (80% - 50%)/50% = 0.6.

The incomplete recovery of activity could probably be attributed to trapping of venturicidin, a highly lipophilic substance, within the membrane (Bizouarn et al., 1990). Without washing, 0.1 μM venturicidin decreases the activity by approximately 50% (not shown), which corresponds to the inhibition of the samples treated with 0.3 μM and washed. The washing procedure would then have eliminated $^2/_3$ of the inhibitor.

Lack of Venturicidin Effect on the Activation of Thiol-Reduced ATPases. A thiol-reduced ATPase which has returned to an inactive state after membrane deenergization needs $\Delta\tilde{\mu}_{H^+}$, i.e., protons, to be reactivated. We have tried to determine whether venturicidin acts on this reactivation process or on the catalytic flow by examining the effectiveness of the time of inhibitor addition. If protons blocked by venturicidin were necessary to activate the enzyme, the presence of this molecule during the reactivation stage should have an additional inhibitory effect on the final activity, compared to its simple addition at the end of this stage. The oxidized enzymes catalyzing phosphorylation are inhibited in 1 s or less after venturicidin addition in the light and to the same extent as enzymes preincubated with venturicidin in the dark (Bizouarn, unpublished data). However, this gives no indication of the nature of inhibition, because the activation/deactivation process and the catalytic turnover of oxidized ATPases are both in the millisecond range (Schlodder et al., 1982; Junesch & Gräber, 1987). In the case of thiol-reduced enzymes, the half-time of activation, short at high $\Delta\tilde{\mu}_{H^+}$, is in the tens of seconds range at low $\Delta\tilde{\mu}_{H^+}$ (Biaudet et al., 1988), and the half-time of deactivation is around 10–50 s (Rumberg & Becher, 1984; Biaudet & Haraux, 1986; Biaudet et al., 1988; this report). Nevertheless, the catalytic turnover remains in the millisecond range, as for oxidized ATPases (Junesch & Gräber, 1987). This indicates that a rapid inhibition of thiol-reduced ATPases upon venturicidin addition would reveal a slowing down of the catalytic cycle, because deactivation cannot occur in short times. On the other hand, a slow effect would mean a decrease of the number of active enzymes. We have thus compared the initial rate of uncoupled ATP hydrolysis catalyzed by samples partially inhibited with venturicidin, added before or at the end of the activation stage by $\Delta\tilde{\mu}_{H^+}$. Thiol-reduced, dark-adapted thylakoids were reactivated in the light for 30 s, in the presence of ADP plus arsenate, but without ATP, to maintain a continuous proton flow through the enzymes. Afterward, the light was switched off and ATP and nigericin were injected to trigger ATP hydrolysis. Venturicidin was added 20–30 s before turning on or 5 s before switching off the light. Table II shows that the remaining ATP hydrolase activity does not depend on the presence of venturicidin during the activation stage. Controls of Table II also indicate that the experiments with venturicidin were made in conditions where neither the light intensity

Table II: Dependence of ATP Hydrolase Activity on Duration and Intensity of Light Reactivation and Absence of Effect of the Time of Venturicidin Addition[a]

time of illumination (s)	light intensity (W m^{-2}) 15	30	1500	presence of venturicidin
	53	69	74	none
30	16 ± 3	26 ± 4	nd	20–30 s before light on
	19 ± 3	26 ± 4	nd	5 s before light off
60	nd	nd	100	none

[a] Single thylakoid preparation, thiol-reduced in light and stored in darkness as described under Materials and Methods. Activities were measured after reactivation in the light under different conditions, in the presence of ADP plus arsenate. Venturicidin, 0.2 μM, was added either in the dark, 20–30 s before light on, or in the light, 5 s before light off. Hydrolase activity is expressed in percentage of maximal activity, which was, for 60 s and 1500 W m^{-2}, 272 μmol of ATP (mg of chlorophyl)$^{-1}$ h^{-1}. ΔpH magnitudes (unaffected by venturicidin) were 3.55 ± 0.04 (100% light), 2.26 ± 0.04 (2% light), and 2.09 ± 0.08 (1% light). Even when both light intensity (15–30 W m^{-2}) and duration (30 s) were limiting, the time where venturicidin was added did not affect the subsequent hydrolase activity (average of three assays).

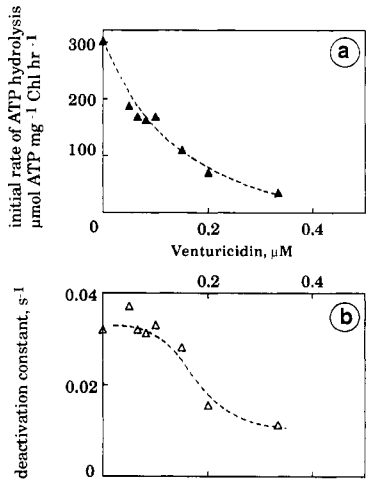

FIGURE 4: Initial rate of ATP hydrolysis (a) and first-order rate constant of deactivation (b) as a function of venturicidin concentration. Conditions were as described under Materials and Methods.

(15–30 W m^{-2}) nor the light duration (30 s) was saturating, which is the best condition to observe an effect, if any, of a hindered proton flow through F_o. On the other hand, ΔpH was unaffected by the presence of venturicidin during the activation, probably due to the fact that, at these low ΔpHs, the contribution of the phosphorylating flow to the total transmembrane current was rather weak (de Kouchkovsky et al., 1982). Finally, this experiment shows that the inhibitory effect of venturicidin is rapid (<5 s) and involves the catalytic cycle itself and not the activation process. This result strongly suggests that catalytic and activating protons bind onto different sites.

Effect of Venturicidin and Phloridzin on the Deactivation of Thiol-Reduced ATPase. Another way to discriminate catalytic and activating protons is to check the effect of venturicidin on the deactivation of thiol-reduced ATPases during the ATP hydrolysis which follows membrane deenergization. If the protonatable sites involved in these two kinds of processes are identical or at least very close together, one would expect that periodical blocking of F_o by venturicidin retains, on the activating site, protons coming from ATP hydrolysis (Figure 1b). This should increase the lifetime (k decrease) of the active form of ATPase after discharging the membrane. We have thus studied the kinetics of ATP hydrolysis at different venturicidin concentrations. The inhibitor was added just before

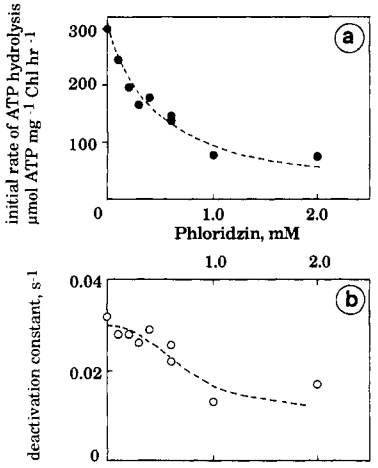

FIGURE 5: Initial rate of ATP hydrolysis (a) and the first-order rate constant of deactivation (b) as a function of phloridzin concentration. Same chloroplast preparation and conditions as in Figure 4.

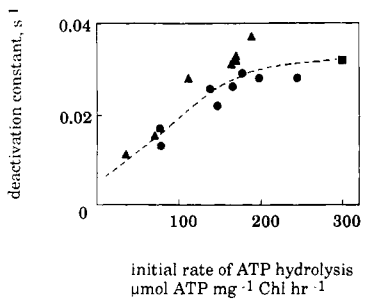

FIGURE 6: First-order rate constant of deactivation as a function of the initial rate of ATP hydrolysis without inhibitor (■) and with variable venturicidin (▲) or phloridzin (●). Data of Figures 4 and 5.

the light reactivation stage (of 1-min duration) and ATP in its middle (i.e., 30 s after turning on the light). Figure 4 shows the effect of venturicidin on the initial rate of ATP hydrolysis upon uncoupling (a), obtained as in Figure 2, and the corresponding first-order rate constant of deactivation (b). The slowing down of the enzyme deactivation by venturicidin is quite obvious. On first sight, this could mean that, contrary to our previous suggestion, regulatory and catalytic protons could bind to identical or close sites.

To clarify this point, we have checked the effect of phloridzin, a reversible F_1 inhibitor (McCarty, 1980), on the deactivation of the ATP hydrolase. F_1 inhibitors are not expected to retain protons in F_o and therefore slow down the enzyme deactivation, whatever the interactions between catalytic and regulatory protonatable sites may be (see Discussion for the question of inhibitor sites). However, Figure 5 shows that, in the same way as venturicidin, phloridzin decreases the deactivation constant of the ATPase. More precisely, the relationship between the initial rate of ATP hydrolysis and the first-order rate constant of deactivation is the same when the enzymes are progressively inhibited either by venturicidin or by phloridzin (Figure 6). This indicates that an inhibitor may slow down the deactivation by an effect which has nothing to do with burying protons.

Since the F_1 specificity of phloridzin has been superficially documented in the literature, we have checked a possible effect of this inhibitor on the proton channel F_o in F_1-depleted thylakoids. EDTA-extracted thylakoids were prepared for this purpose, and the PS2–PS1 mediated redox activity was measured simultaneously to the light-induced fluorescence quenching of 9-aminoacridine. As expected, this preparation

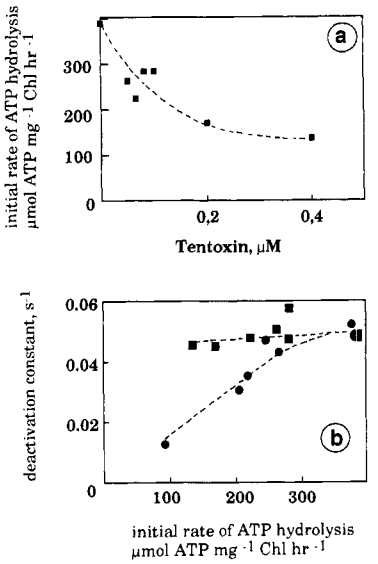

FIGURE 7: Effect of F_1 inhibitors on the initial rate of ATP hydrolysis and on the first-order rate constant of deactivation. Conditions were as described under Materials and Methods. (a) Initial rate of ATP hydrolysis as a function of tentoxin concentration. (b) First-order rate constant of deactivation versus the initial rate of ATP hydrolysis, with variable tentoxin (■) or phloridzin (●, 0–2 mM, same chloroplast preparation).

exhibited a high rate of ferricyanide photoreduction [1.2 mmol (mg of chlorophyll)$^{-1}$ h^{-1}] but no membrane energization, since it became leaky after F_1 extraction. Phloridzin at the highest concentration used here (2 mM) inhibited this activity by at most 10%, but without restoring any proton gradient, which shows that this small inhibition was not due to an increase of the membrane H^+ resistance. On the contrary, 0.5 μM venturicidin had exactly the effect expected from closing F_o (which was, in this condition, the main site of H^+ leaks): it dramatically slowed down the electron flow (by more than 75%), giving rise to a large proton gradient as probed by the fluorescence of 9-aminoacridine. These results confirm that phloridzin is a pure F_1 inhibitor. Since the effects of venturicidin and phloridzin on the deactivation of the ATPase are quantitatively identical (Figure 6), it is tempting to look for a common mechanism, different from a proton retention at the F_o level.

Lack of Tentoxin Effect on the Deactivation of Thiol-Reduced ATPase. Since the deactivation of the ATPases seems strictly related to the rate of ATP hydrolysis modulated by two different inhibitors, one may suspect that a product of the reaction accelerates the deactivation. This could be reasonably ADP, known indeed to inhibit ATPases (Carmeli & Lifshitz, 1972; Shoshan & Selman, 1979; Gräber et al., 1977; Bar-Zvi & Shavit, 1980; Dunham & Selman, 1981; Strotmann et al., 1987; Lohse et al., 1989; Bizouarn et al., 1989; Kleefeld et al., 1990). The higher the concentration of inhibitors, the lower the ADP production, and this could reduce the decay of activity, hence the rate constant k. This behavior is expected for whatever type of inhibitor is used. For this reason, we have examined the effect of tentoxin, an irreversible F_1 inhibitor. Figure 7 shows that, in contrast with phloridzin, tentoxin does not affect the rate constant of the ATPase deactivation. This would mean that this process is insensitive to bulk ADP production. In other words, it is possible that an ADP molecule produced by a given enzyme may inactivate only this enzyme, i.e., does not pass through the medium. Tentoxin indeed, contrary to venturicidin and phloridzin, fully inhibits a given fraction of the ATPases—hence it lowers the initial rate of hydrolysis—but keep the other enzymes completely functional,

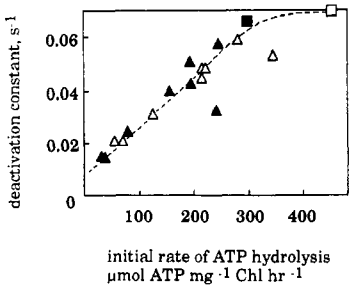

FIGURE 8: First-order rate constant of deactivation as a function of the initial rate of ATP hydrolysis, modulated by venturicidin (0–0.67 μM), without phosphate (□, △) or with 2.5 mM phosphate (■, ▲): (□, ■) no venturicidin; single chloroplast preparation. Conditions were as described under Materials and Methods.

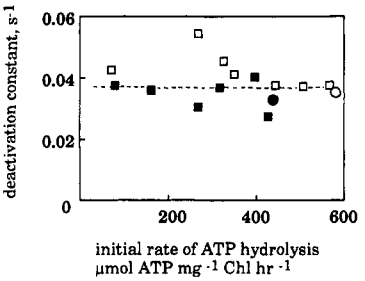

FIGURE 9: First-order rate constant of deactivation as a function of the initial rate of ATP hydrolysis, modulated by tentoxin (0–1 μM; □, ■), without (○, □) or with 2.5 mM phosphate (●, ■): (○, ●) no tentoxin; single chloroplast preparation. Conditions were as described under Materials and Methods.

with an unchanged k. This hypothesis would mean that, at variance with sites involving protons, catalytic and regulatory sites occupied by ADP are confined in the same microspace or even are merged.

Inability of Phosphate To Change the Relationship between the Initial Rate of ATP Hydrolysis and the Rate Constant of Deactivation. Inorganic phosphate is known to protect ATPases against ADP-induced deactivation (Carmeli & Lifshitz, 1972; Dunham & Selman, 1981). It is then interesting to establish if it also changes the relationship between the activity and the kinetics of decay. Figure 8 shows an experiment where progressive inhibition of the ATPase by venturicidin was carried out in the absence or in the presence of 2.5 mM phosphate. First of all, phosphate decreased the activity of control (no inhibitor) by around 35%, probably by a simple back-pressure effect (phosphate is a product of ATP hydrolysis). More important is the fact that phosphate did not alter the relationship between the initial rate of ATP hydrolysis and the deactivation constant varied by venturicidin. Schematically, to add inorganic phosphate is equivalent to increase the concentration of a reversible inhibitor.

Figure 9 shows that when the activity is varied by tentoxin, 2.5 mM phosphate does not affect the deactivation constant, as would have occurred when a reversible inhibitor diminishes the activity by 35% (see the plateau of Figure 6). This is fully consistent with data of Figure 8 and indicates that, in these conditions, phosphate again acts more as a product of the reaction than as an effector of regulatory sites.

Discussion

Reversibility of the Inhibition and Related Problems. The rationale of the present experiments implies that all ATPases interact with venturicidin molecules but at different times. Indeed, a permanent and complete inhibition of a given fraction of enzymes, as with tentoxin, cannot affect the ability of the remaining ones to be reduced by DTT, activated by ΔpH or deactivated after membrane deenergization. In addition, this "collisional" process with a reversible inhibitor must be comparable to the catalytic turnover; otherwise all would happen as in the irreversible case. Direct evidence for this is not easy to obtain, but such a collisional behavior is fully consistent with the following facts, established in the case of thylakoid membranes: (1) while it preserves the Michaelian nature of the kinetics of phosphorylation, venturicidin decreases the K_m for ADP (Bizouarn et al., 1990); (2) the inhibition may be partly reversed by washing the membranes (Bizouarn et al., 1990; this report); (3) the inhibition is almost instantaneous upon venturicidin injection in a thylakoid suspension (data not shown). This indicates a permanent—and probably fast—equilibrium between free and bound molecules of venturicidin. Interestingly, this behavior, indirectly suggested here, was demonstrated by patch-clamp analysis in the case of subunit III of F_0 reconstituted into vesicles. Though transporting cations instead of protons, this channel remained fully sensitive to venturicidin, with opening and closing times in the millisecond range (Schönknecht et al., 1989).

Other inhibitors raise no particular problems. Phloridzin is a fully reversible inhibitor, which decreases the K_m for ADP (Bizouarn et al., 1990). Tentoxin is an "all-or-nothing" irreversible inhibitor, which changes neither the Michaelis constant K_m for ADP (Bizouarn et al., 1990) nor the deactivation constant (this report).

Catalytic and Regulatory Protons. Our data suggest that venturicidin blocks only protons involved in the catalytic process, not those regulating the activity of the enzymes. However, concerning the identical effect of venturicidin and phloridzin on the enzyme deactivation during ATP hydrolysis (Figure 6), one could imagine that phloridzin, although a pure F_1 inhibitor, actually prevents the deprotonation of catalytic sites located on the internal face of the enzyme, close to F_o. The effect of reversible inhibitors could then be interpreted as a mean proton retention on the regulatory site, which might thus be merged with the catalytic one. But this does not explain the lack of venturicidin effect on activation (Table II). Moreover, if activating protons are retained in the catalytic site, one would expect that, in the absence of the substrate ATP, the regulatory site would not be maintained protonated, and the enzyme would deactivate very rapidly. On the contrary, without ATP, the deactivation after membrane deenergization occurs with a low first-order kinetic constant, approximately 10^{-2} s^{-1} (P. Biaudet, unpublished data). This value is in accordance with that reported for the deactivation of ADP-free thiol-reduced ATPase, i.e., 7×10^{-3} s^{-1} (Fromme & Gräber, 1990). Interestingly, this corresponds, in Figures 6 and 8, to the value of the deactivation constant extrapolated to zero activity. To deplete the enzyme in nucleotides or to diminish its rate of catalytic turnover by reversible inhibitors has thus the same effect. The common point between these different conditions is the absence of ADP, externally added or internally produced by ATP hydrolysis. So ADP, and not protons, seems to govern the kinetics of deactivation in the experiments of Figures 6 and 8.

Catalytic protons are also different from those involved in the unmasking of the disulfide bond of the γ subunit, as shown by the lack of effect of venturicidin on the light-induced thiol reduction (Table I). However, that $\Delta\tilde{\mu}_{H^+}$-induced activation and $\Delta\tilde{\mu}_{H^+}$-induced disulfide bond unmasking are independent of the catalytic protons does not imply that they themselves are related. Until now, nevertheless, it is implicitly assumed that activation and disulfide bond unmasking are two aspects of the same phenomenon (Bakker-Grunwald & Van Dam,

1974; Schlodder et al., 1982; Pick, 1983).

The present discrimination between catalytic and regulatory protons is apparently inconsistent with a previous report (Reimer & Selman, 1978), which has shown that two F_o inhibitors, DCCD and triphenyltin chloride, prevented the light-induced exchange of tightly bound ADP, a process classically related to activation. But these inhibitors, which were used at very high concentrations, 0.5 mM triphenyltin (i.e., 0.5 mol/mol of chlorophyll) and 5 mM DCCD (i.e., 5 mol/mol of chlorophyll), are not devoid of side effects. Trialkyltin and triphenyltin chlorides dissipate transmembrane proton gradients [see, for example, Selwyn et al. (1970)]; in chloroplasts, significant uncoupling by triphenyltin chloride may occur at an inhibitor/chlorophyll molar ratio of 0.1 (Gould, 1976), or even 0.03 (Watling-Payne et al., 1974). On the other hand, at relatively high concentrations, DCCD inhibits not only F_oF_1 but also other proton pumps (Azzi et al., 1984). In chloroplasts, it inhibits the chloroplast electron transfer at the b_6f level (Sane et al., 1979), and it short-circuits the proton pumping at the PS2 level (Jahns & Junge, 1989). Moreover, uncoupling effects have been reported in mitochondria (Beattie & Villalobo, 1982). In our hands, even purified DCCD at 40 μM or 2 mol/mol of chlorophyll significantly diminishes the PS1-driven ΔpH (unpublished). To conclude, it is not certain that the reported inhibition of the energy-dependent release of ADP (Reimer & Selman, 1978) can be related to the blocking of F_o, since the proton gradient was not controlled. In addition, the regulatory nature of $\Delta\tilde{\mu}_{H^+}$-induced of ADP is disputed (Rosen et al., 1979; Zhou et al., 1988).

Catalytic and Regulatory ADP. Different explanations may be given for the slowing down of ATPase deactivation during ATP hydrolysis by phloridzin and venturicidin. Their common basis is that these reversible inhibitors, by acting on specific steps of the catalytic cycle, diminish the steady-state concentration of ADP bound to the enzyme and produced by the ATP hydrolysis.

In a first approach, one may simply assume that, upon membrane deenergization, the successive states undergone by the enzymes during the catalytic cycle of hydrolysis (E, E^{ATP}, E_P^{ADP}, and E^{ADP}) irreversibly deactivate at different rates, the E^{ADP} state being the least stable. This would give a linear relationship between the initial activity (V_0) and the apparent kinetic constant of deactivation (k).

Another possibility is a direct transfer of ADP from the catalytic site to a regulatory site, without passing through the medium, which would increase the rate of deactivation of all states. The direct transfer of nucleotides from catalytic to regulatory sites, without equilibration with the medium, has been debated for more than ten years (Rosen et al., 1979; Aflalo & Shavit, 1982). This would lead to an hyperbolic relationship between V_0 and k, maybe closer to the experimental situation (Figures 6 and 8).

The effect of phosphate (Figures 8 and 9) may be simply explained in all cases: by a back-pressure mechanism, it diminishes the concentration of the E^{ADP} state, as phloridzin and venturicidin do. Finally, the lack of effect of tentoxin on the deactivation constant could be explained by its all-or-nothing action and by a relative irreversibility of the release of ADP, which makes the noninhibited enzymes insensitive to ADP production. This quasi-irreversibility of the release of ADP would also account for the first-order decay of the activity, independently of the time of the reaction and, thence, of the concentration of ADP produced (Figure 3). Without speculating any further, plausible and single schemes may account, at least qualitatively, for the effect of the different inhibitors and of inorganic phosphate on the kinetics of ATPase deactivation.

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