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Activity equilibria of the thiol-modulated chloroplast H^+-ATPase as a function of the proton gradient in the absence and presence of ADP and arsenate

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The 9-aminoacridine fluorescence signal was calibrated for quantitative determination of transmembrane ΔpH of isolated thylakoids by imposing defined phosphate potentials at static head in the absence of Δψ. This technique was subsequently employed in the investigation of actual equilibrium activities of the thiol-modulated H^+-ATPase as a function of ΔpH in the absence and presence of ADP and arsenate which was used as a substitute for phosphate. Significant quantitative and qualitative differences were observed depending on the compounds present in the medium. In the presence of ADP alone, virtually no ATPase activity was detected at ΔpH values less than 3; the sigmoidal dependence of activity on intrathylakoidal H^+ concentration indicated positive cooperativity. In contrast, in the presence of arsenate (±ADP) substantial activity was found at much lower proton gradients and a different $[H^+_{in}]$ dependence was observed. In the absence of both, ADP and arsenate, an intermediate activity–ΔpH relationship was obtained. The equilibrium levels of tightly bound ADP (±arsenate) as function of ΔpH were essentially inverse to ATPase activity, indicating the significance of ADP and arsenate (= phosphate) binding for enzyme control. A model was established which describes the process of activation by maximally three consecutive reversible protonation reactions taking place from the lumen phase of the thylakoid, and the interaction of ADP and arsenate/phosphate from the medium phase at defined protonation stages of the enzyme. This model explains the experimental findings in a quantitative manner and allows for relevant results reported in the literature. The model proposes a simple interpretation of active and inactive states of the chloroplast H^+-ATPase.

Introduction

The activity of the chloroplast H^+-ATPase is controlled by co-operation of multiple regulatory factors including thylakoid energization, thiols, adenine nucleotides and phosphate. The ATPase is inactive in chloroplasts kept in the dark. Upon energization by light or an artificial pH or electrical gradient, rapid enzyme activation precedes ATP synthesis [1–4]. In its 'demodulated' (= oxidized) form, the ATPase is immediately inactivated with relaxation of the transmembrane electrochemical proton gradient, whereas in its 'thiol-modulated' (= reduced) state the enzyme remains active under certain conditions and is capable of hydrolyzing ATP after decay of the proton gradient [5–8]. Thiol modulation is accomplished by DTT [5] or the natural thiol donor protein thioredoxin [9]. The target for thiol modulation is a specific disulfide group in γ-subunit which is exposed by a conformational change related with activation [10,11]. The activation/deactivation reactions are regarded all-or-nothing processes, i.e., the overall activity is thought to be determined by the fraction of active ATPase molecules [2,12].

Several workers have studied the effect of pretreatment to break the disulfide bridge in γ-subunit on ΔpH dependence of ATP formation. This treatment caused a shift towards lower ΔpH values at a given rate of phosphorylation [13–17]. It was concluded that in the reduced state of the enzyme the ΔpH curve reflects the actual energy requirement of the catalytic process whereas in non-modulated thylakoids the ΔpH curve

Abbreviations: As_i, arsenate; DTT, dithiothreitol; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; PEP, phospho*enol*-pyruvate; P_i, inorganic orthophosphate; PMS, phenazine methosulfate; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

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indicates the energy requirement for activation of the oxidized enzyme. For complete understanding of the context between catalysis and regulation, however, the regulatory roles of ADP, phosphate and ATP in addition to their function as substrates have to be considered, too.

At transition from light to dark, inactivation of the thiol-modulated ATPase is slow in a nucleotide-free medium, but is strongly accelerated by addition of micromolar concentrations of ADP [7]. Acceleration of deactivation is effected by tight binding of one ADP per ATPase [18–20] to a β-subunit of CF_1 [21,22]. This reaction is slowed down by inorganic phosphate [18,23,24] or phosphate analogues like arsenate [25]. Binding of ATP on the other hand, leads to stabilization of an active ATPase [26].

Tightly bound ADP is re-released by membrane energization [27–31]. The initial rate of liberation of ADP was shown to match the rate of phosphorylation [2,4,32,33]. Hence we may conclude that the equilibrium between active and inactive ATPase molecules in the light depends on $\Delta\tilde{\mu}_{H^+}$ but also on the concentrations of ADP and phosphate (if ATP is experimentally excluded) and that the equilibrium level of tightly bound ADP is essentially inversely related to enzyme activity.

In the present study, equilibrium activities and equilibrium levels of bound ADP were analyzed as a function of ΔpH (at $\Delta\psi = 0$) in the absence and presence of ADP, arsenate (as a substitute for phosphate) and ADP + arsenate. The ΔpH curves reveal significant differences depending on the medium composition. The results are interpreted by a model which can describe the experimental data in a quantitative manner.

Methods

Class II chloroplast (thylakoids) were isolated from spinach leaves as reported in Ref. 30. In all experiments fluorescence of 9-aminoacridine was registrated as a measure of transmembrane ΔpH. The experiments were conducted in a cylindrical glass cuvette ($\varnothing = 12$ mm) with magnetic stirrer which was placed in a self-constructed fluorometer. The central part of the instrument was a thermostated (20 °C) metal bloc containing a bore for the cuvette. Fluorescence excitation and emission light was guided through a side bore by a branched light pipe. Fluorescence emission was received by a photodiode detector (HUV 4000 B, Polytec) and monitored by a recorder. Photosynthesis light was conducted through a lower side bore by a second light pipe and reflected by a 45° mirror through the bottom of the cuvette. The bloc was mounted on a magnetic stirring motor. Fluorescence excitation light was filtered through a 400 nm interference filter (Schott). Fluorescence emission light was passed through a combination of a glass and interference filter (490 nm, Schott) to absorb fluorescence excitation as well as photosynthesis light. Photosynthetic light was passed through a red cut-off filter (RG 630, Schott).

The final reaction volume was 2.5 ml. The medium contained 10 mM Tricine buffer (pH 8.0), 50 mM KCl, 50 nM valinomycin, 5 mM $MgCl_2$, 10 mM DTT, 50 μM PMS, and thylakoids corresponding to 25 μg chlorophyll/ml. After registration of basal fluorescence, 9-aminoacridine (5 μM) was added. The resulting fluorescence increase was termed ϕ_0. For thiol modulation, maximal intensity of photosynthesis light was given for 2 min. The steady-state fluorescence obtained in the light was designated Φ. As a measure of ΔpH the term $\log(\Phi_0 - \Phi)/\Phi$ was computed [34] and calibrated as described in detail in the Results section. For measurements of steady-state ATPase activities as function of ΔpH, light was subsequently attenuated to variable intensities; where indicated, 10 μM ADP and/or 5 mM arsenate was added at the same time. After 3 min, a pulse consisting of [^{32}P]ATP (0.5 mM), phospho*enol*-pyruvate (0.5 mM), pyruvate kinase (salt-free, 15 U/ml) and nigericin (0.2 μM) was employed together with turning off the light. 0.2 ml samples were deproteinized by $HClO_4$ (0.5 M) after 10, 20 and 30 s. The rate of ATP hydrolysis was calculated from the contents of [^{32}P]P_i of the samples which were analyzed as in Ref. 35. Synthesis of [^{32}P]ATP by photophosphorylation was also described in Ref. 35.

Levels of tightly bound ADP as a function of ΔpH were measured in analogous experiments using 10 μM [^{14}C]ADP instead of unlabeled ADP. For termination of the reaction, an isotope dilution quench technique [36] was employed. The quench solution consisting of 5 mM unlabeled ADP and 50 μM FCCP (final concentrations) was added after 3 min reaction. The free [^{14}C]ADP was removed by three washing steps [30]. Aliquots of the resuspended pellets were used for chlorophyll determination and measurement of radioactivity in a scintillation counter [30].

Results

Calibration of 9-aminoacridine fluorescence for determination of ΔpH

The project designed in the Introduction requires a quantitative technique for the determination of transthylakoidal proton gradients. Fluorescence quenching of 9-aminoacridine [34] is a convenient but contested method; the proposed mechanism [34] as well as the quantitative application was challenged [37,38]. However, the reaction mechanism is unimportant when the fluorescence signal is used as an arbitrary indicator of ΔpH for the employed experimental conditions (pH, chlorophyll concentration etc.) and calibrated by an independent standard. The principle of the here conducted standardization is based on the energetic equiv-

alence of the electrochemical proton potential and the phosphate potential in the 'static head' state and assumes complete delocalization of the proton gradient [39]. In equilibrium the relationship between the two energetic parameters is expressed by

$$\Delta G_P = n(2.303 \cdot RT\Delta pH + F\Delta\Psi) \quad (1)$$

The 9-aminoacridine signal observed at a certain equilibrium ΔG_p can be assigned to ΔpH when n (= H^+/ATP) is known and $\Delta\Psi$ is zero. Actually in illuminated thylakoids $\Delta\Psi$ can be neglected at steady-state energization, especially in the presence of K^+ and valinomycin [40]. The H^+/ATP stoichiometry of the H^+-coupled thylakoid-ATPase is assumed to be 3, a value which has been established by different experimental approaches [40–45]. The phosphate potential ΔG_p is composed of a standard term ΔG_p^0 and a variable term including the actual concentrations of ATP, ADP and phosphate:

$$\Delta G_P = \Delta G_P^0 + 2.303 \cdot RT \log \frac{[ATP]}{[ADP][P_i]} \quad (2)$$

ΔG_P^0 was determined by Rosing and Slater [46] under a variety of experimental conditions. Taking into account the temperature (20 °C), pH (8.0), ionic strength of the medium (approx. 0.1 M) and the concentration of Mg^{2+} (5 mM), their data permit to estimate for our conditions a value of 32.2 kJ · mol^{-1}. At $\Delta\Psi = 0$, combination of Eqns. 1 and 2 and insertion of the constant terms yields:

$$\Delta pH = 1.91 + \frac{1}{3} \log \frac{[ATP]}{[ADP][P_i]} \quad (3)$$

To get a calibration curve, 9-aminoacridine signals obtained at the respective equilibrium phosphate potentials are plotted versus ΔpH which is calculated according to Eqn. 3. The advantage of this method of standardization is that the internal thylakoid volume (which is difficult to determine precisely [38]) does not enter in the calculation of ΔpH when the experimental conditions are kept constant.

'Internal' equilibrium phosphate potentials can be determined by analyzing the equilibrium concentrations of the participating substrates in illuminated thylakoid suspensions [47]. Alternatively, the equilibrium state may be determined by 'externally imposed' phosphate potentials as follows [45].

After preillumination of the thylakoids in the presence of DTT, varying ΔpH values are adjusted by changing the intensity of the photosynthetic light and monitored by 9-aminoacridine fluorescence. After reaching the steady state, a mixture consisting of ATP, ADP and P_i of a defined phosphate potential is added. Depending on the light intensity employed, the 9-aminoacridine signal either decreases (indicating net phosphorylation) or increases (indicating net ATP hydrolysis) or does not change at all (equilibrium state). Usually the equilibrium state is not encountered exactly, but can be ascertained by interpolation between positive and negative fluorescence changes obtained at different light intensities [45]. Addition of adenine nucleotides causes some instantaneous fluorescence quench which is unrelated with ΔpH [45,48]. For correction, controls are run under conditions which exclude ATP synthesis and ATP hydrolysis. The nucleotide dependent fluorescence quench can be separated from the ΔpH signal in the presence of EDTA or Ca^{2+} instead of Mg^{2+}.

TABLE I

Logarithm of fluorescence quenching over fluorescence of 9-aminoacridine at an equilibrium phosphate potential of 49 kJ · mol^{-1} adjusted by different concentrations of ATP, ADP and phosphate

The ratio of [ATP]/[ADP][P_i] was 1000 M^{-1} in any case. The equilibrium state between ΔpH and ΔG_P was ascertained as described in the text.

Concentrations (μM)			$\log \frac{\Phi_0 - \Phi}{\Phi}$
ATP	ADP	P_i	
100	100	1000	−0.14
200	200	1000	−0.15
200	50	4000	−0.15
100	200	500	−0.14
50	100	500	−0.14

In an experiment shown in Table I, a constant initial phosphate potential of 49 kJ · mol^{-1} was maintained with varying concentrations of the three substrates. As expected theoretically, the corrected 9-aminoacridine fluorescence signals (expressed as log $(\Phi_0-\Phi)/\Phi$) were identical irrespective of the substrate concentrations employed. This result justified the use of all possible ratios of substrate concentrations for adjustment of different phosphate potentials. An essential point is the correct determination of the substrate concentrations: we found that the commercial preparations of ATP contained up to 4.2% ADP and up to 6% P_i, while ATP contained 1.5% ADP. Of course these contaminations must be taken into account for adequate calculation in particular when low or high ΔG_P values are required.

The collected data of a number of independent calibration experiments are shown in Fig. 1. The two techniques for determination of the equilibrium state gave rather consistent results. Under the employed conditions a linear relationship between log $(\Phi_0-\Phi)/\Phi$ and ΔpH is evident in a ΔpH range from 2.5 to 3.5. For practical application the curve was linearly extrapolated below ΔpH = 2.5 and above ΔpH = 3.5 although scattering is large in these ranges. It must be emphasized that a thus established calibration curve is applicable for the employed conditions only (external

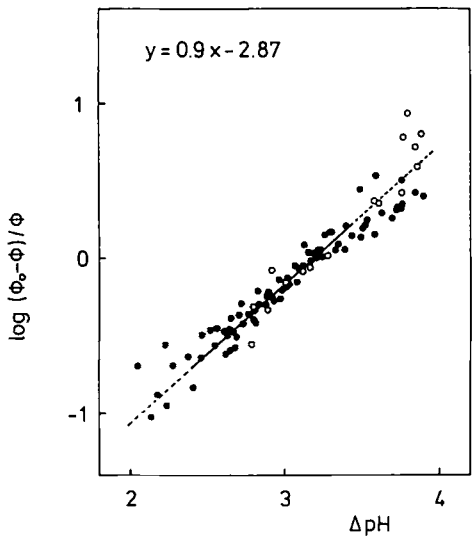


Fig. 1. Relationship between the logarithm of fluorescence quenching over fluorescence and transthylakoidal ΔpH at pH_{out} = 8.0. The ΔpH values were computed from equilibrium ΔG_p values determined according to Eqn. 3. Equilibrium phosphate potentials were either imposed externally (filled symbols) as described in the text or assayed analytically (open symbols). In the latter case thiol-modulated thylakoids were illuminated with 50 μM [^{14}C]ADP and 100 μM phosphate at different light intensities until equilibrium. The concentrations of labeled AMP, ADP and ATP were determined after thin-layer chromatography as in Ref. 49. The inserted equation resulted from linear regression analysis of the data between pH 2.4 and 3.4 and was employed for the assessment of ΔpH in all subsequent experiments.

pH = 8.0, 25 μg chlorophyll/ml and the indicated medium composition). The scattering of the data seems to permit an accuracy of about 0.2 ΔpH units. The relatively large error is mainly due to deviations between the individual experiments. In the single experiments the points are usually located above or below the mean line drawn in Fig. 1 and can be connected by parallels to the mean line. In order to improve comparison between the ΔpH scales in the individual experiments described below, 3–4 calibration points taken at the end of every experiment are used for scale normalization.

ATPase activity as function of transmembrane ΔpH

In this study, activation states of the thiol-modulated H^+-ATPase are investigated as function of ΔpH in the absence or presence of the effectors ADP, arsenate and ADP plus arsenate. The phosphate analogue arsenate was employed in order to prevent the formation of ATP which would complicate the experimental system.

After thiol modulation by 2 min preillumination of the thylakoids in the presence of DTT, varying ΔpH values were adjusted in the presence of the indicated compounds by changing the light intensity, and were monitored by the calibrated 9-aminoacridine fluorescence. After reaching a steady state, the enzymatic activity was assayed by the initial rate of ATP hydrolysis under conditions which were optimal and equal in every sample. For this purpose, a pulse consisting of [^{32}P]ATP, PEP, pyruvate kinase and nigericin was given together with turning off the photosynthetic light. The ATP-regenerating pyruvate kinase system was employed to exclude any subsequent regulatory effects of ADP [49]. Uncoupling by nigericin together with darkening immediately provided the optimal thermodynamic conditions for ATP hydrolysis. ATP hydrolysis was followed kinetically by analyzing [^{32}P]P_i in consecutive samples taken within the first 30 s after the pulse. In

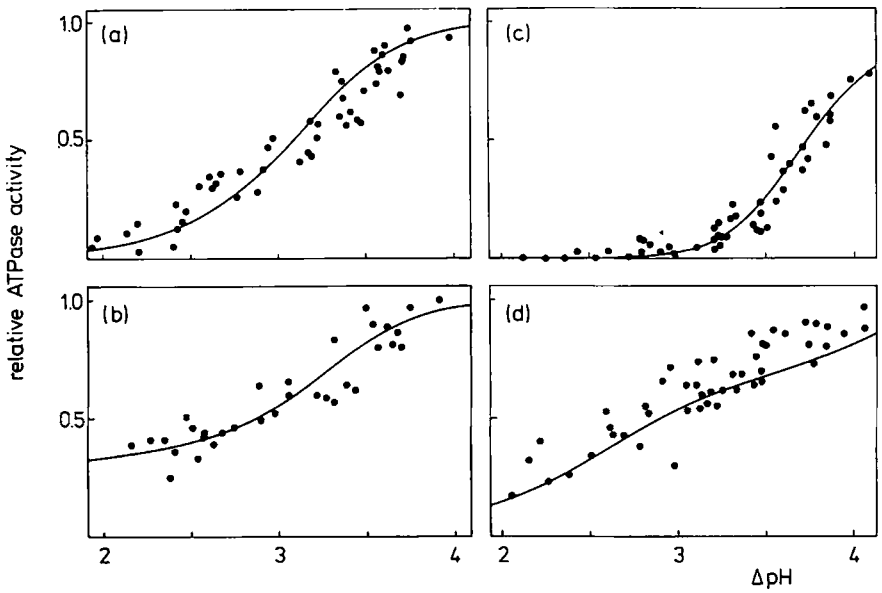


Fig. 2. Relative equilibrium activities of the H^+-ATPase as function of ΔpH in the absence of ADP and arsenate (a), in the presence of 5 mM arsenate (b), in the presence of 10 μM ADP (c) and in the presence of 10 μM ADP + 5 mM arsenate (d). The curves were calculated by the model of Scheme I using the constants indicated in Discussion.

this time range the dilution of specific radioactivity could be neglected.

The time for achievement of the equilibrium state of ATPase activity was measured at low and high ΔpH values in the absence and presence of ADP, arsenate and ADP plus arsenate. It was established that 3 min were sufficient in any case (not shown). Therefore a standardized equilibration time of 3 min was employed in all subsequent experiments. Fig. 2 shows the obtained equilibrium activities as function of ΔpH in the absence of any additions (a), in the presence of 5 mM arsenate (b), in the presence of 10 μM ADP (c) and in the presence of 10 μM ADP + 5 mM arsenate (d). The data of a number of independent experiments were collected and normalized as fractions of maximal activities. In the individual experiments the maximal rate of ATP hydrolysis varied between 280 and 450 μmol P_i/mg chlorophyll per h. The drawn curves were computed according to a model shown in Scheme I which is discussed below.

In spite of the scattering, the results show significant qualitative and quantitative differences between the four variants. The ΔpH values for half-maximal activity are about 3 in the absence of any additions, less than 3 in the presence of As_i or ADP + As_i, but about 3.8 in the presence of ADP alone. At ΔpH = 2.5 no activity is detected if the medium contained ADP alone, about 10% activity is observed in the ADP- and As_i-free medium, but 30–40% of maximal activity is found when As_i with or without ADP is present. At ΔpH = 4 almost full activity is measured under all conditions except for the presence of ADP alone.

ATPase activity as a function of ΔpH was also measured after equilibration in the presence of 1 and 100 μM ADP (not shown). At 1 μM ADP the activity curve was located between the curves obtained at 0 and 10 μM ADP. Points taken in the presence of 100 μM ADP indicated that the activity curve was not further shifted significantly towards higher ΔpH values at ADP concentrations greater than 10 μM. Hence the dissociation constant for ADP must be considerably lower than 10 μM, a conclusion which is in accordance with kinetic measurements [30].

It is evident that the ΔpH curves are different in shape depending on the components present in the medium. For a more detailed analysis the ATPase activities of Fig. 2 were replotted as functions of the intrathylakoidal H^+ concentrations calculated from the ΔpH values (Fig. 3). In the absence of both effectors a more or less hyperbolic curve is apparent. In the presence of ADP, the activity curve is clearly sigmoidal. Assuming that activation involves the interaction of the ATPase with protons from the lumen side, this result suggests positive cooperativity, i.e., more than one H^+ per ATPase is necessary for activation in the presence of ADP. Different $[H^+_{in}]$-activity relationships are observed in the presence of arsenate + ADP and arsenate alone. In particular in the latter case a biphasic curve is evident. A hyperbolic phase saturated at a very low internal H^+ concentrations, is followed by another hyperbolic phase with a half-saturating concentration of about 20 μM H^+_{in}.

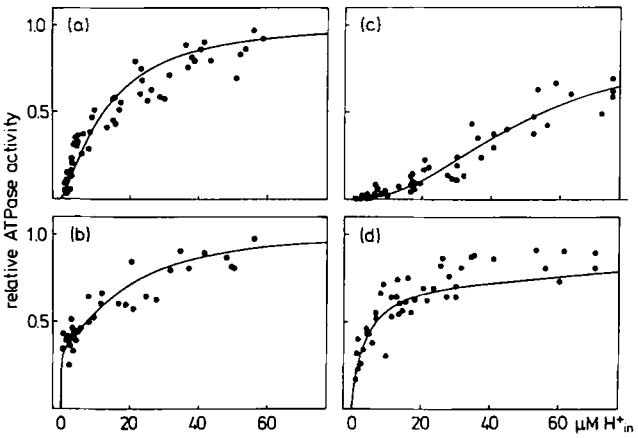


Fig. 3. Relative equilibrium activities of the H^+-ATPase as function of the intrathylakoidal H^+ concentration. Replot of the data presented in Fig. 2.

Levels of tightly bound ADP as function of transmembrane ΔpH

Equilibrium levels of ADP tightly bound to CF_1 as function of ΔpH were measured in analogous experiments by using ^{14}C-labeled ADP as effector. The amounts of tightly bound ADP were assayed by employing a previously developed isotope dilution quench technique [36]. The quench solution consisting of a high excess of unlabeled ADP and FCCP, provides displace-

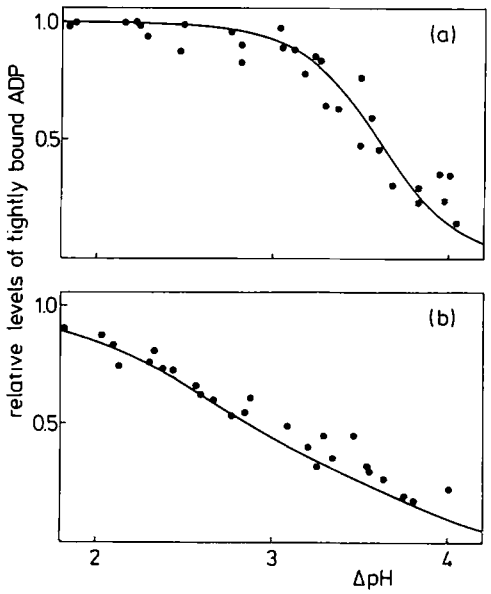


Fig. 4. Relative equilibrium levels of tightly bound ADP as function of ΔpH in the absence (a) and presence (b) of 5 mM arsenate. The concentration of [^{14}C]ADP was 10 μM.

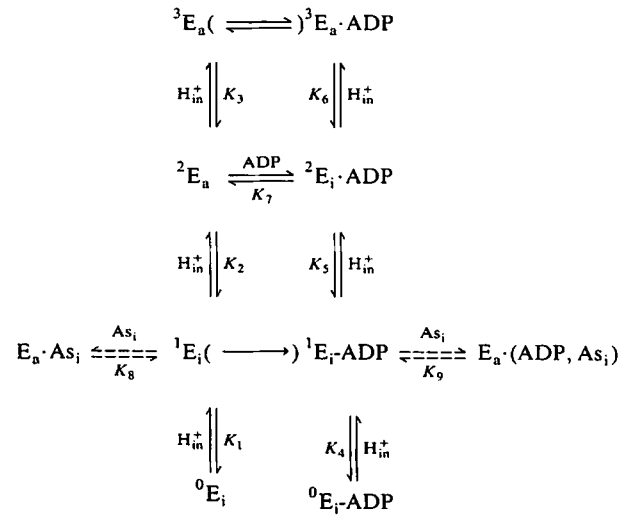
ment of all labeled ADP from the membranes except the molecules which are non-exchangeable, i.e., tightly bound, and furthermore prohibits $\Delta\tilde{\mu}_{H^+}$-dependent re-release of bound ADP molecules because of the rapid uncoupling by FCCP [36]. Maximal levels of tightly bound ADP were ascertained in every single experiment by performing the reaction for 30 min in the dark after preillumination. Under those conditions binding of ADP is complete and irreversible [29,30,36]. The mean of the dark controls was 1.1 nmol/mg chlorophyll. This value is in agreement with previously reported results [30] and corresponds to about 1 mol tightly bound ADP per mol CF_1.

In Fig. 4 the relative equilibrium levels of tightly bound ADP obtained in several independently conducted experiments are plotted as function of ΔpH in the absence and presence of arsenate. Under both conditions the levels of bound ADP decrease with increasing ΔpH, in the presence of arsenate, however, the point of 50% ADP binding is shifted towards lower ΔpH values. The drawn curves are computed by the model described below (Scheme I).

Discussion

$\Delta\tilde{\mu}_{H^+}$-dependent conformational changes leading to activation of the chloroplast H^+-ATPase were attributed to different pH optima at the inner pole (accessible from the thylakoid lumen) and the outer pole (accessible from the medium) of the enzyme [31,50]. The protonation and deprotonation reactions at the two sides may occur in a concerted mode, so that the protonation state of the inner protonable groups affect the pK values of the outer groups and vice versa [51,52]. However, if reasonable assumptions are made [51], the activation state is essentially a function of the intrathylakoidal H^+ concentration when the external proton concentration is kept constant, as in the here reported experiments. In an equilibrium model (Scheme I) the process of reversible activation is interpreted by reversible protonation reactions at the inner pole of the enzyme at the given external pH of 8.0. Starting from some basic assumptions, the model was modified stepwise so that the simulated curves matched the measured data under all experimental conditions (six variants) with an identical set of constants. It turned out that the experimental results could be best accommodated to a model which includes altogether three consecutive protonation steps. As the number of catalytic entities per ATPase is three, we assume that one proton is guided to each of the catalytic units. Hence a successive activation of the catalytic units is proposed. The apparent pK values of the three protonable groups are affected by each other and also by the functional state of the nucleotide and phosphate (= arsenate) binding sites. It is further assumed that each unit contains one nucleotide binding site which is responsible for both, catalysis and regulation. In fact it was demonstrated by photolabeling experiments with 2-azido-ADP, that tightly bound nucleotides are on catalytic sites [53]. Although up to three more nucleotide binding sites were detected in isolated CF_1 [54], their functional meaning in situ remains unclear. In this context it must be mentioned that the targets for binding of photoreactive nucleotide analogues were found to be rather different in membrane-bound and isolated CF_1 [55,56]. In the membrane-bound enzyme the three relevant sites may be closed or open, occupied by a nucleotide molecule or non-occupied. If a closed site is occupied, the nucleotide molecule appears 'tightly bound'. Activation of a catalytic unit is suggested to be related with opening of the corresponding site. The number of sites which have to be available in order to get a catalytically active enzyme depends on the presence or absence of ADP and arsenate/phosphate (see below). Without claiming definitiveness, the employed equilibrium model is capable of describing the reported results in a more or less pertinent way. It is regarded as a working model which can be scrutinized further by analysis of the proposed reaction steps. The main features of the model are the following.

(1) An individual enzyme molecule is either fully active or fully inactive [13,14]; however, multiple active and inactive forms may be discriminated by the state of protonation and binding of regulatory ligands as ADP and phosphate (= arsenate), respectively.



Scheme 1. Model of regulation of the chloroplast H^+-ATPase by interaction of H^+ from the intrathylakoidal phase (H^+_{in}) and ADP and As_i binding from the medium phase. The indices a and i signify active and inactive enzyme species, respectively. The binding state of ADP and arsenate is marked by a bar (tightly bound) or a dot (exchangeable). The constants K_1 to K_9 are equilibrium constants of the respective steps. Reactions put in parentheses were disregarded in the computations.

(2) In dark-adapted chloroplasts two different inactive ground forms exist: one with an ADP tightly bound in one of the three β-subunits or $\alpha-\beta$ interfaces (^0E_i-ADP), respectively, and one which is lacking the bound ADP (^0E_i). All sites are closed towards the medium phase, so that actually no interactions between the protein and the free nucleotides are possible. This may explain inactivity of the ATPase in the ground state.

(3) In ^0E_i one internal protonable group is available with a pK not far below the pH of the medium. In ^0E_i-ADP the respective H^+ binding site exhibits a lower pK. Binding of one H^+ creates still inactive forms with one open non-occupied site (^1E_i, ^1E_i-ADP) which are capable of binding arsenate/phosphate. ^1E_i may be able to slowly bind ADP in an irreversible manner [18,30,36]. In ^1E_i-ADP the ADP-containing site is still closed.

(4) Upon transfer of the two ground states to ^1E_i or ^1E_i-ADP another inner protonable group is becoming accessible at a second catalytic unit. In ^1E-ADP the protonable group is at the unit containing the tightly bound ADP and exhibits a very low pK. Protonation of ^1E_i yields an active species ^2E_a, protonation of ^1E_i-ADP transfers the tightly bound to an exchangeable ADP; this form ($^2E_i \cdot$ ADP) is still inactive, but conversion into the active species ^2E_a would be possible by dissociation of the 'loosely' bound ADP. $^2E_i \cdot$ ADP can undergo exchange of the bound ADP with medium ADP [30]; since this process is known to require dissociation of the bound ADP and simultaneous reassociation of medium ADP to another site [57], we can assume that rebinding of medium ADP takes place at the site which was opened by the first protonation step.

(5) Upon formation of $^2E_i \cdot$ ADP, the H^+ binding site of the third catalytic unit is opened. Protonation of this group creates a species with a loosely bound ADP which is catalytically active ($^3E_a \cdot$ ADP) [30,36,57]. Analogously, ^2E_a may associate a third proton to form an alternative active species ^3E_a. ^2E_a and ^3E_a show different properties with regard to rebinding of ADP in as much as ^3E_a binds ADP at a higher rate than ^2E_a. It has been shown that thylakoid energization accelerates the initial velocity of tight ADP binding [18], a result which could be explained by the above assumption.

(6) On the basis of the non-competitive inhibitory effect of phosphate on tight ADP binding it was concluded that phosphate interacts with an ADP-free as well as with an intermediate ADP-containing enzyme form [24,25]. By phosphate interaction, tight binding of ADP and ADP-dependent inactivation is retarded [18,23–25]. These facts as well as the here reported results were taken into account by postulating that active species are formed by binding of As_i or P_i to both, ^1E_i and ^1E_i-ADP. Possibly more than one step (as drawn in the scheme) is involved. We may speculate that a second and third protonation occurs (as in the absence of phosphate), which are indetectable in our experiments because the pK values of the respective H^+ binding groups might be strongly increased by arsenate/phosphate binding.

For mathematical simulation, the connected equilibrium equations (Scheme I) were solved by a computer program to yield the fraction of all active enzyme species and the fraction of enzymes with tightly bound ADP as a function of ΔpH. K_1 to K_9 were accommodated so that the curves fitted the experimental data in the best way. The curves in Figs. 2–4 were computed with dissociation constants for internal protonable groups $K_1 = 10^{-7}$ M, $K_2 = K_3 = K_4 = K_6 = 2 \cdot 10^{-5}$ M, $K_5 = 10^{-4}$ M. The interactions of ADP and arsenate are characterized by the dissociation constants $K_7 = 2 \cdot 10^{-6}$ M, $K_8 = 10^{-2}$ M and $K_9 = 1.43 \cdot 10^{-3}$ M. Conversion of the dissociation constants K_1 to K_8 into apparent pK values at an external pH of 8.0 yields $pK_1 = 7$, $pK_2 = pK_3 = pK_4 = pK_6 = 4.7$ and $pK_5 = 4$. The curves are not significantly changed by raising pK_1 to values close to 8, i.e., the first protonation step of the ADP-free enzyme may occur at an extremely low pH gradient.

In the absence of ADP, the life time of the coupled ATP hydrolyzing activity of the thylakoids was found to be much larger than the relaxation time of the proton gradient [7]. In the presence of an uncoupler, i.e., when rate limitation by H^+ efflux is abolished and enzyme reactivation during ATP hydrolysis by the developing proton gradient is made impossible, the dark decay of ATP hydrolyzing activity is faster [58], but not as fast as the relaxation of the proton gradient. This fact requires to assume that the structural change induced by protonation of ^1E_i to yield ^2E_a or ^3E_a is not easily reverted when the pH of the internal environment is brought to the initial level. (This may be the most important functional difference between the thiol-modulated and the non-modulated enzyme which falls into its inactive state immediately with relaxation of the gradient). As protonation/deprotonation reactions are fast, we have to conclude that the respective proton binding site disappears upon protonation and gets exposed again to the lumenal phase only after slow reconversion to the initial conformational state. For computation of the equilibrium model this objection is irrelevant; however, we have to admit that the equilibrium reactions in reality may be more complex.

The most significant differences were observed when activity-ΔpH relationships were compared in the presence of ADP and ADP plus arsenate (Fig. 2). At $\Delta pH < 3$, the ATPase activity is nearly zero in the presence of ADP alone. In contrast, substantial activity is found even at $\Delta pH = 2$ when arsenate is also included in the medium. Increase of the fraction of active enzymes in the presence of arsenate is matched by a corresponding decrease of the level of tightly bound

ADP. The results confirm the importance of arsenate/phosphate to counterbalance the effect of ADP as deactivator of the chloroplast ATPase [24]. As pointed out above, we think that regulation by nucleotides and phosphate occurs at the same sites which are responsible for catalysis. To understand the context, we may imagine that occupation of one site with ADP in the absence of arsenate/phosphate triggers closure of all three sites when ΔpH decreases below 3, thus creating an inactive enzyme molecule. On the other hand, binding of both, ADP and arsenate/phosphate, to one of the sites may keep the sites open, i.e., ready for catalysis, even at lower ΔpH values, and retard closure of the sites upon complete deenergization, respectively. Usually $\Delta\tilde{\mu}_{H^+}$-dependent ATPase activation and $\Delta\tilde{\mu}_{H^+}$-driven ATP formation are considered to be independent processes. Nevertheless, the two processes could be traced back to the same primary event, the opening of catalytic sites induced by protonation of groups accessible from the internal phase and deprotonations at the opposite pole. Energy-linked cyclic alteration of substrate affinities of the catalytic sites as proposed in Boyer's 'binding change mechanism' [59], means essentially the same. Measurements of the activation state as performed in this paper yield information about the condition of the enzyme to be ready for catalysis. Our results indicate that this is dependent on the absence or presence of ADP and arsenate/phosphate and suggest that at least two catalytic sites must be freely available, i.e., open and non-occupied (e.g., ^2E_a and $^3E_a \cdot ADP$). After initiation of the catalytic reaction by addition of the substrates, the third site might also be integrated in the catalytic cycle. If the site is covered with ADP ($^3E_a \cdot ADP$), this ligand may be expelled in pace with the first catalytic cycle, since it was shown that the initial rate of release of tightly bound ADP corresponds to the initial rate of ATP formation when ADP and phosphate is in the medium [32,61]. The formation of tightly bound nucleotides would be a compulsory consequence of deenergization of a catalytic unit containing a nucleotide molecule. Although this may be a repetitive step in every cycle [59], a tightly bound nucleotide can be experimentally detected only when the condition of the enzyme excludes continuation of the catalytic cycle.

It appears necessary to comment on the method of ΔpH measurement employed in this study. The confidence of the used calibration relies on the validity of Eqn. 1 which may not be doubted, and the correctness of the values ΔG_p^0 and n. Recently it was suggested that the H^+/ATP ratio may be higher than 3 [60]. With $n = 4$ (Rumberg, B., personal communication) the calibration scale would be altered significantly and hence the x-axes in Figs. 2–5 would be changed, too. Nevertheless, the data could be explained by the model (Scheme I); in this case the constants were $pK_1 = 7.0$, $pK_2 = 5.0$, $pK_3 = pK_4 = pK_6 = 5.3$, $pK_5 = 4.2$ and $K_7 = 2.10^{-6}$M, $K_8 = 3.3 \cdot 10^{-2}$ M, $K_9 = 5 \cdot 10^{-3}$ M.

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References

1 Junge, W. (1970) Eur. J. Biochem. 14, 582–592.
2 Gräber, P., Schlodder, E. and Witt, H.T. (1977) Biochim. Biophys. Acta 461, 426–440.
3 Harris, D.A. and Crofts, A.R. (1978) Biochim. Biophys. Acta 502, 87–102.
4 Schlodder, E. and Witt, H.T. (1981) Biochim. Biophys. Acta 635, 571–584.
5 McCarty, R.E. and Racker, E. (1968) J. Biol. Chem. 243, 129–137.
6 Petrack, B., Craston, A., Sheppy, F. and Farron, F. (1965) J. Biol. Chem. 240, 906–914.
7 Carmeli, C. and Lifshitz, Y. (1972) Biochim. Biophys. Acta 267, 86–95.
8 Bakker-Grunwald, T. and Van Dam, K. (1974) Biochim. Biophys. Acta 347, 290–298.
9 Mills, J.D., Mitchell, P. and Schürmann, P. (1981) FEBS Lett. 112, 173–77.
10 Ketcham, S.T., Davenport, J.W., Warncke, K. and McCarty, R.E. (1984) J. Biol. Chem. 259, 7286–7293.
11 Schumann, J., Richter, M.L. and McCarty, R.E. (1985) J. Biol. Chem. 260, 11817–11823.
12 Mills, J.D. and Mitchell, P. (1982) Biochim. Biophys. Acta 347, 290–298.
13 Mills, J.D. and Mitchell, P. (1984) Biochim. Biophys. Acta 764, 93–104.
14 Junesch, U. and Gräber, P. (1985) Biochim. Biophys. Acta 809, 429–434.
15 Junesch, U. and Gräber, P. (1987) Biochim. Biophys. Acta 893, 275–288.
16 Biaudet, P. and Haraux, F. (1987) Biochim. Biophys. Acta 893, 544–556.
17 Rumberg, B. and Becher, U. (1984) in H^+-ATPase (ATP Synthase): Structure, Function, Biogenesis (Papa, S. et al., eds.), pp. 421–430, Adriatic Editrice. Bari.
18 Schumann, J. and Strotmann, H. (1981) in Photosynthesis II. Electron Transport and Photophosphorylation (Akoyunoglou, G., ed.), pp. 881–892, Balaban International Science Services, Philadelphia, PA.
19 Dunham, K. and Selman, B.R. (1981) J. Biol. Chem. 256, 212–218.
20 Bar-Zvi, D. and Shavit, N. (1982) Biochim. Biophys. Acta 681, 451–458.
21 Czarnecki, J.J., Abbott, M.S. and Selman, B.R. (1982) Proc. Natl. Acad. Sci. USA 79, 7744–7748.
22 Abbott, M.S., Shavit, N., Selman-Reimer, S. and Selman, B.R. (1986) FEBS Lett. 209, 157–161.
23 Dunham, K. and Selman, B.R. (1981) J. Biol. Chem. 256, 10044–10049.
24 Strotmann, H., Niggemeyer, S. and Mansy, A.R. (1987) in Progress in Photosynthesis Research (Biggins, J., ed.), Vol. III, pp. 29–36, Martinus Nijhoff Publishers, Dordrecht.

25 Minkov, J.B. and Strotmann, H. (1989) Biochim. Biophys. Acta 973, 7–12.
26 Schumann, J. (1987) Biochim. Biophys. Acta 890, 326–334.
27 Harris, D.A. and Slater, E.C. (1975) Biochim. Biophys. Acta 387, 335–348.
28 Strotmann, H., Bickel, S. and Huchzermeyer, B. (1976) FEBS Lett. 61, 194–198.
29 Magnusson, R.P. and McCarty, R.E. (1976) J. Biol. Chem. 251, 7417–7422.
30 Strotmann, H. and Bickel-Sandkötter, S. (1977) Biochim. Biophys. Acta 460, 126–135.
31 Huchzermeyer, B. and Strotmann, H. (1977) Z. Naturforsch. 32c, 803–809.
32 Strotmann, H. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. II, pp. 477–484, Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht.
33 Hangarter, R.P., Grandoni, P. and Ort, D.R. (1987) J. Biol. Chem. 262, 13513–13519.
34 Schuldiner, S., Rottenberg, H. and Avron, M. (1972) Eur. J. Biochem. 25, 64–70.
35 Shigalowa, T., Lehmann, U., Krevet, M. and Strotmann, H. (1985) Biochim. Biophys. Acta 809, 57–65.
36 Strotmann, H., Bickel-Sandkötter, S. and Shoshan, V. (1979) FEBS Lett. 101, 316–320.
37 Fiolet, J.W.T., Bakker, E.P. and Van Dam, K. (1974) Biochim. Biophys. Acta 368, 432–445.
38 McCarty, R.E. (1976) in Encyclopedia of Plant Physiology, New Series, Transport in Plants III (Stocking, C.R. and Heber, U., eds.), Vol. 3, pp. 347–376, Springer, Berlin.
39 Davenport, J.W. and McCarty, R.E. (1986) Biochim. Biophys. Acta 851, 136–145.
40 Schröder, H., Muhle, H. and Rumberg, B. (1971) in: Proceedings of the 2nd International Congress of Photosynthesis Research (Forti, G., Avron, M. and Melandri, A., eds.), pp. 919–930, Dr. W. Junk Publishers, Dordrecht.
41 Junge, W., Rumberg, B. and Schröder, H. (1970) Eur. J. Biochem. 14, 575–581.
42 Rathenow, M. and Rumberg, B. (1980) Ber. Bunsenges. Phys. Chem. 84, 1059–1062.
43 Portis, A.R. and McCarty, R.E. (1981) J. Biol. Chem. 256, 8947–8954.
44 Davenport, J.W. and McCarty, R.E. (1981) J. Biol. Chem. 256, 8947–8954.
45 Strotmann, H. and Lohse, D. (1988) FEBS Lett. 229, 308–312.
46 Rosing, J. and Slater, E.C. (1972) Biochim. Biophys. Acta 267, 275–286.
47 Kraayenhof, R. (1969) Biochim. Biophys. Acta 180, 213–215.
48 Pick, U. and McCarty, R.E. (1980) Methods Enzymol. 69, 538–546.
49 Strotmann, H., Kleefeld, S. and Lohse, D. (1987) FEBS Lett. 221, 265–269.
50 Mitchell, P. (1981) in Mitochondria and Microsomes (Lee, C.P. et al., eds.), pp. 427–457, Addison-Wesley, Reading, MA.
51 Mills, J.D. and Mitchell, P. (1984) Biochim. Biophys. Acta 764, 93–104.
52 Biaudet, P., De Kouchkowsky, F. and Haraux, F. (1988) Biochim. Biophys. Acta 933, 487–500.
53 Zhou, J.M., Xue, Z., Du, Z., Melese, T. and Boyer, P.D. (1988) Biochemistry 27, 5129–5135.
54 Xue, Z., Zhou, J.M., Melese, T., Cross, R.L. and Boyer, P.D. (1987) Biochemistry 26, 3749–3753.
55 Bar-Zvi, D., Tiefert, M.A. and Shavit, N. (1983) FEBS Lett. 160, 233–238.
56 Bar-Zvi, D. and Shavit, N. (1984) Biochim. Biophys. Acta 765, 340–346.
57 Schumann, J. (1984) Biochim. Biophys. Acta 766, 334–342.
58 Komatsu-Takaki, M. (1986) J. Biol. Chem. 261, 1116–1119.
59 Boyer, P.D. and Kohlbrenner, W.E. (1981) in Energy Coupling in Photosynthesis (Seman, B.R. and Selman-Reimer, S., eds.), pp. 231–240, Elsevier/North-Holland, Amsterdam and New York.
60 Gräber, P., Junesch, U. and Thulke, G. (1987) in Progress in Photosynthesis Research (Biggins, J., ed.), Vol. III, pp. 177–184, Martinus Nijhoff Publishers, Dordrecht.
61 Lohse, D. and Strotmann, H. (1989) Biochim. Biophys. Acta 976, 94–101.