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Kinetics and energetics of redox regulation of ATP synthase from chloroplasts

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The rate of ATP hydrolysis catalyzed by the membrane-bound CF_0F_1 ATP synthase from chloroplasts served as a probe for the determination of the reduction grade of the enzyme treated with dithiothreitol (DTT) or thioredoxin. Rate constants for reduction were obtained. It turns out that reduction by thioredoxin is about a factor of 6,000 more effective than DTT reduction. The activation profiles with respect to ΔpH were obtained for reduced and oxidized ATPases. The activation curve of reduced enzyme turns out to have its half-maximum degree of activation at ΔpH = 1.65, which is considerably lower than reported hitherto. The corresponding value of the oxidized enzyme has been obtained from the rate of ATP hydrolysis in the case of incomplete reduced ATPases, taking into account the aforementioned rate constants, and comes to ΔpH = 3.35.

Chloroplast; ATP synthase; Latent ATPase; Thiol regulation; Thioredoxin; Enzyme activation

1. INTRODUCTION

The CF_0F_1 ATP synthase from chloroplasts is a latent ATPase; by procedures like heat activation [1], trypsin [2] or methanol treatment [3], or by chemical reduction with a suitable agent, the enzyme can be stimulated to perform transient ATP hydrolysis.

Such a reduction takes place when thiols like dithiothreitol (DTT) [4] or the ubiquitous redox protein thioredoxin [5,6] are used. The altering of the catalytic properties of the native ATP synthase to a reversible synthase/hydrolase is due to the reduction of a disulfite group in the γ subunit of CF_1 [7].

Investigations of ATP hydrolysis by the membrane-bound enzyme have so far mainly been carried out with DTT as the reductive agent. The reduction occurs sufficiently quickly and to completion only if the membrane is previously energized by $\Delta\mu(H^+)$ [8]. The kinetics of thiol reduction have not been investigated so far; this will be part of this paper.

Thiol modulation does not only allow ATP hydrolysis to occur but also diminishes the energy requirement of ATP synthesis [9]. Within a widely accepted model of the redox regulation of the chloroplastic ATPase [10,11] this is explained by the assumption that the reduced form of the enzyme requires a facilitated activation by $\Delta\mu(H^+)$ which is a prerequisite of all catalytic events [12]. It is assumed that this stimulation of ATP synthesis plays an important regulatory role in vivo [5] with thioredoxin as the modulating thiol.

The experimental procedure which allows the enzyme to perform ATP hydrolysis is therefore a multistep process (see Fig. 1): $\Delta\mu(H^+)$-dependent activation of the oxidized (native) enzyme, reduction by thiols in the presence of energizing conditions (light) and hydrolysis of ATP in the absence of the latter one.

In this paper we confirm the concept of redox regulation of ATP synthase by re-investigating the activation profiles of reduced and oxidized enzymes. The rate of uncoupled ATP hydrolysis under reduced conditions which occurs after activation with $\Delta\mu(H^+)$ reflects the activation degree of the reduced enzyme. Because of the inability of the oxidized enzyme to perform ATP hydrolysis the $\Delta\mu(H^+)$ dependence of ATP synthesis has so far been interpreted as the activation profile [13]. Here the procedure is put down to measurements of ATP hydrolysis of not completely reduced ATPases which can be interpreted in terms of the activation profile of the oxidized enzyme taking into account the kinetics of thiol-mediated reduction.

2. EXPERIMENTAL

Experiments were performed with suspensions of envelope-free chloroplasts (thylakoids) isolated from spinach. The suspension medium contained final concentrations of 50 mM KCl, 3 mM free $MgCl_2$, 1 mM tricine, 15 μM pyocyanin, 3 mM ATP, 1 mM phosphate, 50 μM ADP and chloroplasts equivalent to 10 μM chlorophyll. The intensity of the red actinic light was at least 200 W/m^2, the temperature was 20°C, the reaction volume of the stirred suspension was 6 ml and its pH was 8.0 ± 0.05. The rate of ATP hydrolysis was obtained from the rate of the coupled pH changes which were continuously monitored by means of a glass electrode. The variation of the light-induced

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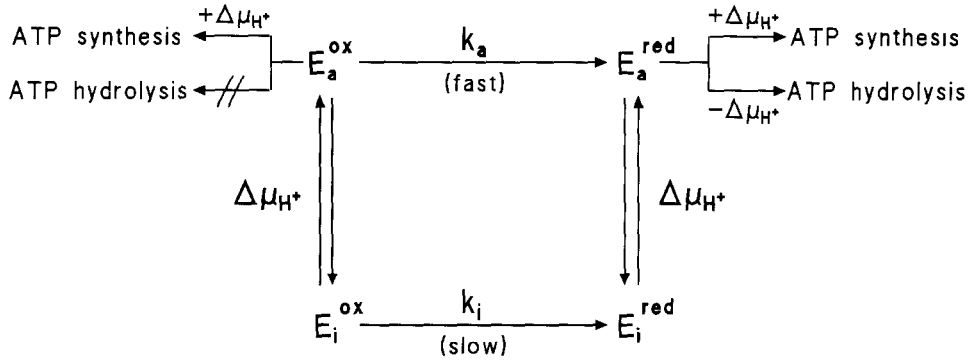

Fig 1. Reaction scheme of redox regulation of CF_0F_1 ATP synthase.

internal pH changes as well as the complete uncoupling of the thylakoid for stimulating ATP hydrolysis were performed by different amounts of uncouplers, gramicidin D or nigericin. Internal acidification was obtained from the fluorescence quenching of 2 μM *N*-(1-naphthyl)-ethylene-diamin as described elsewhere [14,15].

For determination of the reduction grade of the enzyme as a function of illumination time the rate of hydrolysis was assayed after injection of uncoupler nigericin (100 nM) or gramicidin D (1 μM) at the moment of switching off the actinic light. In the case of thioredoxin small amounts (0.1 mM) of DTT were present to keep the thioredoxin in a reduced state. *E coli* thioredoxin (CAS 52500-60-4), which corresponds to the m-type thioredoxin from spinach chloroplasts [16], was purchased from Calbiochem.

The determination of the activation degree η_{red} of the reduced ATPases was carried out as a two-step experiment: first the enzyme was completely reduced by illumination in the presence of 5 mM DTT over a period of 5 min, then, after a dark time of 2 min for deactivation, substrates were added and a second illumination period with controlled internal pH followed. Variation of internal pH during this second illumination period was achieved by different amounts of uncoupler which were added immediately after the first illumination period in order to accelerate the deactivation process thereafter and to make sure that sufficient complete deactivation occurred in any case. If low and medium ΔpH are employed, ATP hydrolysis started already during the second illumination period. It was allowed to continue until stationarity, indicating an equilibrium amount of active ATPases. Meanwhile released ADP (up to 50 μM) had no effect on the activation degree in the presence of 3 mM ATP (data not shown). After an activation period of 10–100 s, transient ATP hydrolysis was assayed as described already.

The determination of the reduction degree η_{max} of not completely reduced ATPases was carried out by illumination for 5 min in the presence of 5 mM DTT with variation of internal pH and subsequent assay of ATP hydrolysis.

3. RESULTS AND DISCUSSION

3.1. *Kinetics of thiol-mediated reduction*

Fig. 2 shows the relative rate of ATP hydrolysis in relation to the illumination time for the reducing agents DTT and thioredoxin, respectively. The data show an exponential characteristic, heavy lines are calculated according to Eqn. 1.

In correspondence to the reaction scheme (Fig. 1) the rate of ATP hydrolysis v_{Hyd} is proportional to the degree of reduction $[E_a^{red}]/[E_t]$ (where $[E_t]$ = total enzyme concentration) which can be calculated as a function of the illumination time t_{ill} as follows:

$$v_{rel} = \frac{v_{Hyd}}{v_{Hyd}^{max}} = \frac{E_a^{red}}{E_t} = \frac{1}{1+1/K^{red}} \cdot \{1 - \exp[-k_a \cdot [RED] \frac{1}{1+1/K^{ox}} \cdot t_{ill}]\} \quad (1)$$

with $K^{ox} = [E_a^{ox}]/[E_i^{ox}]$ and $K^{red} = [E_a^{red}]/[E_i^{red}]$ being the equilibrium constants for activation at maximum ΔpH conditions, k_a being the rate constant of reduction and [RED] the concentration of the reducing agent. The derivation of Eqn. 1 is based on the assumption of excess reducing agent (first order reaction). To achieve accordance to the experiment it is necessary to set k_a/

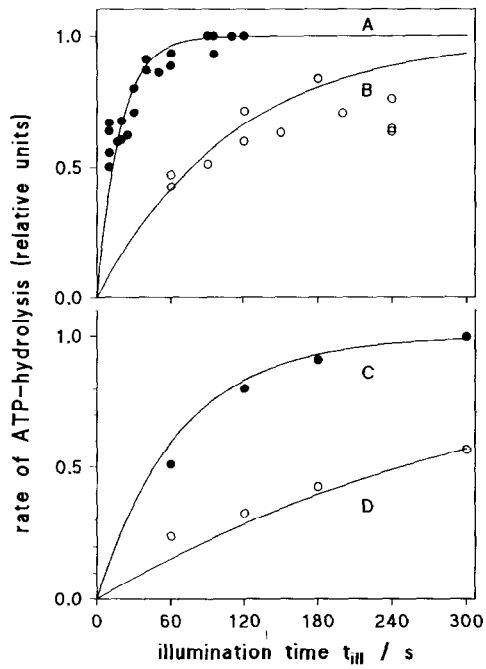

Fig 2. Relative rate of ATP hydrolysis in relation to illumination time. (A) 3 μM thioredoxin, 0.1 mM DTT; (B) 0.5 μM thioredoxin, 0.1 mM DTT; (C) 5 mM DTT; (D) 1 mM DTT. The lines were calculated with Eqn 1. The standard rate was 150 mM ATP (M Chl s)$^{-1}$.

$(1 + 1/K^{ox})$ to 3 l · mol^{-1} · s^{-1} in the case of DTT and to 18,000 l · mol^{-1} · s^{-1} in the case of thioredoxin. It turns out that the natural redox protein thioredoxin is about 6,000-fold more effective than DTT. In order to determine the actual rate constant k_a it is necessary to know more about the degree of activation of the oxidized enzyme (e.g. K^{ox}) under the conditions of maximal ΔpH. With the aid of an estimation for K^{ox} given in the next section, it is also possible to estimate values for k_a. These are given in Table I. Values for the rate constant k_i of reduction in the inactive (dark) state are also considered (data not shown). It turns out that the reduction without activation is about 300 times slower than under light conditions. This difference between light and dark reduction seems to reflect the fact that the reducible disulfite-group on the γ-subunit has to be exposed by a conformational change which accompanies $\Delta\mu(H^+)$-induced activation.

3.2. *Energetics of the $\Delta\mu(H^+)$-induced activation process*

Fig. 3 top shows the experimental data for the activation degree η_{red} in relation to ΔpH (the contribution of an electrical part $\Delta\psi$ to the protonmotive force $\Delta\mu(H^+) = 2.3 \cdot RT \cdot \Delta pH + F \cdot \Delta\psi$ is negligible for our experimental conditions of high salt concentration [17]). The activation profile with a sigmoid feature has its value for half-maximum activation at ΔpH = 1.65 which is significantly lower than reported hitherto [13,18,19]. For the experimental procedure used here it was of great importance that the activation time was long enough, especially for weak internal acidification. Insufficient activation would result in apparently higher activation barriers.

Fig. 3 bottom shows the activation degree η_{ox} in relation to ΔpH which has been calculated from the experimental data of the reduction degree η_{mix} in the case of incompletely reduced ATPases (Fig. 3 top). With $\eta_{mix} = [E_a^{red}]/[E_t]$, it follows from rearrangement of Eqn. 1:

$$\eta_{ox} = \frac{1}{1 + 1/K^{ox}} = -\frac{\ln[1 - (\eta_{mix}/\eta_{red})]}{k_a \cdot [RED] \cdot t_{ill}} \qquad (2)$$

η_{ox}, η_{red} and η_{mix} are functions of ΔpH. For calculation of η_{ox} it is necessary to know k_a, whereas only $k_a/(1 + 1/K^{ox})$ for maximum ΔpH has been determined. To estimate k_a one can discuss two cases:

(1) The oxidized ATP synthase under conditions of maximum ΔpH $\approx$ 3.7 is completely activated, i.e. $K^{ox} = \infty$ and therefore $k_a/(1 + 1/K^{ox}) \approx k_a$. The calculation of η_{ox} with Eqn. 2 then gives an activation curve with a half-maximum activation degree at ΔpH = 3.3. This is the lower limit for the position of the activation barrier and for the value of k_a.

(2) The oxidized ATP synthase under conditions of maximum ΔpH is *not* completely activated. When the equilibrium constant K^{ox} is set to 4 one gets an activation profile with a half-maximum degree of activation at ΔpH = 3.4. This is equal to the highest value reported for the half-maximum rate of ATP synthesis in the case of oxidized ATP synthase [13]. Because of the fact that the activation barrier can be lower but never higher than any energy requirement of ATP synthesis, this turns out to be the upper limit for the activation curve and the corresponding value of k_a.

In summary it appears that the activation profile of the oxidized enzyme has its half-maximum value at a ΔpH between 3.3 and 3.4. The interpretation of the energy requirement of ATP synthesis as the activation barrier of oxidized enzyme seems in essence to be correct.

Fig. 3 bottom shows the activation curve η_{ox} calculated for K^{ox} set to 4. The graph of η_{ox} calculated with

Table I

Rate constants for thiol-induced reduction of the CF_0F_1 ATP synthase

Thiol used	k_a (l(mol · s)$^{-1}$)	k_i (l(mol · s)$^{-1}$)
DTT	3.00–3.75	
Thioredoxin	$1.8 \cdot 10^4$–$2.2 \cdot 10^4$	62

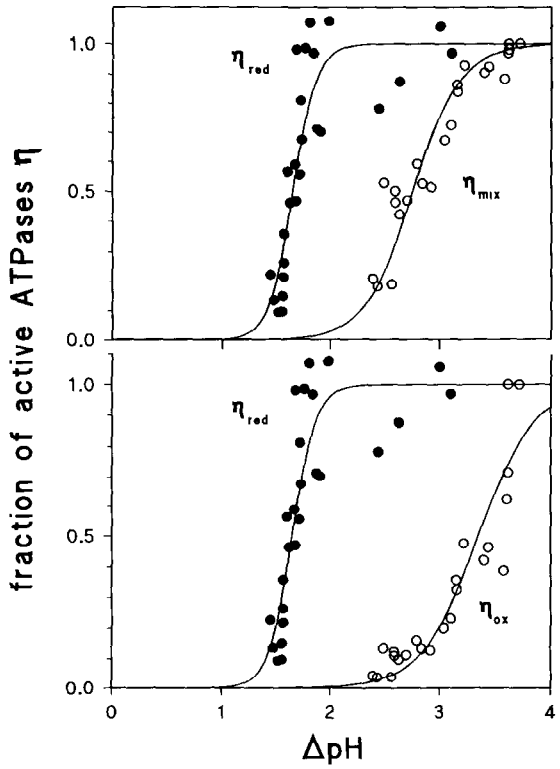

Fig. 3. Top: fraction of active ATPase in relation to ΔpH for the cases of completely reduced (η_{red}) and incompletely reduced (η_{mix}) enzyme. Bottom: fraction of active ATPase in relation to ΔpH in the case of oxidized enzyme (η_{ox}). Datapoints for η_{ox} are calculated from those of η_{mix} by Eqn. 2. Data for η_{red} are depicted for comparison.

the upper limit of $K^{ox} = \infty$ would be confusingly close to the depicted one. The corresponding values of k_a differ by a factor of 1.25 which also matches the precision of the experimental methods.

The experimental results in Fig. 3 have been fitted by Hill plots, the Hill coefficients with respect to the intrathylakoid H^+ concentrations being 4.0, 2.0 and 1.7 for η_{red}, η_{mix} and η_{ox}, respectively. The change of the Hill coefficient seems to indicate a corresponding change of the H^+ cooperativity. However, we do not believe that this is really the case. A specific reaction model which allows simulation of the degree of activation in dependence on ΔpH has been put forward by us previously [18]. The experimental results of η_{red} may be fitted easily if the number of protons involved is 4 [15]. Moreover, by only changing the rate constants, i.e. without changing the H^+ cooperativity, it is, in principle, possible to combine a shift of the half-maximum ΔpH to higher values to a concomitant shift of the Hill coefficient to lower values as is the case if η_{red} is switched to η_{ox}. Corresponding theoretical work is in progress.

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