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From uni-site to multi-site ATP synthesis in thylakoid membranes

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The membrane-bound H^+-ATPase from chloroplasts, CF_0F_1, was brought into the active, reduced state by illumination in the presence of thioredoxin and dithiothreitol. The endogenous nucleotides were removed by a washing procedure so that the active, reduced enzyme contained one tightly bound ATP per CF_0F_1. When [^{14}C]ADP was added in substoichiometric amounts during continuous illumination, ADP was bound to the enzyme, phosphorylated and released as [^{14}C]ATP, i.e., the tightly bound ATP was not involved in the catalytic turnover ('uni-site ATP-synthesis'). The rate constant for ADP binding was $k = (2.0 \pm 0.5) \cdot 10^6$ M^{-1} s^{-1}. The rate of ATP synthesis was measured as a function of the ADP concentration from 8 nM up to 1 mM in the presence of 2 mM phosphate during continuous illumination. A linear increase of the rate was observed up to 100 nM. Above this concentration a supralinear increase was found, indicating the occupation of a second ADP-binding site. A plateau was reached between 1.5 μM and 2.3 μM ADP with a rate of $v_{pl} = 3.7$ s^{-1}. The half-maximal rate from this plateau was observed at 780 nM. Above 2.3 μM ADP up to 1 mM ADP the data were described by Michaelis–Menten kinetics ($v_{max} = 80$ s^{-1}; apparent $K_M = 32$ μM.) These results indicated the participation of at least two different ADP binding sites in ATP synthesis catalyzed by the membrane-bound CF_0F_1.

Introduction

The H^+-ATPase from chloroplasts, CF_0F_1, catalyzes reversibly ATP-synthesis/hydrolysis coupled with a transmembrane proton transport. The enzyme has a hydrophilic F_1-part which contains six nucleotide binding sites and a hydrophobic F_0-part which is inserted into the membrane and is supposed to act as a proton channel. Three of the binding sites have catalytic properties and this raises the question whether the catalytic sites work independently or in a cooperative manner [1–3].

This question was investigated mainly by measuring ATP-hydrolysis catalyzed by the isolated F_1-parts [4–8] or by the membrane-bound F_0F_1 [9–12]. The question of cooperativity can also be investigated by measuring ATP-synthesis. However, only few reports are found in literature. For mitochondrial F_0F_1 two K_M values (6–10 μM ADP and 50–100 μM ADP) were reported. The higher value depends on the way how the membrane was energized (NADH or succinate) [13,14]. For chloroplast F_0F_1 two K_M values (0.6 μM and 20 μM) were reported by Stroop and Boyer [15], whereas other groups reported only one K_M between 10 μM and 70 μM, depending on light intensity and other parameters [16–23].

In an earlier work we have shown ATP-synthesis under uni-site conditions with thylakoid membranes [24]. These measurements of uni-site ATP-synthesis were carried out with membrane-bound CF_0F_1 with two bound ATP per CF_0F_1. In order to investigate the influence of the occupation of non-catalytic nucleotide binding sites on uni-site catalysis we reinvestigated uni-site ATP-synthesis with one bound ATP per CF_0F_1 using 'washed' thylakoid membranes.

Furthermore, we tried to detect the transition from uni-site to multi-site ATP synthesis by measuring the rate of ATP synthesis as a function of ADP concentration from 8 nM to 1 mM in the presence of 2 mM phosphate.

Materials and Methods

Thylakoid membranes were isolated from spinach and stored under liquid nitrogen as described earlier [25]. The ratio of chlorophyll per CF_0F_1 was determined by immune electrophoresis (Chl/CF_0F_1 = 730 100) [26,27]. The H^+-ATPase was activated by continuous illumination and reduced in the presence of dithio-

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Abbreviations: CF_0F_1, H^+-translocating ATPase from chloroplasts; CF_1, hydrophilic part of the H^+-ATPase from chloroplasts; P_i, orthophosphate.

threitol and thioredoxin. The endogenous nucleotide content was reduced by a washing procedure described previously [29]. These 'washed thylakoid membranes' contained one bound ATP per CF_0F_1. They were stored on ice in the dark for up to 4 h. In order to reactivate the inactive reduced enzyme 75 μl thylakoids in buffer 1 (60–80 μM chlorophyll, 5.9 mM Tricine-Na (pH 8.05), 0.6 mM EDTA, 2.95 mM $MgCl_2$, freshly added 3.5 μM thioredoxin, 1.2 mM dithiothreitol and 23.5 μM pyocyanin) was added to 250 μl buffer 2 (100 mM Tricine, 60 mM KOH, 20 mM succinate, 2 mM $MgCl_2$, 2 mM NaH_2PO_4, titrated with NaOH to pH 8.05 and freshly added 40 μM pyocyanin) and illuminated with saturating white light. After 15 s 250 μl buffer 3 (16 nM–2 mM ADP (monopotassium salt, Boehringer), 100 mM Tricine, 60 mM KOH, 2 mM $MgCl_2$, 2 mM NaH_2PO_4 titrated with NaOH to pH 8.3) was added. This is time $t = 0$ for all the following experiments. The resulting mixture is abbreviated in the following as 'reaction medium'. This reaction medium is similar to that used in earlier work on ATP hydrolysis [29].

The following measurements were carried out:

(1) Free nucleotides: During continuous illumination, 50 μl samples were taken at different reaction times t (0–50 s) and free ATP was measured with luciferin/luciferase [28]. In a second sample the sum of free (ATP + ADP) was determined with luciferin/luciferase and pyruvate kinase/phosphoenolpyruvate [28]. Free ADP was calculated from the difference of both measurements. The endogenous concentrations of ADP and ATP were measured after 15 s preillumination without addition of ADP.

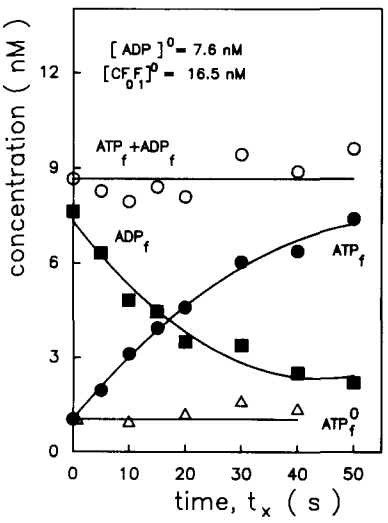


Fig. 1. Uni-site ATP synthesis catalyzed by illuminated washed thylakoids membranes. At zero reaction time 7.6 nM [^{14}C]ADP is added, aliquots of the suspension are filtered at different reaction times and the filtrate is analyzed. Free ATP (full circles) and the sum of free (ADP + ATP) (open circles) were measured with luciferin/luciferase. The concentration of free ADP is calculated from the difference between these two values (full squares). In the control no ADP was added. In this case only free ATP is observed (open triangles).

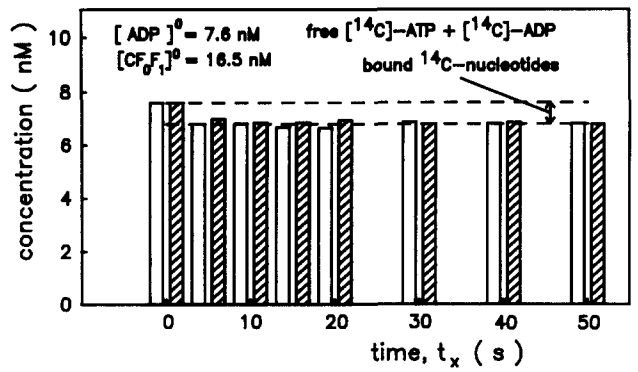


Fig. 2. Free radioactive nucleotides ([^{14}C]ADP+[^{14}C]ATP) during uni-site ATP synthesis. At zero reaction time, 7.6 nM [^{14}C] ADP is added, aliquots of the suspension are filtered at different times and analyzed for total radioactivity. The open and the hatched columns show the concentration of free [^{14}C]nucleotides in two different experiments. The difference between the concentration at $t = 0$ and the following reaction times represent the bound [^{14}C]nucleotides.

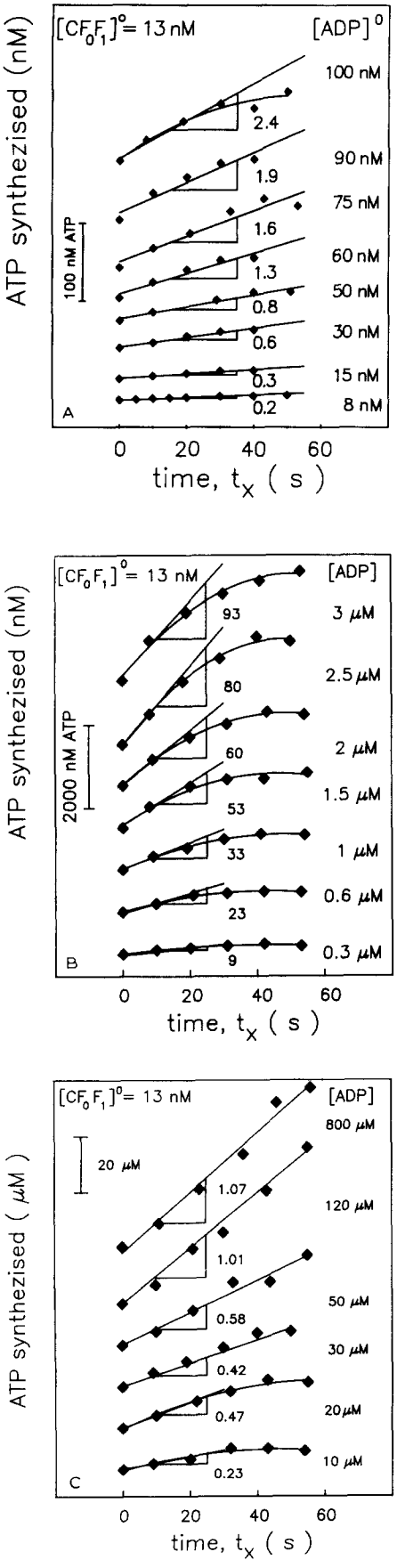
(2) Radioactive nucleotides: Buffer 3 with 16 nM [U-^{14}C]ADP (100 Bq, specific activity 20 GBq/mol, Amersham Buchler) was added after 15 s preillumination. At different reaction times t (0–50 s) the free and enzyme-bound nucleotides were separated by filtration under continuous illumination (filter-tip-technique, see Ref. 24). The filtrate was divided into three parts. Aliquots of 50 μl were used to determine free ATP and free ADP with the luciferase assay as described above. The third aliquot of 50 μl was mixed with 4 ml scintillator (Hionic Fluor (Canberra-Packard)), and the free radioactive nucleotides (ATP + ADP) were measured. The initial (total) ^{14}C activity was obtained by mixing buffer 1 containing no thylakoids with buffer 2. All further steps were performed as described above. The time-courses of endogenous free nucleotide concentrations were measured by mixing buffer 3 without ADP with the reactivated enzyme.

(3) Free and bound nucleotides: After addition of buffer 3 (200 nM–2 mM ADP) every 10 s an aliquot of 50 μl ($t = 0$–50 s) was taken and denatured with the same volume of 4% trichloroacetic acid. The amount of ATP was determined with the luciferase assay. In this case, the sum of free and enzyme-bound ATP was obtained. To obtain ATP at $t = 0$ the thylakoid suspension was first denatured and then buffer 3 was added.

For each set of experiments the amount of active enzymes was determined by measuring the rate of ATP synthesis at 300 μM ADP.

Results

In order to characterize the washed thylakoid membranes, the rate of ATP synthesis was measured in the presence of 300 μM ADP. The reaction conditions



(electron acceptor, light intensity etc.) were the same as for uni-site ATP synthesis. For different preparations it resulted (80 ± 20) M ATP/(M $CF_0F_1 \cdot s$) in accordance with earlier results with thylakoids [24].

In order to measure uni-site ATP synthesis, the preilluminated thylakoids were mixed at reaction time $t = 0$ s with 8 nM [^{14}C]ADP. The thylakoids were filtered at different times t (10–50 s) and the concentrations of free ATP and free ADP and the free ^{14}C activity were measured in the filtrate. In the control experiment, the same steps were performed except that no ADP was added.

Fig. 1 shows the result of these experiments. The concentrations of free nucleotides are plotted as a function of the reaction time. The free ADP concentration decreases exponentially.

The initial rate of ADP disappearance from the medium is 200 pM/s (see Fig. 1). ADP binding is a second order process and the rate constant can be calculated using the concentrations at reaction time $t = 0$, $[CF_0F_1]^0 = 16.5$ nM and $[ADP]^0 = 7.6$ nM. The data shown in Fig. 1 give a rate constant of $1.6 \cdot 10^6$ M^{-1} s^{-1}, the average of four different measurements gives $k = (2.0 \pm 0.6) \cdot 10^6$ M^{-1} s^{-1}.

Free ATP increases with reaction time, whereas the sum of ATP and ADP remains constant. From the initial slope of the ATP_f time-course, a rate of uni-site ATP synthesis of 200 pM/s or a turnover number of 0.012 s^{-1} was determined. This means that ATP is released from the enzyme with the same rate as ADP is bound. When no ADP is added to the thylakoid membranes free ATP remains constant during the reaction time (open triangles) and no ADP is detected. This indicates that all endogenous ADP is phosphorylated to ATP within 15 s preillumination. In washed thylakoid membranes, the initial state for the reaction is as follows [29]: CF_0F_1 contains one bound ATP per CF_0F_1 and no bound ADP; there is no endogenous free ADP detectable and the free ATP concentration is 1 nM (0.06 ATP per CF_0F_1).

Since the enzyme contains still one tightly bound ATP per CF_0F_1 it may be asked whether the free ATP was really newly synthesized or whether ADP-binding to one site leads to ATP release from the tight ATP site according to the binding change mechanism (see review, Ref. 3). In order to investigate this question

Fig. 3. ATP synthesis as function of reaction time at different initial ADP concentrations. The curves are shifted arbitrarily along the ordinate for clearer representation. (A) ADP concentration up to 100 nM. The numbers at the slope give the rates in nM s^{-1}. (B) ADP concentration up to 3 μM. The numbers at the slope give the rates in nM s^{-1}. (C) ADP concentration up to 800 μM. The numbers at the slope give the rates in μM s^{-1}.

[^{14}C]ADP was added and free ^{14}C-labelled nucleotides were measured as a function of the reaction time. If [^{14}C]ADP is bound to one site and the unlabelled ATP is released from a second site the total free radioactivity in the filtrate must decrease to zero with the same kinetics as found for ADP binding. Fig. 2 shows the result. The sum of free [^{14}C]ADP and free [^{14}C]ATP remains constant. This result indicates that [^{14}C]ADP binds to one site, it is phosphorylated and [^{14}C]ATP is released from the same catalytic site. This indicates that the tightly bound ATP is not involved in the catalytic turnover.

Initially, only a small decrease in the concentration of the free radioactive nucleotides was found (0.8 nM), reflecting the enzyme-bound [^{14}C]ADP and [^{14}C]ATP. The different columns represent two different experiments. If we assume that all the bound radioactivity is [^{14}C]ATP, we can calculate the lower limit of the rate constant for ATP release under energized conditions. With the initial rate from Fig. 1 (200 pM s^{-1}) and ATP_{bound} = 0.8 nM (Fig. 2), a rate constant > 0.25 s^{-1} was obtained.

The dependence of the rate of ATP synthesis on the ADP concentration was measured as described in Materials and Methods. At low substrate concentrations (below 90 nM) the free ATP was measured as function of the reaction time. Fig. 3A shows the result. At higher substrate concentrations the thylakoids were denatured and the ATP (free and bound) was measured as function of the reaction time. These data are shown in Fig. 3A,B,C. At reaction time $t = 0$ (control) the enzyme was first denatured before the addition of

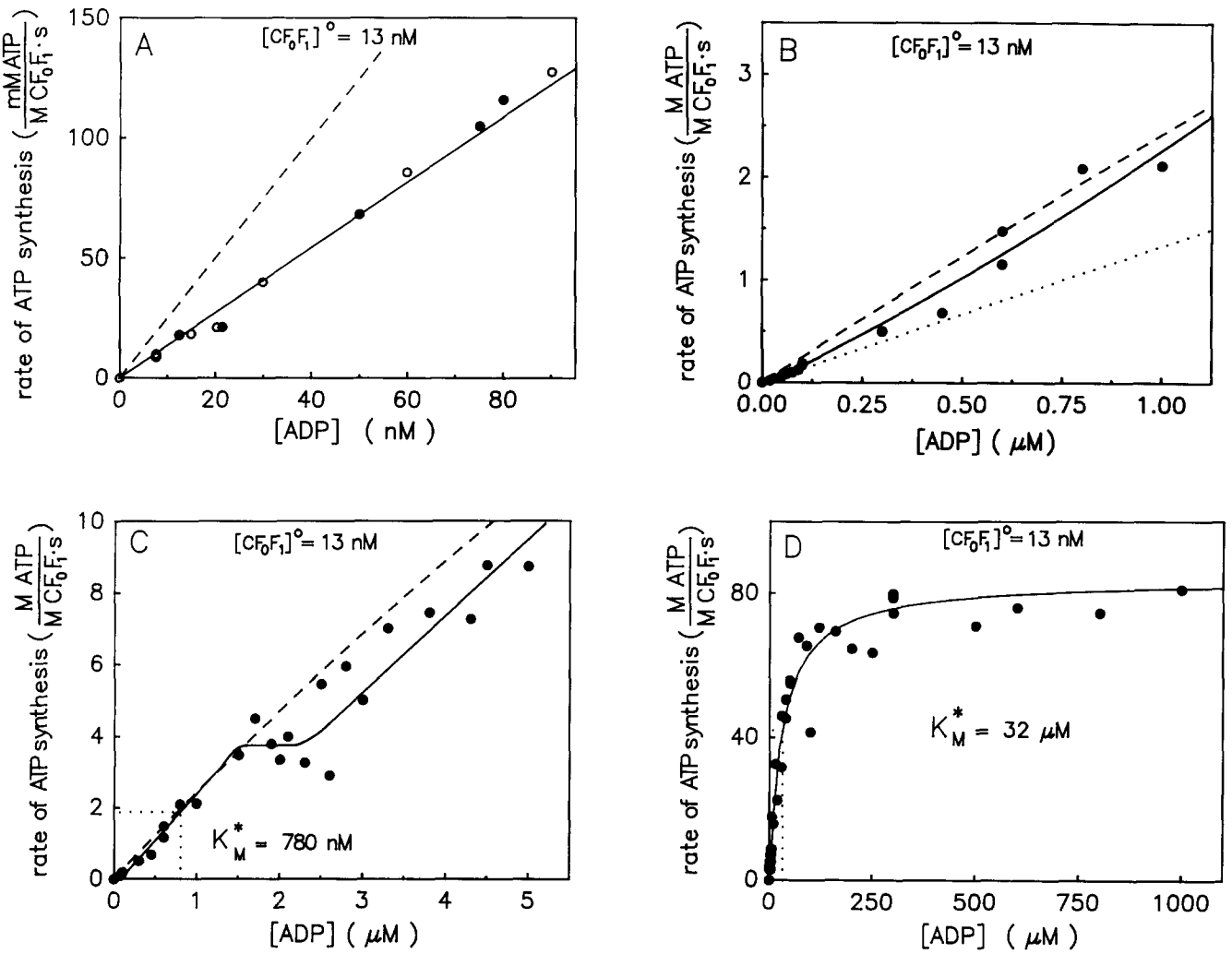


Fig. 4. The rate of ATP synthesis as a function of the ADP concentration. Data from Fig. 3A–C and additional measurements. The full circles indicate data where ATP was measured after denaturing the enzyme, i.e., the sum of bound and free ATP is determined. The open circles represent measurements where free ATP is measured. The dashed line is the extrapolated Michaelis–Menten kinetics from D. (A) ADP concentration up to 90 nM. (B) ADP concentration up to 1 μM. The dotted line is the extrapolated solid line from A to higher concentration. (C) ADP concentration up to 5 μM. (D) ADP concentration up to 1 mM. The solid line is calculated from Michaelis–Menten kinetics (v_{max} = 80 s^{-1}, K_M^* = 32 μM).

ADP. The background ATP level at low ADP concentrations results mainly from the tightly bound ATP which is released after denaturation.

The curves in Fig. 3A–C are arbitrarily shifted along the ordinate for a clearer presentation. A linear increase of ATP synthesized with the reaction time up 50 s is observed in Fig. 3A and C. In the micromolar range (Fig. 3B) a linear increase is observed only during the first 10–20 s. It can be calculated that the flattening of the curves arise from the consumption of ADP. The slopes of these curves (Fig. 3B initial slopes) are the steady-state rates of ATP synthesis for the initial conditions. These rates of ATP synthesis are plotted as function of the ADP concentration in Fig. 4A–D. The rate of ATP synthesis increases linearly with the ADP concentration up to 100 nM (Fig. 4A). In addition to the rates measured by the increase of free ATP (open circles) measurements are shown where the enzyme was denatured, i.e., free and bound ATP were measured (full circles). Both methods give the same rates, although the background ATP is different in each case.

Above approx. 200 nM the rate increases supralinearly and this indicates the participation of a second ADP binding site (Fig. 4B). In order to demonstrate this clearly, the solid curve from Fig. 4A is extrapolated to higher concentrations (dotted line in Fig. 4B). In the range between 0.3 μM and 1 μM ADP the curve has a nearly constant slope which is almost doubled as compared to that in the low concentration range. A plateau is reached between 1.5 μM and 2.3 μM ADP (Fig. 4C). This might indicate that two ADP-binding sites are completely occupied. The rate at the plateau is $v_{pl} = 3.7$ s^{-1} and the half-maximal rate is observed at 780 nM.

At higher ADP concentrations again a linear increase is found (Fig. 4C) and finally in the millimolar range, saturation is observed (Fig. 4D). The data in the millimolar range can be described by Michaelis–Menten kinetics. The Michaelis–Menten function is plotted as a solid line in Fig. 4D with $v_{max} = 80$ s^{-1} and an apparent $K_M^* = 32$ μM as parameters. The latter was obtained from the half-maximal velocity. This Michaelis–Menten kinetics is extrapolated to lower concentrations and it is shown as a dashed line in Fig. 4A, B and C.

All these measurements were carried out with three different thylakoid preparations which differ in v_{max}. The plot v/v_{max} vs. the ADP concentration gives the same result as the non-standardized one.

Discussion

Uni-site catalysis and non-catalytic ATP binding sites

The data from Figs. 1 and 2 demonstrate that under uni-site conditions (7.6 nM ADP, 16.5 nM CF_0F_1) ADP is bound to the catalytic site, it is phosphorylated to ATP and this ATP is released into the medium. The enzyme contains one tightly bound ATP. The nucleotide balance for total free nucleotides (Fig. 1) and radioactive free nucleotides (Fig. 2) indicates that the tightly bound ATP (1 ATP/CF_0F_1) is not involved in the catalytic turnover. At the catalytic site only 0.8 nM enzyme bound nucleotides of exogenous source are observed corresponding to about 0.05 (ATP + ADP) per CF_0F_1. Thus, only 5% of one catalytic site is occupied.

Uni-site ATP synthesis was observed earlier with thylakoids containing 2 tightly bound ATP per CF_0F_1 [24]. Also in that case the two tightly bound ATP's are not involved in the catalytic turnover. The rate constant for ADP-binding was $(2.3 \cdot 0.7) \cdot 10^6$ M^{-1} s^{-1} and that for ATP release was > 0.36 s^{-1}. Within the error limits they are the same as observed in this work and we conclude that occupancy of either one or two non-catalytic site(s) with ATP does not influence uni-site ATP synthesis, i.e., catalytic turnover occurs without participation of tightly bound ATP.

This was demonstrated earlier by Leckband and Hammes [30] using a different approach: isolated CF_1 was loaded with [γ-^{32}P]ATP and reconstituted to depleted thylakoid membranes. The [γ-^{32}P]ATP was not released during the catalytic turnover.

For isolated F_1 from *E. coli* it was reported that the rate of uni-site ATP hydrolysis changes by a factor of 3 when 2 ADP instead of 5 ADP per F_1 are bound on the enzyme [31].

The rate of ADP-binding is diffusion controlled, i.e., the rate constant for ADP-release is small compared to that of the forward direction (ATP release). Therefore, the K_M for ATP synthesis is approximately k(ATP release)/k(ADP-binding). The lower limit for k(ATP release) is $k > 0.25$ s^{-1}. Recently, this rate constant was measured and a value of k(ATP release) = 1 s^{-1} was observed [41]. Using the rate constant for ADP binding $k = 1.6 \cdot 10^6 M^{-1}$ s^{-1} from Fig. 1 the K_M value for uni-site ATP synthesis can be estimated as $K_M =$ 625 nM.

ATP synthesis as function of the ADP concentration

The rate of ATP synthesis can be described by Michaelis–Menten kinetics in the micromolar range with a $K_M^* = 32$ μM and $v_{max} = 80$ s^{-1} (see solid line in Fig. 4D). This is in accordance with earlier results where K_M values between 10–70 μM have been reported [15–23].

Below an ADP concentration of 100 nM, a linear increase of the rate with increasing substrate concentration is observed. In this range the rate is of second order, i.e., $v = k[CF_0F_1]$ [ADP]. If the free enzyme concentration is approximately the same as the total

enzyme concentration, we can estimate the rate constant from the slope in Fig. 4A and B ($[CF_0F_1] = 13$ nM). This was found to be $k = 1.4 \cdot 10^6\ M^{-1}\ s^{-1}$ in the range below 100 nM and $k = 2.6 \cdot 10^6\ M^{-1}\ s^{-1}$ in the range between 0.5 and 1 μM. The supralinear increase above 100 nM requires the presence of at least two ADP-binding sites. A plateau is reached between 1.5 and 2.3 μM. From the rate at the plateau ($v_{pl} = 3.7\ s^{-1}$) and the data in Fig. 4C the substrate concentration for the half-maximal rate is obtained (780 nM) and we take this as the apparent K_M value for these two sites. It is of course clear that the sigmoidal dependence of the rate up to 2.3 μM is not a Michaelis–Menten kinetics. However, for comparison with literature data we characterize both sites by the substrate concentration where the half-maximal rate is observed. Additionally, there is a surprising accordance between the K_M value obtained from the data in Fig. 4C and that estimated from the rate constants under uni-site conditions above. Stroop and Boyer [15] measured ATP synthesis in the range from 100 nM up to 1 mM. They obtained two apparent K_M values (0.6 μM and 32 μM). This is in agreement with the K_M values reported here; however, our data indicate that two sites are already involved at 780 nM.

Measurements of K_M values at constant light intensity (as presented in this work) contain a systematic error as discussed earlier [32,33]. When the ADP concentration is changed the proton efflux changes (at almost constant proton influx) and, thereby, the steady state ΔpH changes [34,35]. In recent measurements of the K_M (in the concentration range above 1 μM ADP) the variation of ΔpH was eliminated by different techniques [36–38,20–23].

At concentrations below 1 μM we have to consider an additional effect. The proton efflux occurs via different pathways: a basal flux via unspecified sites through the membrane, a phosphorylation coupled flux via the H^+-ATPase and a regulated flux via the H^+-ATPase which is not coupled with phosphorylation. The regulated proton flux through the H^+-ATPase can be blocked by binding of ATP ($K_D = 6$ μM) or ADP ($K_D = 1$ μM) [39,40]. The effect is observed under phosphorylating and non-phosphorylating conditions [40]. This implies that with increasing ADP concentration from 0 to about 1 μM the ΔpH increases. When the ADP concentration is further increased above 1 μM under phosphorylating conditions, the increasing phosphorylation coupled proton leads to a decrease in the ΔpH.

The Michaelis–Menten parameters ($K_M^* = 32$ μM, $v_{max} = 80\ s^{-1}$) are determined from the rates between 5 μM and 1 mM ADP (Fig. 4D). When this function is extrapolated to lower ADP concentrations (see dashed line in Fig. 4A,B,C) significant deviations from Michaelis–Menten kinetics can be observed.

The data presented in this work show that more than one site is involved in ATP synthesis. However, according to the discussion above, this result can be explained by different models.

(1) One catalytic–one regulatory site

There is only one catalytic site for proton transport coupled ATP synthesis operating from the nanomolar to millimolar range. In the nanomolar range additionally a regulatory site ($K_D = 780$ nM) is occupied. This leads to a decrease of the regulated proton flux through the enzyme with a corresponding increase of ΔpH and consequently the rate catalyzed by the catalytic site increases. Therefore, a nonlinear increase of the rate with ADP concentration is observed. The plateau between 1.5–2.3 μM indicates the complete occupancy of the regulatory site, and a further increase of ADP lowers the ΔpH due to the increasing phosphorylation coupled proton flux.

(2) Two catalytic–one regulatory site

In the nanomolar range one catalytic site ($K_M = 625$ nM, $V_{max} = 1\ s^{-1}$ estimated from the uni-site rate constants) and one regulatory site ($K_D = 1$ μM [40]) are operating. In the plateau region both are occupied and when they are occupied a second catalytic site ($K_M^* = 32$ μM; $V_{max} = 80\ s^{-1}$) becomes involved.

(3) Three catalytic sites

In the nanomolar range two catalytic sites are operating ($K_M^* = 780$ nM). A third catalytic site ($K_M^* = 32$ μM) is working only when the first two sites are occupied. In this case the rate at the plateau represents two site catalysis ($v_{pl} = 3.7\ s^{-1}$), whereas $v_{max} = 80\ s^{-1}$ represents three site catalysis.

On the basis of the experiments reported here we cannot distinguish between these possibilities. We prefer the 'two catalytic–one regulatory site' model since this one is able to describe all relevant literature data.

The first convincing evidence for a cooperativity between catalytic sites came from measurements of uni- and bi-site hydrolysis of 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP [42,43] and of ATP [4] by MF_1 where uni-site hydrolysis is accelerated up to 10^6-fold when additional sites are occupied. Similar experiments cannot be carried out with CF_1. For CF_0F_1 uni-site ATP hydrolysis is accelerated only by a factor 10 [9,44]. Even less convincing is the evidence for cooperativity between catalytic sites when proton-transport-coupled ATP synthesis is measured [13–15]. The evidence was based mainly on deviations from Michaelis–Menten kinetics and there might be several reasons for such deviations in complex enzyme kinetics. As discussed above the data presented in this work do not require two cooperating catalytic sites: they require the existence of at least two ADP binding sites, one of them

with catalytic properties; however, the second one might have regulatory (regulation of proton translocation through CF_0F_1 [39,40]) or catalytic properties. The existence of a second catalytic site with slow turnover and no cooperativity with the high turnover site has been shown in chloroplasts [17,45,46].

Finally, three remarks should be added. (1) The effect of the regulatory site on the rate cannot be measured when the pH is kept constant (e.g., in a pH-jump experiment at different ADP concentrations. (2) The v_{max} reported here is limited by the magnitude of pH obtained with light which is lower than that used in pH-jump experiments. The maximal turnover of the enzyme is 400–600 s^{-1} [36]. (3) Below 100 nM ADP there is no evidence for the participation of more than one catalytic site, i.e., in this range uni-site ATP synthesis can be measured.

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