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Heterogeneity of ATP-hydrolyzing sites on reconstituted CF_0F_1

Petra Fromme and Peter Gräber+

Max Volmer Institut für Biophysikalische und Physikalische Chemie, Technische Universität Berlin, Str.d. 17. Juni 135 D-1000 Berlin 12, Germany and *+Biologisches Institut, Universität Stuttgart, Pfaffenwaldring 57, 7000 Stuttgart 60, FRG*

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The proton translocating ATP-synthase from chloroplasts, CF_0F_1, was isolated and reconstituted into asolectin liposomes. [γ-^{32}P]ATP hydrolysis was measured under uni-site conditions. When 1 mM unlabeled ATP was added so that all ATP-binding sites were occupied, [γ-^{32}P]ATP bound to the first site, was hydrolyzed with a rate of 0.5 ATP/(CF_0F_1 s). In a second experiment, first cold ATP was hydrolyzed under uni-site conditions and then 1 mM [γ-^{32}P]ATP was added. This allows under otherwise identical conditions the measurement of the rate of ATP hydrolysis catalyzed by the second (and possibly third) site. It resulted in a rate of 80 ATP/(CF_0F_1 s). It is concluded that the catalytic nucleotide binding sites are heterogeneous: there exists one nucleotide binding site which hydrolyzes ATP with a maximal turnover of 0.5/s and another one (or two) which hydrolyze ATP with a turnover of 80/s. The latter one is the catalytic site for maximal turnover.

Chloroplast; ATPase, H^+-; CF_0F_1; Reconstitution; Nucleotide binding site; Uni-site catalysis

1. INTRODUCTION

Membrane-bound ATP-synthases of the F_0F_1-type catalyze ATP synthesis/hydrolysis coupled with a transmembrane proton transport. These ATP-synthases have a hydrophilic part, F_1, which contains the nucleotide binding sites and a hydrophobic membrane-integrated part F_0, which is inserted into the membrane and is supposed to act as a proton channel. The F_1-part has six nucleotide binding sites. Three of them are supposed to have catalytic properties, i.e. they can hydrolyze ATP. The function of the other nucleotide binding sites is still unknown [1–3].

The presence of three catalytic sites raises the question whether they operate independently or in a cooperative manner. It has been suggested that there exists a 'binding change' mechanism, i.e. the release of products from one site occurs only after binding of substrate to a second site [4,5]. Since there are three β-subunits (with a corresponding number of catalytic sites), the binding change mechanism was consequently extended to include three different sites [6]. Independent evidence for a binding change mechanism came from the result that ATP, bound to one site of the mitochondrial F_1, is hydrolyzed extremely slowly, however, binding of additional ATP to a second site accelerates ATP hydrolysis on the first site by orders of magnitude [7,8].

In a previous work we have measured ATP hydrolysis catalyzed by the reconstituted ATP-synthase from chloroplasts, CF_0F_1, under conditions where only one nucleotide binding site is occupied ('single site' conditions) [9]. Under these conditions the enzyme is able to make a complete turnover, with ATP binding and release of ADP and P_i, but the rate constants are too slow to explain the maximal turnover of the enzyme. In this work we investigate the question as to what happens on the first site when the other sites become occupied.

2. MATERIALS AND METHODS

2.1 *Isolation and reconstitution of CF_0F_1*

CF_0F_1 was isolated, purified and reconstituted as described earlier [10–12]. The proteoliposomes finally contained approximately 1 μM CF_0F_1 and 30 g/l asolectin, 10 mM Na-tricine pH 8.0, 0.2 mM EDTA, 2.5 mM $MgCl_2$ and 2.5 mM dithiothreitol.

2.2. *Reduction and activaton of CF_0F_1 in proteoliposomes*

The proteoliposomes were incubated for 1.5 h with 50 mM dithiothreitol (DTT) at pH 8.0 at room temperature. After the incubation, the proteoliposomes can be stored on ice up to 4 h without any change in activity.

The reduced proteoliposomes (usually 10 or 20 μl) were incubated for 15 s with the 5-fold volume of the acidic solution (buffer 1) containing 30 mM Na-succinate pH 4.9, 5 mM NaH_2PO_4, 2 mM $MgCl_2$, 0.5 mM KCl and 1 μM valinomycin (freshly added). The pH during incubation was 5.0. Then, the same volume of an alkaline solution (buffer 2) containing 200 mM Na-tricine pH 8.7, 120 mM KCl, 5 mM NaH_2PO_4, 2 mM $MgCl_2$ was added. The final pH was 8.3. Thereby, a transmembrane pH-difference Δ pH, and an electric potential difference $\Delta\Psi$ were generated. During activation of CF_0F_1

Correspondence address: P. Fromme, Max Volmer Institut für Biophysikalische und Physikalische Chemie, Technische Universität Berlin, Str.d. 17. Juni 135 D-1000 Berlin 12, Germany

Abbreviation: CF_0F_1, H^+-translocating ATPase (ATP-synthase) from chloroplasts

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one nucleotide/enzyme is released [13]. This has been used for the determination of the number of active enzymes as described earlier [9,13]. It was found that about 25% of the total CF_0F_1 can be activated.

2.3. *Kinetic measurements*

ATP hydrolysis was measured after addition of [γ-^{32}P]ATP by $^{32}P_i$ formation. The separation of $^{32}P_i$ and [γ-^{32}P]ATP was performed by complexing the P_i with ammonium molybdate and extracting the complex with isobutanol/toluene as described earlier [9].

3. RESULTS AND DISCUSSION

For all experiments, the enzyme was brought into the reduced, active state as described before. Fifteen seconds after activation by $\Delta pH/\Delta\Psi$, the reaction was started ($t_x = 0$) by addition of a solution containing ATP (either radioactive or unlabeled) and NH_4Cl in the same buffer as the proteoliposomes after activation, i.e. buffer 1 + buffer 2. The resulting mixture is called the reaction medium and finally contains 10 mM NH_4Cl for uncoupling. The initial nucleotide concentrations are the sum of the nucleotides released by activation and added nucleotides. The resulting ATP concentration is determined by luciferin/luciferase for every set of experiments as described elsewhere [9]. The initial concentrations are 33 nM ATP and 27 nM active enzyme (total enzyme concentration 93 nM). The rate constant for ATP binding to the inactive enzyme is only

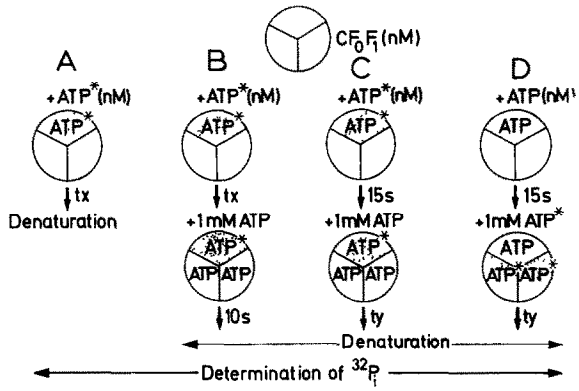

Fig.1. Scheme of the experiments for determination of the rate of ATP hydrolysis catalyzed by different nucleotide binding sites of the enzyme. The circle symbolizes the active, reduced CF_0F_1; each third of the circle represents one α/β pair with one of the three catalytic nucleotide binding sites. The dotted fields show the nucleotide binding sites which are investigated in the corresponding set of experiments. ATP* indicates [γ-^{32}P]ATP (for further details see text).

2×10^3/M per s in comparison to 1×10^6/M per s for the active enzyme. Therefore, ATP binding to the inactive enzymes can be neglected [9].

From this reaction medium samples were taken at different times t_x and then treated differently for the following experiments. Fig.1 shows the scheme of the measurements (A–D); the results are shown in fig.2:

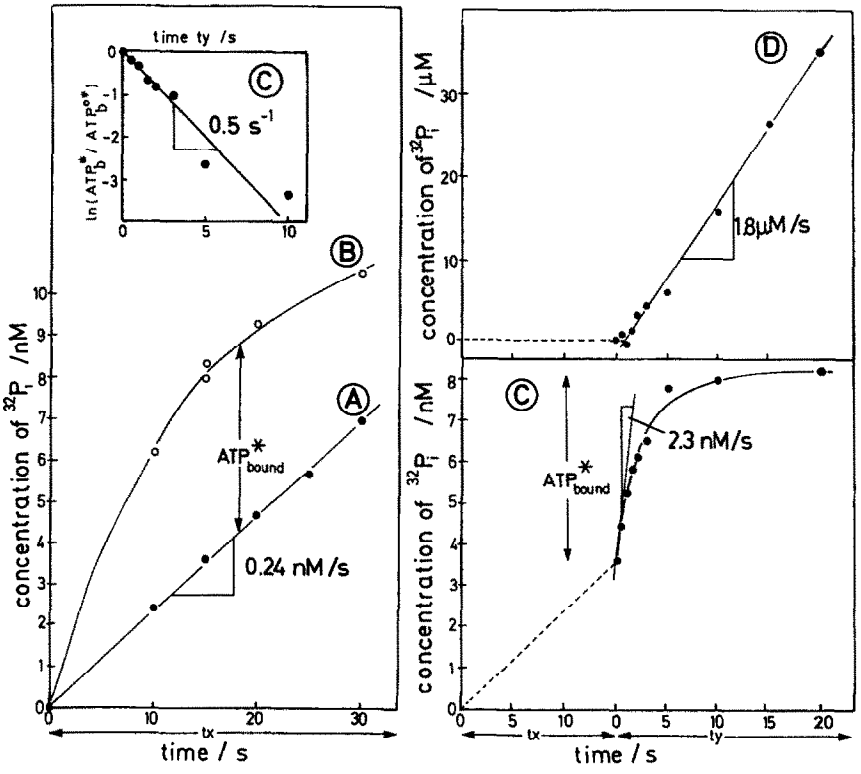

Fig.2. Concentration of $^{32}P_i$ as a function of time determined by measurements (A–D). (A) CF_0F_1 + [γ-^{32}P]ATP → t_x denaturation (Acid Quench). (B) CF_0F_1 + [γ-^{32}P]ATP → t_x + 1 mM ATP → 10 s denaturation (Cold Chase). (C) CF_0F_1 + [γ-^{32}P]ATP → 15 s + 1 mM ATP → t_y denaturation. Inset: first-order plot of the data. (D) CF_0F_1 + ATP → 15 s + 1 mM [γ-^{32}P]ATP → t_y denaturation (for details see text).

(A) In measurement A (see fig.1) (Acid Quench) [γ-^{32}P]ATP is added to the active, reduced CF_0F_1 (final concentration 33 nM ATP, 27 nM CF_0F_1). The ATP is bound to one site of the enzyme and hydrolyzed. After the reaction time, t_x, the enzyme was denatured by addition of trichloroacetic acid (final concentration 2% (w/v)). Finally, unlabeled Mg-ATP (1 mM) was added and the amount of $^{32}P_i$ was measured.

Fig.2A shows the result: the curve labeled (A) gives the amount of $^{32}P_i$ as a function of the reaction time t_x. The data represent the concentration of free and enzyme-bound P_i, i.e. $[P_i(A)] = [P_{i_f}] + [P_{i_b}]$. The rate of ATP hydrolysis is 0.24 nM/s. Since only the active enzymes (27 nM) can catalyze ATP hydrolysis, it results in a rate of 0.009 ATP/(CF_0F_1 s).

(B) In measurement B (see fig.1) (Cold Chase) first [γ-^{32}P]ATP is added to the active CF_0F_1 in the reaction medium. After the reaction time, t_x, unlabeled Mg-ATP was added (final concentration 1 mM) in a buffer identical with the reaction medium. The isotope dilution after the addition of 1 mM cold ATP is so high that there is no further binding of [γ-^{32}P]ATP to the enzyme after the chase. The [γ-^{32}P]ATP already bound to the enzyme was allowed to hydrolyze for $t_y = 10$ s. Then the samples were denatured as described in (A) and the amount of $^{32}P_i$ was determined.

Fig.2B shows the result: there is more $^{32}P_i$ detected in these samples than in the samples denatured after reaction time t_x (curve A). This difference must be due to hydrolysis of [γ-^{32}P]ATP, which was already bound on the enzyme before the addition of cold ATP. Curve B represents the sum of free and enzyme-bound $^{32}P_i$ and enzyme-bound [γ-$^{32}P_i$]ATP, i.e. $[ATP_b] = [P_i(B)]-[P_i(A)]$. At reaction time $t_x = 15$ s, it resulted in $[ATP_b] = 4.5$ nM. It should be mentioned that after the chase no radioactivity, i.e. $^{32}P_i$ and [γ-^{32}P]ATP was found on the enzyme. This has been measured with centrifugation columns as described in [14] (data not shown).

(C) In measurement C (see fig.1) the rate of hydrolysis of enzyme-bound [γ-^{32}P]ATP was measured. The reaction was performed as described in (B), but the reaction time t_x was constant (15 s) and the time between addition of 1 mM cold ATP and denaturation was varied: the enzyme was incubated with [γ-^{32}P]ATP for $t_x = 15$ s. After this time ($t_y = 0$ s) 1 mM cold Mg-ATP was added. This prevented further binding of [γ-^{32}P]ATP and, additionally, all other nucleotide binding sites were filled with cold ATP. At different times after addition of the cold ATP ($t_y = 0.5$–20 s), the enzyme was denatured and the amount of ^{32}P was determined.

Fig.2C shows the result: the bound [γ-^{32}P]ATP is hydrolyzed with an initial rate of 2.3 nM/s. The formation of $^{32}P_i$ is due to enzymes which had bound [γ-^{32}P]ATP already at the time of the cold chase, i.e., in measurement C the hydrolysis of [γ-^{32}P]ATP bound to the first site is measured under conditions where all nucleotide binding sites are occupied. In order to obtain the rate constant for ATP hydrolysis, the rate must be divided by the concentration of enzyme-bound ATP, i.e. [E–ATP] = 4.5 nM. It results in a rate constant of 0.5/s. Since the whole reaction cannot be faster than its slowest step, this is the maximal turnover rate of the first site when all other sites are occupied by ATP. Fig.2 (C, inset) shows a first-order plot of the data from fig.2C. An exponential decay of the concentration of enzyme-bound ATP can be seen giving again a rate constant of 0.5/s. Since the curve extrapolates to zero, all enzyme-bound ATP is hydrolyzed with the same rate constant and the existence of a rapid initial phase is excluded.

(D) In measurement D (see fig.1) the rate of ATP hydrolysis catalyzed by the other sites was measured under the same conditions as in measurement C: the enzyme was incubated with unlabeled ATP (33 nM) for $t_x = 15$ s. During that time cold ATP binds to the first site and is hydrolyzed. Then 1 mM of Mg-[γ-^{32}P]ATP was added ($t_y = 0$) and the enzyme was allowed to hydrolyze the labeled ATP. After reaction time t_y = 0.5–20 s, the enzyme was denatured and the amount of $^{32}P_i$ was determined.

Fig.2D shows the result: The rate of ATP hydrolysis is 1.8 μM/s. Based on the total amount of active enzymes, a rate of 80 ATP/(CF_0F_1 s) is obtained. This is practically identical with the maximal turnover rate found in chloroplasts [15].

A comparison of measurements C and D indicates that during the first 2 s after addition of 1 mM ATP, only about 50% of the ATP bound to the first site is hydrolyzed; whereas, the other sites of the enzyme have performed more than 100 turnovers in the same time.

These results demonstrate that there exists one catalytic nucleotide binding site on the active, reduced CF_0F_1 which has different kinetic properties than the others. It is able to hydrolyze ATP, but with slow rates. The maximal rate is 0.5/s, when the other sites become occupied by ATP. Therefore, this site cannot be involved in the catalytic turnover which occurs under the same conditions on one or the two other sites with a rate of 80/s.

The rate obtained in measurement C is about a factor 10 higher than the rate observed in measurement A. In both cases ATP hydrolysis catalyzed by the first ATP-binding site is measured, however, in (A) the other sites are empty, whereas in (C) they are occupied by cold ATP. Therefore, we have to conclude that there is an interaction between the different sites which increases the rate of hydrolysis on the first site. However, as discussed above, this rate is still a factor 100 lower than required for maximal turnover of the enzyme.

It has been shown with chloroplasts that there are two different types of catalytic sites. One shows a slow turnover and is also called 'regulatory site'. The other

one shows, parallel to the slow turnover, a rate which is about a factor 30 higher [16]. This result is quite similar to that obtained here. Data with the isolated CF_1 show also a heterogeneity of catalytic sites [17].

Data obtained with the isolated MF_1 are different: the ATP on the first site, which is hydrolyzed very slowly under uni-site conditions, is hydrolyzed with the maximal turnover when the other sites are filled with ATP [7,8,18,19]. However, results obtained with MF_1 are not internally consistent: it has been shown [20,21] that under appropriate conditions the uni-site turnover is about a factor 100 higher than found in [7]. Furthermore, a heterogeneity of catalytic sites has been proposed also for MF_1 [22]. Although there exist quite a number of differences between experimental conditions for MF_1 and CF_0F_1 (e.g. different origin of enzymes, involvement of protonation/deprotonation reaction in CF_0F_1, different nucleotide contents of the enzymes, etc.) we are not able to resolve these contradictions.

It has been shown that in CF_1 there are two different nucleotide binding sites on each β-subunit. One has catalytic properties, i.e. it can hydrolyze ATP, and one has no catalytic properties. Since CF_1 has three β-subunits, it was concluded that there exist three catalytic and three non-catalytic binding sites [23,24].

If this is true, the nucleotide binding site investigated in this work which slowly hydrolyzes ATP must be one of the three catalytic sites. We suggest that one of the three catalytic sites has a regulatory function in CF_0F_1, whereas the other two catalytic sites are involved in the catalytic turnover. Therefore, a binding change mechanism which is based on several independent lines of evidence (for review see [25]) can operate in CF_0F_1 only between two sites.

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