Journal of Bioenergetics and Biomembranes, Vol. 24, No. 5, 1992

Minireview

Proton Transport-Coupled Unisite Catalysis by the H^+-ATPase from Chloroplasts

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Received April 27, 1992; accepted May 20, 1992

Proton transport-coupled unisite catalysis was measured with the H^+-ATPase from chloroplasts. The reaction was measured in the ATP hydrolysis direction under deenergized conditions and in the ATP synthesis direction under energized conditions. The equilibrium constant of the enzyme does not change upon energization, whereas the dissociation constants of substrates and products change by orders of magnitude. This indicates that the Gibbs free enthalpy derived from proton translocation is used to change binding affinities of substrates and products, and this results in synthesis of free ATP.

KEY WORDS: Chloroplasts; H^+-ATPase; unisite catalysis.

INTRODUCTION

Membrane-bound H^+-ATPases (F_0F_1-ATPases; Pedersen and Carafoli, (1987a,b) catalyze ATP synthesis/hydrolysis coupled with transmembrane proton transport in bacteria, mitochondria, and chloroplasts (Mitchell, 1961). The hydrophilic F_1 part contains six nucleotide-binding sites; the membrane integrated F_0 part is assumed to act as a proton channel.

The H^+-ATPase from chloroplasts, CF_0F_1, has an intermediate structural complexity between the bacterial enzyme (8 subunits) and the mitochondrial enzyme (> 11 subunits). The CF_1 part has five subunits with the stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon$ (Moroney *et al.*, 1983), and the CF_0 part has four subunits (Fromme *et al.*, 1987a) and has presumably the stoichiometry $I\text{–}II\text{–}III_{12}\text{–}IV$ (Fromme *et al.*, 1987b).In functional respect, CF_0F_1 has several peculiarities.

1. *The activity of CF_0F_1 is strongly regulated.* The enzyme occurs in different redox and activation states (Junesch and Gräber, 1987). The redox state (exactly the redox state of a –S–S– bridge in the γ-subunit (Nalin and McCarty, 1984) can be changed by incubation with dithiothreitol (*in vitro*) or thioredoxin (*in vivo*). In both redox states the enzyme is catalytically inactive, E_i^{red} and E_i^{ox}. It can be converted into the metastable active enzyme states E_a^{red} and E_a^{ox} by membrane energization. These two forms can catalyze ATP synthesis and ATP hydrolysis. They differ in two respects. The inactive–active transition requires a lower ΔpH for the reduced enzyme than for the oxidized enzyme. After dissipation of the membrane energization, the half-lifetime of the metastable E_a^{red} is in the range between seconds and minutes (depending on reaction conditions), and that of E_a^{ox} is presumably in the millisecond range. Since the activation and the catalytic reaction both depend on the membrane energization, kinetic investigations of the catalytic reaction are to be carried out with the enzyme in the state E_a^{red}. Otherwise, interfering activation/inactivation processes are superposed.

2. *CF_1 differs from F_1 parts of other H^+-ATPases.* The F_1 parts of F_0F_1-ATPases can be removed from the membrane and have been used frequently for functional and structural investigations. When CF_1 is removed from the membrane, it does not catalyze ATP hydrolysis as the F_1 parts from other

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0145-479X/92/1000-0493$06.50/0

sources. Only after some special (mis-) treatments (usually called activation) does CF_1 catalyze Ca^{2+}-dependent ATP hydrolysis, whereas CF_0F_1 catalyzes Mg^{2+} dependent ATP hydrolysis as the other H^+-ATPases. We conclude, therefore, that CF_1 is not an appropriate model for the CF_0F_1-catalyzed reactions.

3. *The reconstituted CF_0F_1.* CF_0F_1 has been isolated and purified (Pick and Racker, 1979). It can be reconstituted into liposomes and shows nearly the same rate of ATP synthesis and ATP hydrolysis as in thylakoid membranes (Schmidt and Gräber, 1987). Therefore, our investigations of the kinetics of proton transport-coupled ATP synthesis/ATP hydrolysis were carried out with the holoenzyme using either thylakoid membranes or the CF_0F_1 reconstituted into liposomes and, additionally the enzyme was always brought into the active, reduced state E^{red}.

COUPLING BETWEEN PROTON TRANSPORT AND ATP SYNTHESIS

Currently, two hypotheses are discussed:

1. The protons translocated through the enzyme are involved directly in the chemical reaction ("direct coupling," Mitchell, 1974). In this case protons from the internal aequeous phase can reach the catalytic site, leading to a threefold protonation of the bound phosphate. A nucleophilic attack of the bound ADP (which is not protonated at the catalytic site) leads via a pentavalent transition state of the phosphorus atom to the elimination of water and formation at ATP.

2. The protons translocated through the F_0 part lead to conformational changes which are transmitted to the catalytic site in the F_1 part, and this leads to a change of the binding affinities of the nucleotides ("indirect coupling," Boyer, 1975, 1989). In a simplified way this mechanism works as follows: ADP and phosphate are bound to an open binding site. This is followed by a conformational change leading to a closing of the binding site, so that nucleotides and phosphate cannot exchange with the medium. Under those conditions ATP is formed spontaneously and it remains tightly bound at the catalytic site. Protonation of the enzyme from the inside gives rise to a conformational change; the catalytic site is opened again and ATP is released. This implies that the dissociation constants of substrates and/or products are drastically changed by membrane energization, whereas the ratio between bound ADP and bound ATP does not change significantly.

In respect to enzyme kinetics of the H^+-ATPase the phrase "membrane energization" means protonation/deprotonation reaction of the enzyme. We call this in the following a "proton-induced binding change" (Boyer *et al.*, 1973; Slater, 1974). The cooperativity between two catalytic binding sites in the binding change mechanism was introduced later, first with two cooperating sites (Boyer, 1979) and then with three sites (Boyer and Kohlbrenner, 1981, Boyer, 1989). We call this "nucleotide-induced binding change" in order to distinguish it clearly from the effects connected with membrane energization. In order to distinguish between nucleotide-induced binding changes and the proton-induced binding changes, we have carried out measurements under unisite conditions. In this minireview we describe some recent experiments dealing with the following questions:

1. Are the protons involved directly or indirectly in ATP synthesis? One consequence of the direct coupling mechanism is that the ratio between bound ADP and bound ATP must depend strongly on the membrane energization: increasing the internal proton concentration will directly lead to an increase of bound protonated phosphate and consequently to an increase of the bound ATP. In this case the ratio between bound ATP and bound ADP must change by orders of magnitude. Therefore, we have measured bound ADP and bound ATP under energized and deenergized conditions.

2. Which step is influenced in case of a proton induced binding change?

THE PROTON-INDUCED BINDING CHANGE

In a first series of experiments we have measured unisite ATP hydrolysis with reconstituted CF_0F_1 (Fromme and Gräber, 1989, 1990a). The enzyme was brought into the active, reduced state. The inactivation under the experimental conditions was measured and it was shown that kinetics can be measured up to about 30–60 s without significant inactivation. Under these conditions the enzyme binds ATP, an equilibrium between enzyme-bound substrates and products is established, and P_i and ADP are released from the enzyme. The rate constants and equilibrium constant were measured.

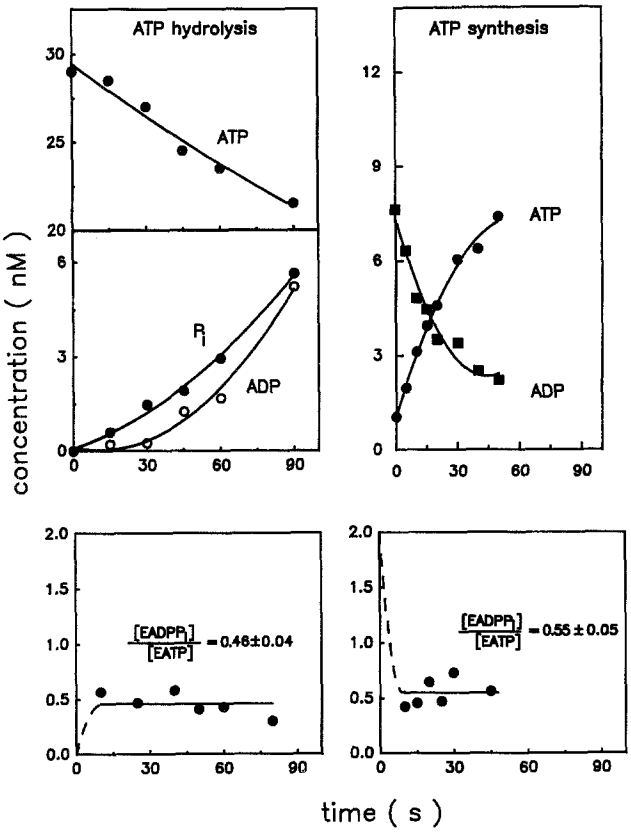

Fig. 1 Single-site catalysis by CF_0F_1 bound at thylakoid membranes $[CF_0F_1] = 15$ nM (Fromme and Gräber, 1990b; Labahn, 1991).

Left: ATP hydrolysis under deenergized conditions. Top: Time course of the free species. Bottom: Time course of the ratio of bound nucleotides. Right: ATP synthesis under energized conditions. Top: Time course of the free species. Bottom: Time course of the ratio of bound nucleotides.

In a second series of experiments, unisite ATP hydrolysis was measured with thylakoid membranes (Fromme and Gräber, 1990b). The enzyme is brought into the active, reduced state; inactivation of the enzyme plays no role during the reaction time. Again the enzyme binds ATP, the equilibrium on the enzyme is established, and P_i and ADP are released. The equilibrium constant and the rate constants are almost identical for both systems. This excludes mutually artefacts which might arise, on the one hand, by the use of the purified well-defined reconstituted system and, on the other hand, by the use of the natural but highly complex system.

Thylakoids can be easily energized by illumination and, therefore, ATP hydrolysis under energized conditions (Fromme and Gräber, 1990c) and ATP synthesis (Labahn *et al.*, 1990) was measured under unisite conditions. Recently, we have extended these measurements (Labahn, 1991). Figure 1 shows a summary at several measurements: At the top the free nucleotides are shown during unisite catalysis. Left: hydrolysis leads to a decrease of free ATP, and after a lag P_i and ADP are released; right: ATP synthesis under the same conditions except for energization leads to ADP binding and ATP release. At the bottom, the enzyme bound species are shown. We have plotted the ratio between EADP-P_i and EATP. During ATP hydrolysis (left) a constant ratio of 0.4 is observed. During ATP synthesis (right) a constant ratio of 0.5 is also observed. Both numbers are the same within the error limits of the measurements.

We can conclude from these data that during single-site catalysis the same equilibrium constant between enzyme-bound substrates and products is found irrespective of whether the reaction occurs in the direction of ATP hydrolysis or ATP synthesis. This shows that the equilibrium on the enzyme is not shifted by membrane energization, i.e., the transported protons are not directly involved in the chemical step at the nucleotide-binding site. (Of course the "chemical" proton must participate at this site.)

The next question is at which step the protons are involved. In order to approach this problem, the rate constants for all steps of the catalytic cycle were measured under deenergized and energized conditions, and the equilibrium constant and the dissociation constants were calculated. Table I shows the result. It can be seen that energization leads to an increase of the dissociation constant for ATP and a decrease of the dissociation constant for ADP and P_i, whereas the equilibrium constant on the enzyme does not change. The biggest effect is seen for the dissociation constant for P_i which changes by 12 orders of magnitude. We have to conclude that phosphate binding is the step which requires most energy. For comparison, we have collected in Table I also the corresponding data from MF_1, EF_1, and MF_0F_1. The equilibrium constants and dissociation constants from CF_0F_1 (deenergized) and the data from the other systems are in surprisingly good accordance in view of the different enzymes and the difficult measurements. These results demonstrate directly that the energy derived from proton transport is used to change binding affinities of substrates and products.

Table I. Equilibrium and Dissociation Constants for Unisite Catalysis under Deenergized and Energized Conditions for CF_0F_1, and the Corresponding Data for MF_1, EF_1, and MF_0F_1 (Deenergized)

Reaction	CF_0F_1, deenergized[a]	CF_0F_1, energized[b]	MF_0F_1, deenergized[c]	MF_1[d]	EF_1[e]
$E + ATP \leftrightharpoons EATP$	≤ 14 nM	5 μM	1.3 pM	1.2 pM	390 pM
$EATP \leftrightharpoons EADP \cdot P_i$	0.4	0.4	0.5	0.5	1.2
$EADP\,P_i \leftrightharpoons EADP + P_i$	$\leq 3.8 \times 10^4$ M	13 nM	87 M	8×10^2 M	2.3×10^3 M
$EADP \leftrightharpoons E + ADP$	0.6 μM	7 nM	0.15 μM	1 nM	0.7 μM

[a] Fromme and Gräber (1990b); Labahn (1991).
[b] Labahn (1991).
[c] Penefsky (1985a, b); Al-Shawi *et al.* (1989).
[d] Grubmeyer *et al.* (1982); Cunningham and Cross (1988).
[e] Al-Shawi *et al.* (1989).

THE NUCLEOTIDE-INDUCED BINDING CHANGE

Under our experimental conditions only one nucleotide-binding site is involved and only about 2% of this site is occupied. This is different from the experiments with MF_1 and EF_1, and it is due to the higher rate constants for ADP and P_i release (Fromme and Gräber, 1990a, b). Thus, the energetics of coupling seems to us to have been clarified; however, the way in which proton transport is coupled with ATP synthesis is not yet completely understood.

The results with the proton-induced binding change were obtained with only one catalytic site in operation. The occupation of the noncatalytic sites was as follows: tightly bound ADP is removed from CF_0F_1 upon activation and the enzyme contains either one ATP (reconstituted CF_0F_1) or two ATP (thylakoids) or one ATP (washed thylakoids). No difference in rate constants was observed for the different nucleotide occupation.

When one catalytic site is partly occupied (2%) by radioactive labeled substrates and the other sites are filled by using high concentrations of cold ATP, the rate constant for the P_i release is increased (from $0.2\,s^{-1}$ to about $1\,s^{-1}$; Fromme and Gräber, 1989, 1990b). This indicates the interaction between different catalytic nucleotide-binding sites and corresponds to a nucleotide-induced binding change. It must be mentioned, however, that the rate constant for P_i release from the first site is about $1\,s^{-1}$ when all the other sites are filled, whereas the rate constant from the other catalytic site(s) is at least $40\,s^{-1}$ under exactly the same conditions. We must conclude, therefore, that at least two catalytic sites have different properties.

ACKNOWLEDGMENTS

This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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