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Rapid report

Blocking one non-catalytic ADP binding site results in complete inhibition of the F-type ATPase from the thermophilic *Bacillus* PS3

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Abstract

The F-type ATPase, TF_0F_1, from the thermophilic *Bacillus* PS3, which is free of nucleotides after isolation, was specifically loaded with one 2-azido ADP on a non-catalytic site. The enzyme was covalently modified to various extents and the rate of ATP synthesis and ATP hydrolysis was measured. Both ATP synthesis and ATP hydrolysis extrapolated to zero for covalently binding one nucleotide per enzyme. This was interpreted such that the non-catalytic sites are involved in the coupled catalytic process.

Keywords: ATPase, F-type; ATPase, H^+-; ATPase TF_0F_1; 2-Azido nucleotide; Adenosine binding site; (thermophilic *Bacillus* PS3)

F-type ATPases use a transmembrane electrochemical potential difference to drive ATP synthesis [1]. This process is reversible. F-type ATPases consist of a membrane embedded F_0 part for the ion transport and a hydrophilic F_1 part for the catalytic reaction. F_0 and F_1 are connected by a small stalk. F_1 has the subunit stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$, independent of its origin, and six nucleotide binding sites which are located on the α- and β-subunits. The structure of the binding sites is well known since the structure of the $\alpha_3\beta_3\gamma$ complex from the mitochondrial F_1 is known at atomic resolution [2]. To identify the role of the six binding sites, the use of 2-azido nucleotides is a useful tool since they are true substrates and can be used for covalent labeling. When the 2-azido nucleotide is bound to a catalytic site it labels the β-tyrosine in the conserved sequence GIYPAVDPLDSXS [3] which is in the β-subunit. When the azido nucleotide is bound to a non-catalytic site it labels the β-tyrosine in the conserved sequence GXEHYXXA. This site is found in the α-subunit and the tyrosine of the β-subunit is on a loop which reaches into the α-subunit [2]. Blocking one catalytic site with a $2N_3$-nucleotide results in complete inhibition of the catalytic process of both hydrolysis and synthesis of ATP [4]. The roles of the non-catalytic sites remains unknown. In this communication, the effect of covalent modification of specifically one non-catalytic site of the TF_0F_1 is described.

Preparation and reconstitution of TF_0F_1. The TF_0F_1 was isolated from the thermophilic *Bacillus* PS3 as described [5]. It was reconstituted into artificial liposomes together with the light driven proton pump bacteriorhodopsin following the protocol of [6].

Kinetic measurements. All the kinetic measurements with the TF_0F_1 were performed at 40°C. ATP synthesis was measured after reconstitution of the enzyme into artificial liposomes together with bacteriorhodopsin in a buffer containing 50 mM Na_2SO_4, 50 mM K_2SO_4, 25 mM Na_2HPO_4, 1 mM $MgCl_2$ (pH 7.3). The proteoliposomes were preilluminated for 15 minutes to establish a stable electrochemical potential difference for protons. ATP synthesis was started by the addition of 1 mM ADP. During a period of 20 min, samples were quenched in trichloroacetic acid (20 g/l final concentration) and the ATP content was measured using the luciferin luciferase assay [7]. ATP hydrolysis was measured with a coupled assay in a stirred and thermostated cuvette. The assay mixture contained 50 mM Na_2SO_4, 50 mM K_2SO_4, 25 mM Na_2HPO_4 (pH 7.3), 1 mM $MgCl_2$, 5 g/l Triton X-100, 1 mM phospho*enol*pyruvate, 0.3 mM NADH, 0.1 mg/ml pyruvate kinase, 0.1 mg/ml lactate dehydrogenase, 2 μg/ml TF_0F_1. 2 mM ATP was added to start the reaction. After a few minutes a constant rate of hydrol-

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ysis was established. The rate was then measured over a period of 10 min.

Synthesis of $2N_3[\beta\text{-}^{32}P]ADP$. $2N_3$-adenosine was synthesized from 2-chloroadenosine after [8]. $2N_3$AMP was made from $2N_3$-adenosine after [9]. $2N_3[\beta\text{-}^{32}P]$ADP was made by incubating $2N_3$AMP with $[\gamma\text{-}^{32}P]$ATP and myokinase over night at room temperature. The assay contained 0.2 mM EDTA, 100 mM Pipes-NaOH (pH 7.6), 2 mM $MgCl_2$, 1.2 mM $2N_3$AMP, 1 mM $[\gamma\text{-}^{32}P]$ATP and 100 μg/ml myokinase (Boehringer). The $2N_3[\beta\text{-}^{32}P]$ADP was purified by DEAE column chromatography (column 1 cm × 28 cm) with a linear gradient from 0 to 0.5 M triethylamine bicarbonate (pH 7). The fractions containing the compound were combined, the solvent removed by evaporation and the 2 $N_3[\beta\text{-}^{32}P]$ADP resuspended in water. The concentration of $2N_3[\beta\text{-}^{32}P]$ADP was approx. 2 mM as determined by the optical density [10].

Labeling and estimation of the fraction of covalent modified enzyme. The TF_0F_1, at a concentration of 3 g/l, was incubated in 100 μM $2N_3[\beta\text{-}^{32}P]$ADP for 15 min at 40°C and subsequently passed through 3 successive 'Penefsky' centrifugation columns [11]. The buffer for incubation and centrifugation columns contained 50 mM Na_2SO_4, 50 mM K_2SO_4, 25 mM NaH_2PO_4, 1 mM $MgSO_4$, 5 g/l Triton X-100 (pH 7.3) (adjusted with NaOH). Fractions of the enzyme solution were placed in the cap of an eppendorf tube and covered with quartz glass and illuminated for periods between 15 s and 15 min. The UV-lamp (Spectroline ENF 24/F, Spectronics, Westbury, NY) was placed 1 cm above the sample. Protein concentration was measured with the Bio-Rad protein assay using BSA as a standard. After acid precipitation and washing, the amount of label which was covalently bound was calculated from the remaining radioactivity in the protein. A molecular mass of 550000 kDa for the TF_0F_1 was assumed.

The TF_0F_1 was nearly free of nucleotides after isolation and purification. It was found that 0 ATP and 0.2 ADP were bound per molecule TF_0F_1. In order to bind one nucleotide the TF_0F_1 was incubated in 100 μM $2N_3[\beta\text{-}^{32}P]$ADP followed by three successive centrifugation columns. Under these conditions 1 ADP is bound to a non-catalytic site [6]. $2N_3[\beta\text{-}^{32}P]$ADP has the same affinities and behaves exactly as ADP [12,13]. The enzyme was then photoirradiated for various periods so that various fractions of TF_0F_1 were covalently modified. The protein concentration and the fraction of covalent modified protein (i.e., the amount of label stable with respect to acid precipitation) were measured for different illumination times. Fig. 1 gives the fraction of covalently modified enzyme as a function of the illumination time. After about 10 min a maximum level of labelling was reached. The maximal labelling was 0.6–0.7 nucleotides per enzyme. This is in accordance with earlier reports where, due to a tautomerisation, not more than 60% of the 2-azido nucleotides can react to covalently label the enzyme [10]. The

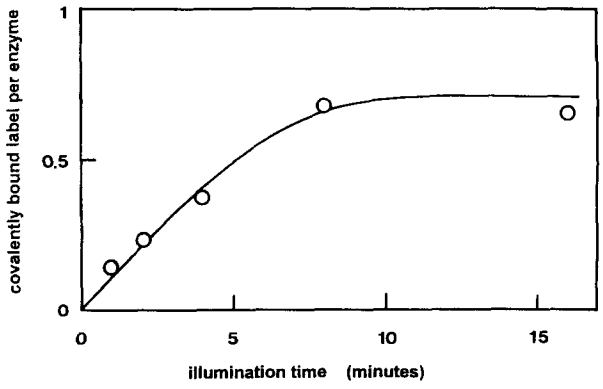

Fig. 1. Fraction of covalently modified TF_0F_1 versus the illumination time with UV light. The fraction of covalently modified enzyme is given in mol of nucleotide per mol of enzyme.

0.2 ADP per enzyme initially present might have exchanged during incubation with the $2N_3$ADP. In a control, where ADP instead of $2N_3$ADP was used, UV irradiation led to a decrease of the enzyme activity. Irradiation of 10 min led to about 10% decrease in activity, this was taken into account for all further measurements.

The UV-irradiated enzyme was then reconstituted into artificial liposomes together with bacteriorhodopsin and the rate of ATP synthesis measured. Care was taken to measure only the initial rate of ATP synthesis, since accumulation of ATP can lead to a stimulation of ATP synthesis [6]. In Fig. 2 the rate of ATP synthesis is plotted versus the fraction of covalently labelled enzyme. The rate of ATP synthesis was normalized to the rate when TF_0F_1 was not modified. The maximal rate of ATP synthesis for the non-modified enzyme was 0.2 ATP $TF_0F_1^{-1}$ s^{-1} or 22 nmol ATP mg^{-1} min^{-1}. The fraction of covalent labelled enzyme is given as the molar fraction of nucleotide per enzyme. The relative rate of ATP synthesis decreased with an increasing amount of covalent modified enzyme. At 0.6 nucleotides per enzyme the residual relative rate of ATP

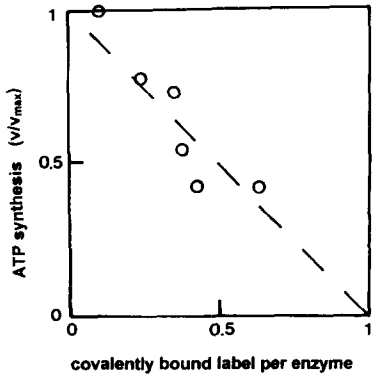

Fig. 2. Normalized rate of ATP synthesis of TF_0F_1 covalently labeled, by differing amounts, with $2N_3$ADP on a non-catalytic site. ATP synthesis was measured in the light with proteoliposomes, where TF_0F_1 was co-reconstituted with bacteriorhodopsin into artificial liposomes.

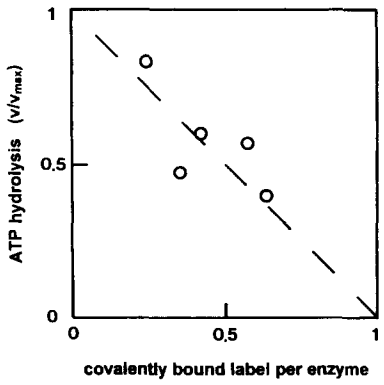

Fig. 3. Normalized rate of ATP hydrolysis with covalently labeled TF_0F_1 as in Fig. 2. ATP hydrolysis was measured with a coupled assay as described in the text.

synthesis is only 0.4. At 100% labelling – i.e., 1 ADP per enzyme – the rate of ATP synthesis extrapolates to zero.

To measure the rate of ATP hydrolysis the enzyme was added to a coupled assay and the reaction was started by adding ATP. Hydrolysis was measured with an enzyme where different fractions were covalently labelled with the 2-azido ADP by different amounts. The results are shown in Fig. 3. The representation is similar to the Fig. 2. The rate of ATP hydrolysis is normalized relative to the rate when the enzyme was not covalently modified. The rate of hydrolysis the non-modified enzyme was 26 ADP TF_0F_1 $^{-1}$ s^{-1} which corresponds to 2.8 μmol mg^{-1} min^{-1}. Covalent modification caused inhibition of the ATP hydrolysis. Covalent binding of 0.6 nucleotides per enzyme resulted in an inhibition of hydrolysis of 60%. For a labelling of one ADP per enzyme the rate of ATP hydrolysis extrapolates to zero. This situation is similar to that observed for ATP synthesis.

2-Azido nucleotides can bind to two conserved sequences in F-type ATPases when the holoenzyme is treated. This was observed for the F-type ATPases from mitochondria, chloroplast, *E. coli* [3] and thermophilic *Bacillus* PS3 [14]. These two sequences were named catalytic and non-catalytic [3]. These two conserved sequences are both part of the β-subunits. After the structure of a F-type ATPase was solved at atomic resolution, these two sequences were identified in the three-dimensional structure. The catalytic site is in the β-subunit. The non-catalytic site is in a space which is filled with amino acids mainly from the α-subunit. A loop from the β-subunit with the sequence, which can be labelled with 2-azido nucleotides, is reaching into this binding site [2].

In TF_0F_1 there is an obvious difference between catalytic and non-catalytic sites in that the non-catalytic sites are stable to gel filtration [6]. More general characteristics are that the non-catalytic sites are adenosine specific, strongly Mg^{2+}-dependent, and bind $MgPP_i$ [15]. However, the physiological role of the non-catalytic sites remains obscure. Recently, Richard et al. [6] observed that ATP, bound to a non-catalytic site, stimulated the rate of ATP synthesis in the TF_0F_1. The concentration required to achieve stimulation of ATP synthesis was approx. 10 μM, a concentration which is physiological relevant only under starvation conditions.

The three non-catalytic sites of the TF_0F_1 consist of one ADP site and two ATP sites [6]. To obtain conditions with only one $2N_3$-nucleotide per enzyme the ADP site rather than an ATP site was chosen. This is because $2N_3$ATP hydrolyses and exchanges with the ADP so that the enzyme would contain $2N_3$ADP and $2N_3$ATP. Another advantage of using the ADP site is that there is only one site, so it can be completely filled with absolutely no occupation of any other site as would be the case with the ATP sites. Also, in TF_0F_1 only the non-catalytic sites are stable to gel filtration and TF_0F_1 has only one ADP site which is stable to gel filtration [16], this makes the ADP site of this enzyme an ideal candidate for blocking specifically one non-catalytic site.

There are differing reports in the literature concerning covalent labelling of the TF_1. Schäfer et al. [17] found that one 8-azido ATP per TF_1, which was covalently bound to a non-catalytic site, was sufficient for complete inhibition of ATP hydrolysis. Bar-Zvi et al. [18] found complete inhibition after binding two BzADP to non-catalytic sites of the TF_1. Also, using $2N_3$-adenine nucleotides for different F-type ATPases gave different results. Melese et al. [19] found complete inhibition of the CF_1 when one $2N_3$ nucleotide was bound to a catalytic site or to a non-catalytic site, whereas Cross et al. [20] found for MF_1 that one nucleotide on a catalytic site was sufficient for complete inhibition but two nucleotides were required on a non-catalytic sites to achieve the same inhibition.

There are far fewer reports about labelling the F_0F_1 holoenzyme. Martins and Penefsky [4] reported that labelling one catalytic site of the MF_0F_1 with $2N_3$ADP was sufficient for complete inactivation of hydrolysis as well as synthesis. The authors claimed that this supported the theory of a cooperative interaction of the catalytic sites. In this report one non-catalytic site of a F_0F_1 ATPase is specifically labelled. The labelling of one nucleotide per enzyme led to complete inactivation of synthesis and hydrolysis. This supports the idea that a single catalytic pathway is used for both enzymatic reactions, synthesis and hydrolysis. It further supports the idea that the non-catalytic sites are involved in the catalytic process. The cooperative interaction between the catalytic sites might be mediated through the non-catalytic sites. According to Abrahams et al. [2] the γ-subunit is able to rotate in the $\alpha_3\beta_3$ core complex. In this scheme the different occupation of non-catalytic sites may facilitate or impair this rotation. The involvement of the non-catalytic sites in catalysis may be different for the different F-type ATPases. In the TF_0F_1, ATP can stimulate the catalytic reaction [6], in the CF_0F_1, ADP is involved in the activation/inactivation process [21]. Covalent modification,

however, blocks the coupled process in both directions, synthesis and hydrolysis.

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