Volume 321, number 1, 46–50 FEBS 12324 April 1993

AT(D)PMg-induced dissociation of the $\alpha_3\beta_3$ complex of the F_1-ATPase from a thermophilic *Bacillus* PS3 into $\alpha_1\beta_1$ heterodimers is prevented by mutation β (Y341C)

Chitose Kaibara[a], Masafumi Odaka[b], Toru Hisabori[c] and Masasuke Yoshida[a]

[a]*Research Laboratory of Resources Utilization, Tokyo Institute of Technology, Nagatsuta 4259, Yokohama 227, Japan,* [b]*The Tokyo Metropolitan Institute of Medical Science, Honkomagome, Tokyo 113, Japan* and [c]*Department of Biology, Yokohama City University, Seto, Yokohama 236, Japan*

Received 9 February 1993; revised version received 1 March 1993

AT(D)PMg induces dissociation of the $\alpha_3\beta_3$ complex of F_1-ATPase from a thermophilic *Bacillus* strain, PS3, into the $\alpha_1\beta_1$ heterodimers [(1991) Biochim. Biophys. Acta 1056, 279–284] but the location of the AT(D)PMg binding site responsible is not known. From the analysis of AT(D)PMg binding properties of the isolated mutant β subunit, β(Y341C), and the stability of the $\alpha_3\beta$(Y341C)$_3$ complex in the presence of AT(D)PMg, we conclude that binding of AT(D)PMg to the Tyr-341 site of the β subunit(s) in the $\alpha_3\beta_3$ complex triggers the dissociation of the $\alpha_3\beta_3$ complex into the $\alpha_1\beta_1$ heterodimers.

F_1-ATPase; $\alpha_3\beta_3$ complex of F_1; $\alpha_1\beta_1$ heterodimer of F_1; ATP-induced dissociation

1. INTRODUCTION

F_1-ATPase, a peripheral sector of H^+-ATP synthase, has a subunit structure of $\alpha_3\beta_3\gamma\delta\varepsilon$ [1–4]. Reconstitution of active F_1-ATPase from isolated subunits has been demonstrated for F_1-ATPases from a thermophilic *Bacillus* strain, PS3 (TF_1) [5,6], and *Escherichia coli* (EF_1) [7,8]. Isolated α and β subunits can bind adenine nucleotides individually but both have only a trace amount of ATP hydrolyzing activity [8–11]. The $\alpha_1\beta_1$ heterodimer was recently isolated from *Rhodospirillum rubrum* chromatophore membranes as the minimum unit of a subunit complex that has ATPase activity [12]. In the case of TF_1, the ATPase activity appears when the α and β subunits are assembled into the $\alpha_3\beta_3$ complex [6], however, the $\alpha_3\beta_3$ complex is not stable during ATP hydrolysis; it tends to dissociate into three pairs of $\alpha_1\beta_1$ heterodimers [13]. The dissociation is reversible and dependent on AT(D)PMg. ATPMg is more effective than ADPMg in causing dissociation, and non-hydrolyzable ATP analogs are not effective [13,14]. Covalent binding of 3′-*O*-(4-benzoyl)benzoyl ATP to the β subunits in the $\alpha_3\beta_3$ complex results in the dissociation of the complex [9], but covalent binding of 3′-*O*-(4-benzoyl)benzoyl ADP does not [15]. The implication of this dissociation could be important since it may reflect the AT(D)PMg-triggered conformational change occurring to H^+-ATPase during the catalytic cycle, and the effect of AT(D)PMg on the interconversion of $\alpha_3\beta_3$ to $\alpha_1\beta_1$ should be understood with more clarity. For example, the location of the AT(D)PMg binding site responsible for the dissociation should be identified.

In a previous report, we showed that the $\alpha_3\beta_3$ complexes of TF_1 containing mutant β subunits, in which Tyr-341 was replaced by Cys (β(Y341C)), retained significant ATPase activity [16]. This was an unexpected result because, based on chemical modification of F_1-ATPases with affinity analogs of nucleotides and other inactivating reagents, this tyrosine residue in the β subunit had been thought to be essential for catalysis [17–22]. We concluded that a tyrosine residue at this position is not involved in bond making–breaking steps of catalysis. Wise and his colleagues carried out more extensive studies on EF_1 containing β subunits with various mutations at Tyr-331 (a corresponding residue to $TF_1\beta$ Tyr-341) and reached the same conclusion. In addition, they indicated that the residue β331 is part of the rather non-polar adenine binding sub-domain [23,24]. Here we report that the binding affinity of the isolated β(Y341C) to AT(D)PMg is very weak and that the $\alpha_3\beta$(Y341C)$_3$ complex does not dissociate into $\alpha_1\beta$(Y341C)$_1$ heterodimers, even in the presence of rather high concentrations of AT(D)PMg. The indication of this result is that the AT(D)PMg binding site

Correspondence address: M. Yoshida, Research Laboratory of Resources Utilization, R-1, Nagatsuta 4259, Yokohama 227, Japan. Fax: (81) (45) 922-5279.

Abbreviations: CD, circular dichroism; HPLC, high performance liquid chromatography; TF_1 and EF_1, F_1-ATPases from a thermophilic *Bacillus* strain PS3, and *E. coli*, respectively.

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at βTyr- 341 is responsible for dissociation of the complex.

2. EXPERIMENTAL

2.1. *Materials*

TF_1 was prepared as described previously [25]. The α, β(WILD), and β(Y341C) subunits of TF_1 were individually expressed in *E coli* and purified as described [16,26]. Purified preparations were stored in 75% ammonium sulfate suspension at 4°C. Prior to use, precipitated β(Y341C) was solubilized in a minimum volume of 20 mM Tricine NaOH (pH 8.0) containing 1 mM dithiothreitol, and applied onto a centrifuge-gel filtration column equilibrated with an appropriate buffer.

2.2. *Difference spectra*

Circular dichroism (CD) spectra were measured at 25°C with a J-600 CD spectrophotometer (JASCO, Tokyo) equipped with a computerized data processor using a quartz cuvette with a 2-mm light path. CD spectra were measured after a 5 min preincubation, and 10 scans at wavelengths from 250 to 300 nm were averaged. The sample solution contained 40 μM β(WILD) or β(Y341C) in 20 mM Tricine NaOH buffer (pH 8.0), 100 μM cyclohexyl diaminetetraacetic acid and, when indicated, 250 μM AT(D)P and 5 mM $MgSO_4$. In each case, the CD spectra of protein solution, adenine nucleotide solution, and protein-adenine nucleotide solution were measured and stored in the data processor and the difference spectrum was computed as follows: (β subunit + adenine nucleotide) − (β subunit) − (adenine nucleotide). Difference absorbance spectra were measured with a Double beam spectrophotometer UV-2200 (Shimazu, Kyoto) using a pair of matched double cells with a light path length of 4 mm for each compartment, as described [27]. The sample solution contained 5 μM of the isolated β(WILD) or β(Y341C) in the 20 mM Tricine NaOH buffer (pH 8.0), 100 μM cyclohexyl diaminetetraacetic acid and indicated amounts of AT(D)P and $MgSO_4$. Spectra were measured after a 5 min incubation at 25°C and three scans were averaged.

2.3. *Equilibrium dialysis*

Equilibrium dialysis was performed in lucite cells (2 × 100 μl) using a dialysis membrane (cutoff molecular weight 12,000–14,000 Da, Hoefer Scientific Instruments), as described previously [28] Briefly, the solution containing 40 mM Tricine NaOH (pH 8.0), 2.5 mM $MgSO_4$, 0.5 mM dithiothreitol, 100 mM NaCl, and 10, 20, 40, 60, 80, 100, 150, or 300 μM ATP was placed in a control cell and the solution containing 12 μM β(WILD) or 19 μM β(Y341C) in the above solution without ATP was placed in a sample cell. After dialysis for 6 h at 25°C with gentle agitation, the dialyzed solution was removed from each cell and a 1/50 volume of 24% perchloric acid was added. The precipitated proteins were removed by centrifugation at 4°C, and a 1/2 volume of 1 M Tricine-NaOH (pH 8 0) containing 2 M KCl was added to the supernatant fraction. After brief centrifugation at 4°C, the adenine nucleotide content in supernatant fractions was analyzed by HPLC with a reverse-phase column (Cosmosil $5C_{18}$-AR, Nakarai, Kyoto) equilibrated and eluted with 100 mM sodium phosphate (pH 6.9) [29]. When the molar ratio of added ATP per β subunit was higher than one, almost all ATP added initially was recovered without decay after dialysis. When the ratio was 1:3 and 1:2, about 25% and 10% of added ATP was hydrolyzed during dialysis due to trace ATPase activity of the isolated β subunit [6]. Attainment of equilibrium under the above conditions was confirmed in a parallel experiment by equal distribution of AMP between two cells which was added in a control cell at the start of dialysis.

2.4. *Gel permeation HPLC analysis of the complex*

Reconstitution of the $\alpha_3\beta_3$ complex from isolated subunits, and purification of the complex with a HPLC gel permeation column (G3000SWXL, Tosoh, Tokyo), were carried out as described [16] Specific ATPase activities of the purified $\alpha_3\beta(\text{WILD})_3$ complex and the $\alpha_3\beta(\text{Y341C})_3$ complex were 0.71 and 0.34 U (μmol ATP hydrolyzed/min) per mg protein at 25°C Dissociation of the $\alpha_3\beta_3$ complex into $\alpha_1\beta_1$ heterodimers was measured with the same gel permeation HPLC system. The purified complex (4 μM) was preincubated for 1 min in 10 μl of 10 mM NaOH piperazine-*N,N'*-bis[2-ethanesulfonic acid) buffer (pH 7.0) containing 0.2 M Na_2SO_4, and indicated concentrations of ATPMg or ADPMg, and then applied to a G3000SWXL column equilibrated with the same solution used for preincubation. The elution was carried out with the same buffer at room temperature (22°C) and at a flow rate of 0.5 ml/min, and was monitored with absorbance at 280 nm. The $\alpha_3\beta_3$ complex and the $\alpha_1\beta_1$ heterodimer were eluted at 14.5 min and 17 5 min, respectively, as separated peaks. The peak areas of proteins were calculated with a computer.

3. RESULTS

3.1. *Difference spectra of the isolated β(WILD) and β(Y341C)*

The binding of adenine nucleotides to TF_1 and to the isolated α and β subunits can be detected by CD and UV spectral change [11]. The isolated β(WILD) and β(Y341C) showed almost the same CD spectra at 210–250 nm (data not shown) and at 250–300 nm (Fig. 1A), indicating that loss of an aromatic residue at position 341 did not affect the CD spectrum itself. When 250 μM ATPMg was added to the solution of β(WILD), a difference CD spectrum with a large negative ellipticity at 267 nm was induced (Fig. 1B, left) [10,30]. However, β(Y341C) mixed with 250 μM ATPMg did not show any difference CD spectrum (Fig. 1B, right). Therefore, ATPMg cannot bind to β(Y341C) with high affinity. Similarly, difference UV absorbance spectrum, characterized by a peak at 278 nm and a trough at 254 nm, was induced when 250 μM ATPMg was added to β(WILD). In contrast, β(Y341C) did not show any peak or trough in the difference spectrum (Fig. 1C). When 250 μM ADPMg was used to induce difference CD and UV spectrum, the results were almost the same as those observed for ATPMg: for β(WILD), ADPMg induced difference CD and UV spectra that were indistinguishable from those induced by ATPMg but it did not induce any significant difference CD and UV spectra to β(Y341C) (data not shown). Also ATP or ADP without Mg^{2+} induced difference spectra to β(WILD) but not to β(Y341C) (data not shown). The indication of these results is that the binding affinity of the adenine nucleotides to the isolated β(Y341C) should be very weak.

3.2. *Equilibrium dialysis*

In order to eliminate the possibility that AT(D)PMg binds to β(Y341C) without giving difference spectrum, equilibrium dialysis, a direct method of measuring ligand binding, was applied to the isolated β subunit. As shown in Fig. 2, β(WILD) has a single binding site for ATPMg. The dissociation constant (K_d) is roughly calculated to be 25 μM, however, under the same condi-

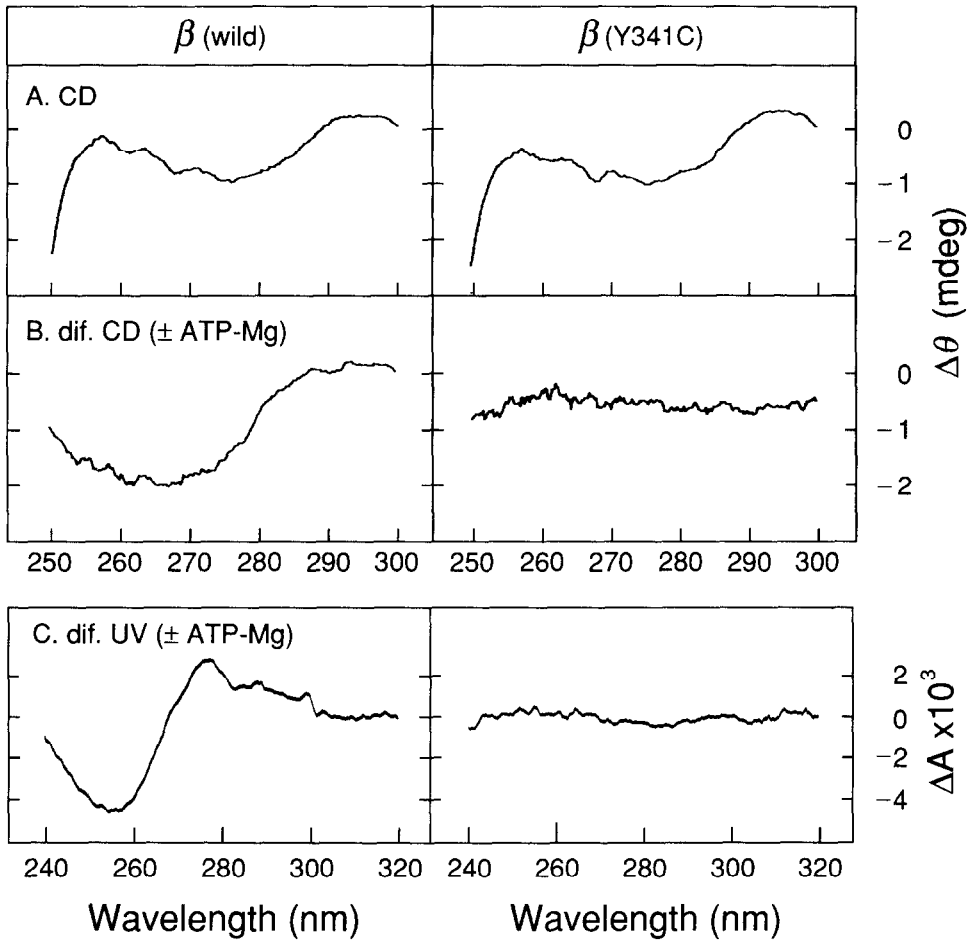

Fig. 1. Difference spectra induced when ATPMg was added to the isolated β(WILD) and β(Y341C). (A) CD spectra of the isolated β subunits. (B) Difference CD spectra observed when 250 μM ATPMg was added to β(WILD) or β(Y341C). (C) Difference UV absorbance spectra induced when 20 μM ATPMg was added to the isolated β(WILD) and β(Y341C). Other experimental details are described in section 2.

tions, β(Y341C) failed to bind ATPMg and the K_d value, if it exists, would be larger than 500 μM. Similarly, ADPMg bound to β(WILD) but not to β(Y341C) (data not shown). Thus spectroscopically 'invisible' binding did not occur with β(Y341C).

3.3. *Effect of AT(D)PMg on dissociation of the $\alpha_3\beta_3$ complex*

Consistent with the report by Harada et al. [13], the $\alpha_3\beta$(WILD)$_3$ complex mostly dissociated into $\alpha_1\beta$(WILD)$_1$ heterodimers when exposed to 5 μM ATPMg or 100 μM ADPMg for 1 min and eluted from a gel permeation HPLC with the buffer containing the same concentration of ATPMg or ADPMg (Fig. 3). In contrast, as shown clearly in Fig. 3, the $\alpha_3\beta$(Y341C)$_3$ complex did not dissociate into heterodimers under the same conditions. The $\alpha_3\beta$(Y341C)$_3$ complex was resistant to ATPMg-induced dissociation even at 100 μM ATPMg.

4. DISCUSSION

The position corresponding to β Tyr-341 of TF_1 is occupied by Tyr in all F_1-ATPases which have been sequenced to date. Although the $\alpha_3\beta$(Y341C)$_3$ complex of TF_1 and some of the EF_1 mutants lacking aromatic side chains at this position have significant ATPase activity, and hence this Tyr may not be involved directly in catalysis [16,23], there is general agreement that this residue is located at the adenine nucleotide binding site. This report shows that the binding affinity of the isolated β(Y341C) to AT(D)PMg was too weak to be detectable by usual binding assays (Figs. 1 and 2), and that the $\alpha_3\beta$(Y341C)$_3$ complex does not dissociate into $\alpha_1\beta_1$ heterodimers in the presence of ATPMg or ADPMg at concentrations where dissociation of the $\alpha_3\beta$(WILD)$_3$ complex is readily induced (Fig. 3). The parallel loss of AT(D)PMg binding affinity to the isolated β(Y341C) and

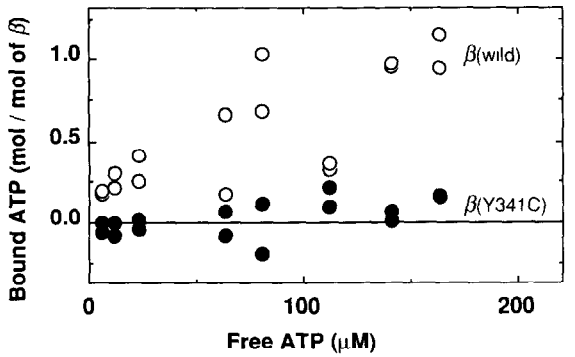

Fig. 2. Equilibrium dialysis of the isolated β(WILD) and β(Y341C) with ATP. Concentrations of free ATP in the medium (ATP concentration in a control cell) are indicated in abscissae. Open and closed circles represent β(WILD) and β(Y341C), respectively. Other experimental details are described in section 2.

AT(D)PMg-induced dissociation of the $\alpha_3\beta$(Y341C)$_3$ complex indicates that the former is the reason of the latter; occupation of the adenine nucleotide binding site at Tyr-341 of β(WILD) by ADPMg or ATPMg (or its subsequent hydrolysis) induces some conformational change which ends up with dissociation of the $\alpha_3\beta$(WILD)$_3$ complex into three pairs of $\alpha_1\beta$ (WILD)$_1$ heterodimers, but, in the case of β(Y341C), adenine nucleotides cannot bind to β(Y341C) in the $\alpha_3\beta$(Y341C)$_3$ complex with enough affinity and, hence, does not induce subsequent conformational changes in the complex. The effect of chemical labeling of the $\alpha_3\beta_3$ complex on the dissociation is well interpreted in this context; covalent modification of βTyr-307 by 7-chloro-4-nitrobenzofurazan does not induce the dissociation of the $\alpha_3\beta_3$ complex [31], but covalent binding of 3′-*O*-(4-benzoyl)benzoyl ATP results in dissociation [9]. The binding site of 3′-*O*-(4-benzoyl)benzoyl ATP in the $\alpha_3\beta_3$ complex has not been determined but it is most likely to be βTyr-341 since, in the case of bovine mitochondrial F_1-ATPase, this analog was incorporated into the peptide segment containing the Tyr equivalent to TF_1-βTyr-341 [15].

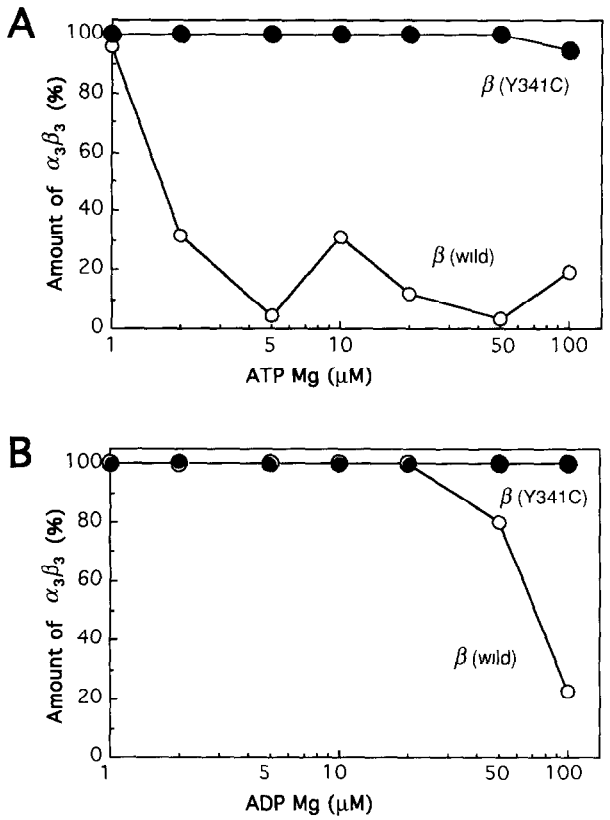

Fig. 3. Effect of various concentrations of (A) ATPMg and (B) ADPMg on the dissociation of the $\alpha_3\beta$(WILD)$_3$, and $\alpha_3\beta$(Y341C)$_3$ complexes Dissociation of the $\alpha_3\beta_3$ complex into $\alpha_1\beta_1$ heterodimers was measured with gel permeation HPLC. Open and closed circles represent β(WILD) and β(Y341C), respectively. The detailed experimental conditions were described in section 2.

One can speculate that binding of AT(D)PMg to the site at βTyr-341 of the H^+-ATPase might induce similar conformational change which diminishes the interactions between adjacent $\alpha_1\beta_1$ pairs in the $\alpha_3\beta_3$ structure of H^+-ATPase, and then this conformational change may trigger the next event in the catalytic cycle. Of course, dissociation does not occur to H^+-ATPase since the $\alpha_3\beta_3$ structure is stabilized by other subunits, especially by the γ subunit [32,33]. It makes no difference whether the adenine nucleotide binding site at Tyr-341 is a catalytic one as widely accepted, or a non-catalytic site, it now becomes clear that the binding of adenine nucleotides to this site triggers the significant change of interactions among subunits.

Acknowledgements: We are grateful for Dr. Eiro Muneyuki and Miss Hiromi Kobayashi for discussion and isolating mutants, respectively. Financial support from a Grant-in-Aid for Scientific Research on Priority Area of 'Energy Coupling in the Cell' to M.Y from the Ministry of Education, Science and Culture of Japan is gratefully acknowledged.

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