Thermodynamic Analyses of Nucleotide Binding to an Isolated Monomeric β Subunit and the $\alpha_3\beta_3\gamma$ Subcomplex of F_1-ATPase

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ABSTRACT Rotation of the γ subunit of the F_1-ATPase plays an essential role in energy transduction by F_1-ATPase. Hydrolysis of an ATP molecule induces a 120° step rotation that consists of an 80° substep and 40° substep. ATP binding together with ADP release causes the first 80° step rotation. Thus, nucleotide binding is very important for rotation and energy transduction by F_1-ATPase. In this study, we introduced a βY341W mutation as an optical probe for nucleotide binding to catalytic sites, and a βE190Q mutation that suppresses the hydrolysis of nucleoside triphosphate (NTP). Using a mutant monomeric βY341W subunit and a mutant $\alpha_3\beta_3\gamma$ subcomplex containing the βY341W mutation with or without an additional βE190Q mutation, we examined the binding of various NTPs (i.e., ATP, GTP, and ITP) and nucleoside diphosphates (NDPs, i.e., ADP, GDP, and IDP). The affinity ($1/K_d$) of the nucleotides for the isolated β subunit and third catalytic site in the subcomplex was in the order ATP/ADP > GTP/GDP > ITP/IDP. We performed van't Hoff analyses to obtain the thermodynamic parameters of nucleotide binding. For the isolated β subunit, NDPs and NTPs with the same base moiety exhibited similar ΔH^0 and ΔG^0 values at 25°C. The binding of nucleotides with different bases to the isolated β subunit resulted in different entropy changes. Interestingly, NDP binding to the $\alpha_3\beta$(Y341W)$_3\gamma$ subcomplex had similar K_d and ΔG^0 values as binding to the isolated β(Y341W) subunit, but the contributions of the enthalpy term and the entropy term were very different. We discuss these results in terms of the change in the tightness of the subunit packing, which reduces the excluded volume between subunits and increases water entropy.

INTRODUCTION

F_1-ATPase is a cytoplasmic portion of the F_oF_1-ATP synthase, which synthesizes ATP from ADP and inorganic phosphate (P_i) by using the energy of the proton flow driven by a transmembrane electrochemical proton gradient ($\Delta\mu H^+$) (1,2). By analyzing the oxygen exchange reactions, Boyer (3-5) established the binding change mechanism, whereby the proton translocation driven by $\Delta\mu H^+$ in the F_o portion is energetically coupled with the substrate binding and product release steps in F_1-ATPase. He further proposed a rotary mechanism in which single-copy subunits rotate within the $\alpha_3\beta_3$ cylinder (3–5) during catalysis. The rotary mechanism was strongly supported by the crystal structure of F_1-ATPase (6) and was finally envisioned by means of single-molecule experiments using the $\alpha_3\beta_3\gamma$ subcomplex (7). Single-molecule experiments further revealed that hydrolysis of one ATP molecule corresponded to a 120° step rotation (8) consisting of 80° and 40° substeps (9–11). ATP binding (11,12) together with ADP release (13) triggered the first 80° substep without cleavage of the chemical bond, which was in harmony with the binding change mechanism. The crystal structure indicated that the catalytic β subunit undergoes a large bending motion upon binding of nucleotides, and an NMR study revealed that a similar structural change occurs in an isolated β subunit (14,15) and a subcomplex in solution (16). These data indicate that nucleotide binding and release are critical for the rotation and hence the energy transduction by F_1-ATPase or F_oF_1-ATP synthase.

Many experimental techniques have been used to investigate nucleotide binding to both F_1-ATPase and ATP synthase. One of the most powerful of these methods involves the use of Trp mutants, whose responses to fluorescence indicate nucleotide binding or nucleotide-binding-induced conformational change (17–21). In this study, Y341 in the β subunit that interacts directly with the base of bound nucleotide was replaced with tryptophan (17–20). The indole ring of the introduced tryptophan stacks the base moiety of the nucleotide bound to the catalytic site, and its fluorescence quenching can serve as an optical probe for nucleotide binding to catalytic sites. In addition, to suppress the hydrolysis of the bound nucleotide during the experiments, we introduced a βE190Q mutation when necessary. The residue βE190 is known to play an indispensable role in catalysis (22), and this mutation suppressed the hydrolytic activity of the $\alpha_3\beta_3\gamma$ subcomplex almost completely. Then, using a mutant monomeric β subunit (βY341W) and a mutant $\alpha_3\beta_3\gamma$ subcomplex containing the βY341W mutation with or without the additional βE190Q mutation, we examined the binding of various nucleoside triphosphates (NTPs, i.e., ATP, GTP, and ITP) and nucleoside diphosphates (NDPs, i.e., ADP, GDP, and IDP). For nucleotide binding to the subcomplex, we focused on the third binding site because during the steady-state turnover, two catalytic sites are always occupied and NTP binding to the third site causes an 80° substep.

Submitted August 9, 2013, and accepted for publication October 15, 2013.

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Editor: Kazuhiro Oiwa.

0006-3495/13/12/2541/8 $2.00

http://dx.doi.org/10.1016/j.bpj.2013.10.018

We obtained thermodynamic parameters by conducting van't Hoff analyses on the temperature dependency of the dissociation constant (K_d). We observed that the Gibbs free-energy changes induced by NDP binding to the β(Y341W) monomer and the $\alpha_3\beta$(Y341W)$_3\gamma$ subcomplex were similar, but binding to the subcomplex resulted in less heat and more entropy production than binding to the β monomer. ADP binding to the β(Y341W/E190Q) monomer and $\alpha_3\beta$(Y341W/E190Q)$_3\gamma$ subcomplex showed a similar tendency. However, NTP binding to the β(Y341W/E190Q) monomer and $\alpha_3\beta$(Y341W/E190Q)$_3\gamma$ subcomplex showed the opposite tendency: NTP binding to the $\alpha_3\beta$(Y341W/E190Q)$_3\gamma$ subcomplex resulted in more heat and less entropy production. We discuss the results in relation to the effect of complex formation and the recently proposed water entropy effect (23,24).

MATERIALS AND METHODS

Strains, plasmids, and preparation of subcomplexes

Escherichia coli strain JM109 was used for plasmid amplification. JM103Δ(uncB-uncD) was used for overexpression of the β subunit and the $\alpha_3\beta_3\gamma$ subcomplex of F_1-ATPase from thermophilic bacterium PS3 (TF_1). The plasmids used were puc β, which carried the gene for the β subunit for both mutagenesis and expression (21), and pTABG1 and pKABG1 (25,26), which carried the genes for the α, β, and γ subunits of TF_1 for mutagenesis and gene expression, respectively. We introduced the βY341W mutation into puc β by ligating the MluI-SmaI fragment of pTABG1, which contained the βY341W mutation for nucleotide-binding measurements in the corresponding site of puc β (18). An α-W463F mutation was also introduced to reduce the background fluorescence during the nucleotide-binding measurements. We introduced the βE190Q mutation, which greatly reduces ATPase activity (27,28), to analyze NTP binding to the $\alpha_3\beta_3\gamma$ subcomplex with or without this mutation. For future studies of single-molecule rotation experiments, the mutations α-C193S, γ-S109C, and γ-I212C for specific biotinylation of the γ subunit (9) and β-His_{10} at the amino terminus (7) for fixation on Ni-NTA-coated glass were introduced.

The mutant β subunit and subcomplex containing the above mutations were overexpressed and isolated as described previously (25,26). Before conducting fluorescence-measurement experiments, we fractionated the proteins on a Superdex 200 gel filtration column (GE Healthcare, Little Chalfont, United Kingdom) using 50 mM 3-morpholinopropane-sulfonic acid-KOH (pH 7.0) buffer containing 50 mM KCl, and 2 mM $MgCl_2$ (MKM buffer) to remove aggregated and/or disassembled subunits.

Fluorescence measurements

Nucleotide binding was monitored by the decrease in fluorescence of the genetically introduced Trp (βY341W). Fluorescence measurements were performed with an FP-6500 spectrofluorometer (JASCO, Tokyo, Japan). Typically, the excitation wavelength was 300 nm and the emission wavelength was 345 nm, with a slit width of 3 and 10 nm, respectively, for time-course measurements. In the case of GTP/GDP binding, the excitation wavelength was set to 312 nm to avoid the absorbance of GTP/GDP at 300 nm. The concentration of the subcomplex was typically 50 nM, and for the GTP/GDP-binding studies, it was increased to 300 nM. The concentration of the isolated β subunit was double that of the subcomplex (100 nM or 600 nM for GTP/GDP). For time-course measurements, aliquots of concentrated nucleotide solutions containing equimolar $MgCl_2$ were injected into solutions of the β subunit or the subcomplex in MKM buffer while stirring. By circulating temperature-controlled antifreezing liquid, we performed the measurements between 2°C and 50°C. Dry air was introduced to prevent condensation at low temperatures.

Data analyses

Fluorescence titration of the isolated β subunit with various nucleotides

Fig. 1 *A* shows the titration of MgATP binding to the isolated β(Y341W) subunit through successive addition of small aliquots of concentrated MgATP solution. After correcting for the small effects of dilution, the fluorescence decrease (ΔF) was plotted against nucleotide concentration (Fig. 1 *B*) and the dissociation constant for MgATP was obtained by curve fitting using Eq. 1:

$$\Delta F = \Delta F_{monomer} \times \frac{[MgATP]}{Kd_{monomer} + [MgATP]} \quad (1)$$

Here, ΔF, $\Delta F_{monomer}$, and $K_{dmonomer}$ represent the fluorescence decrease at each MgATP concentration, the total fluorescence decrease at saturating MgATP, and the dissociation constant for MgATP, respectively. We carried

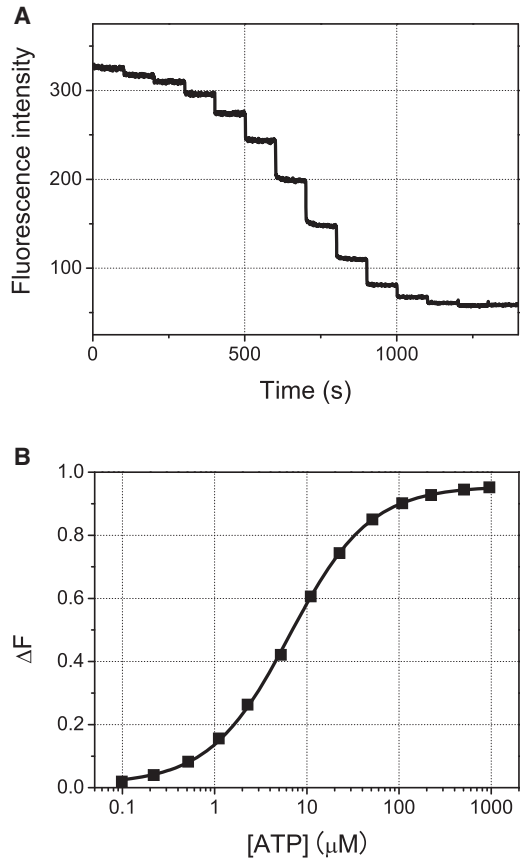

FIGURE 1 Fluorescence titration of MgATP binding to the isolated β(Y341W) subunit. (*A*) Quenching of the fluorescence of the reporter tryptophan by successive addition of concentrated MgATP solutions. The data point at zero concentration was determined with air. The concentration of the subunit was 100 nM and measurement was carried out at 25°C. (*B*) Titration curve constructed after correcting for the small effect of dilution. The curve is a best fit based on Eq. 1.