Kinetic analysis of the inhibition mechanism of bovine mitochondrial F\textsubscript{1}-ATPase inhibitory protein using biochemical assay

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Running title: Inhibition mechanism of IF\textsubscript{1}

Keywords: ATPase inhibitory factor 1 (IF\textsubscript{1}), ATP synthase, enzyme kinetics, F\textsubscript{1}-ATPase, rotary molecular motor

Abbreviations: F\textsubscript{1}, F\textsubscript{1}-ATPase; F\textsubscript{o}F\textsubscript{1}, F\textsubscript{o}F\textsubscript{1}-ATP synthase; pmf, proton motive force; bMF\textsubscript{1}, F\textsubscript{1} from bovine mitochondria; TF\textsubscript{1}, F\textsubscript{1} from thermophilic Bacillus PS3; IF\textsubscript{1}, ATPase Inhibitory factor 1; GFP,
green fluorescent protein; His-tag; histidine tag; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; KPi; potassium phosphate buffer; TEV, tobacco etch virus; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; MALDI-TOF/TOF, Matrix Assisted Laser Desorption / Ionization - Time-of-Flight/Time-of-Flight mass spectrometer.
Abstract

ATPase inhibitory factor 1 (IF\(_1\)) is a mitochondrial regulatory protein that blocks ATP hydrolysis of F\(_1\)-ATPase, by inserting its \(N\)-terminus into the rotor-stator interface of F\(_1\)-ATPase. Although previous studies have proposed a two-step model for IF\(_1\)-mediated inhibition, the underlying molecular mechanism remains unclear. Here, we analyzed the kinetics of IF\(_1\)-mediated inhibition under a wide range of [ATP]s and [IF\(_1\)]s, using bovine mitochondrial IF\(_1\) and F\(_1\)-ATPase. Typical hyperbolic curves of inhibition rates with [IF\(_1\)]s were observed at all [ATP]s tested, suggesting a two-step mechanism: the initial association of IF\(_1\) to F\(_1\)-ATPase and the locking process, where IF\(_1\) blocks rotation by inserting its \(N\)-terminus. The initial association was dependent on ATP. Considering two principal rotation dwells, binding dwell and catalytic dwell, in F\(_1\)-ATPase, this result means that IF\(_1\) associates with F\(_1\)-ATPase in the catalytic-waiting state. In contrast, the isomerization process to the locking state was almost independent of ATP, suggesting that it is also independent of the F\(_1\)-ATPase state.

Further, we investigated the role of Glu30 or Tyr33 of IF\(_1\) in the two-step mechanism. Kinetic analysis showed that Glu30 is involved in the isomerization, whereas Tyr33 contributes to the initial association. Based on the present findings, we propose an IF\(_1\)-mediated inhibition scheme.
Introduction

ATP synthase, also termed as F_oF_1-ATP synthase (F_oF_1), is ubiquitously found in bacterial plasma membranes, chloroplast thylakoid membranes, and mitochondrial inner membranes (1–5). It catalyzes ATP synthesis from ADP and inorganic phosphate (P_i) using proton motive force (pmf) across membranes. F_oF_1-ATP synthase consists of two reversible rotary motors, F_o and F_1. F_o, the membrane-embedded portion of ATP synthase, conducts proton translocation across the membrane, whereas F_1, the water-soluble portion, contains the catalytic center domain for ATP synthesis. In the F_oF_1 complex, F_o and F_1 are connected by the common rotary shaft and the peripheral stalk, enabling the interconversion of pmf and the chemical potential of ATP. Under physiological conditions with sufficiently high pmf level, F_o reverses the rotation of F_1, inducing ATP synthesis. When pmf is low or diminished, F_1 hydrolyzes ATP to rotate F_o in the opposite direction, resulting in active proton pumping to form pmf.

F_1 hydrolyzes ATP to ADP and P_i when isolated from F_o (6, 7). α_3β_3γ is the minimum component of a rotary molecular motor. Three α subunits and three β subunits are arranged alternately to form the α_3β_3 stator ring, of which the central rotary shaft, the γ subunit, is inserted into the central cavity (8, 9). The catalytic reaction centers are located on the αβ interface, mainly on the β subunit. During ATP hydrolysis, the γ subunit of F_1 rotates against the α_3β_3-ring in anticlockwise direction.
when viewed from the F, side (5). Crystal structures of bovine mitochondrial F₁-ATPase (bMF₁) showed asymmetric features of nucleotide occupancy and the conformational states of the three β subunits (9–12). One β subunit, designated β₁TP, preferentially binds to an ATP analog, AMP-PNP, whereas another one, β₁DP, binds to ADP and P, or P, analogs, representing the catalytically active state. The third one, β₁E, has no nucleotides, although some crystal structures show that it has phosphate (9), thiophosphate (11), or sulfate ions (12), suggesting that β₁E represents the phosphate releasing state (13). Both β₁TP and β₁DP adopt a closed conformational state in which the C-terminal domain swings toward the γ subunit, wrapping the bound nucleotide, whereas β₁E assumes an open conformation.

The coupling reaction scheme for the rotation and catalysis of bMF₁ was recently studied in a single-molecule rotation study (14). Similar to the reaction scheme for thermophilic F₁ (TF₁), bMF₁ makes rotation with 120° steps, each resolved into 80° and 40° substeps. The 80° and 40° substeps are triggered by ATP binding and hydrolysis, respectively. Therefore, the dwelling states before the 80° and 40° substeps are referred to as “binding dwell” and “catalytic dwell,” respectively. In addition, bMF₁ exhibits a short transient pause between the binding dwell and the catalytic dwell. The reaction step involved in the short transient pause remains to be identified. Previous studies have shown that the crystal structures of bMF₁ in the ground state or relevant states correspond to the catalytic dwell in the rotation assay (14–16).
There are diverse mechanisms for the suppression of ATP hydrolysis by F₁, which is generally detrimental to cells (17–19). The self-inhibition of F₁, termed ADP inhibition, is the most universal mechanism for F₁ inhibition (14, 20, 21). Inhibitory factor 1 (IF₁) of mitochondrial F₁ inhibits unfavorable ATPase. Under inhibitory conditions, IF₁ associates with F₁ to block ATP hydrolysis by inserting its N-terminal region into FₒF₁-ATP synthase, which results in mechanical blockage of rotation and catalysis. Bovine mitochondrial IF₁ is composed of 84 amino acid residues. IF₁ inhibits the function of FₒF₁-ATP synthase under hydrolytic condition to prevent wasteful consumption of ATP. Thus, it is an unidirectional inhibitor of FₒF₁-ATP synthase (22), although several reports have suggested that IF₁ also has an inhibitory effect under synthesized condition (23, 24). Among the 84 residues in bovine IF₁, the N-terminus is responsible for the inhibition of FₒF₁-ATP synthase, forming a long α-helix when bound to F₁. When isolated from F₁, the N-terminus of IF₁ is intrinsically disordered (25). Native IF₁ forms a homodimer associating at the C-terminal region (26, 27). Deletion of C-terminus residues 61–84 produces a stable monomeric form of IF₁ without the loss of inhibition activity (27, 28). Thus, the C-terminus deleted form of IF₁ (termed IF₁¹⁶⁰ hereafter) provides a simple platform for biochemical and structural analyses of IF₁ (27, 29, 30). Biochemical assay showed that the fusion of green fluorescent protein (GFP) and 6 histidine tag (His-tag) to the C-terminus in IF₁¹⁶⁰ (IF₁¹⁶⁰GFPHis) has little impact on its inhibitory capacity (29).
The first crystal structure of the complex of bMF₁ and IF₁₁⁻⁶⁰ showed that the N-terminal helix of IF₁₁⁻⁶⁰ was deeply buried in the αDP/βDP interface in its locked state (27). Following this, the crystal structure of the bMF₁-(IF₁₁⁻⁶⁰)₃ complex was resolved, revealing that each of the three α/β interfaces was occupied with IF₁ (30). Each IF₁ bound to the α/β interface differed in conformation: IF₁ at the αDP/βDP interface showed the most folded state, as found in the first bMF₁-IF₁ structure; IF₁ at αTP/βTP showed partially folded α-helix with unfolded N-terminal region; and IF₁ at αE/βE was largely disordered. These findings suggest progressive conformational isomerization of IF₁ from a disordered state to the α-helical state, accompanying the conformational transition of the α/β pair from αE/βE to αDP/βDP. As the conformational transition of the α/β pair is tightly coupled with γ rotation, the progressive conformational isomerization model inevitably assumes that IF inhibition accompanies γ rotation (Fig. 1A). Comprehensive studies of mutagenesis based on the crystal structure revealed that Glu30 and Tyr33 of IF₁ are particularly critical for IF₁ inhibition. Glu30 and Tyr33 form a salt bridge and hydrophobic interaction with a residue of the β subunit, respectively. Ala substitution causes a loss of the inhibitory activity of IF₁ (29). However, the roles of these residues in the proposed progressive conformational change model remain to be elucidated.

Biochemical studies have revealed that IF₁ inhibition requires catalytic turnover of F₁₆; in the absence of ATP, IF₁ only shows slow and partial inhibition of F₁ (31, 32). In the presence of ATP, IF₁
shows rapid inhibition during turnover (33). Kinetic analyses showed that the rate constant of IF$_1$-mediated inhibition increases with [ATP], although some complex behaviors of IF$_1$ inhibition were observed, such as a decrease in the inhibition rate at the mM range of [ATP] (31, 32, 34). The correlation of IF$_1$ inhibition with occupancy of the catalytic site was also studied (31, 32, 35). Based on kinetic analyses, an ATP-dependent two-step model has been proposed for IF$_1$-mediated inhibition (34, 36, 37). The two-step model suggests that the rate constant of IF$_1$-mediated inhibition should increase with [IF$_1$], reaching an ATP-dependent plateau. However, comprehensive kinetic analysis covering a wide range of IF$_1$ and ATP concentrations to confirm the expected hyperbolic curves has not been performed yet.

In the present study, we studied the kinetics of bMF$_1$ inhibition by bovine mitochondrial IF$_1$ with an NADH-coupled ATP-regenerating system, using a wide range of [ATP] from 100 nM to 1 mM with [IF$_1$] from 0.05 μM to 40 μM. The resultant rate constant of IF$_1$-mediated inhibition showed typical hyperbolic curves for [IF$_1$] at each [ATP], consistent with the two-step model. We then investigated the IF$_1$ mutants IF$_1$(E30A) and IF$_1$(Y33A) to study the effects of Glu30 and Tyr33, respectively, on the kinetics of IF$_1$ inhibition. Based on the results as well as the established reaction scheme for catalysis and rotation of bMF$_1$ (14), we propose an IF$_1$ inhibition scheme.
Materials and Methods

Construct and purification of IF1

An expression plasmid encoding residues 1-60 of bovine IF1 was constructed as follows: His-tag and TEV site were fused to the N-terminal region, and the linker and mScarlet sequences were fused to the C-terminal region. The resulting artificially synthesized construct was introduced into the pRSET-B plasmid, which encoded the protein IF1-60 mScarlet (hereafter referred to as wild-type IF1-60 or IF1).

IF1-60(ΔmS) was generated by deleting the linker and mScarlet sequences from the wild-type IF1-60 plasmid. The single point amino acid mutants, E30A and Y33A, were introduced into the wild-type IF1-60 plasmid. The sequence encoding IF1-60 or a mutation was amplified by PCR. After gel electrophoresis and purification, the product was digested with two restriction enzymes and cloned into the vector wild-type IF1-60 plasmid, digested with the same restriction enzymes. The sequences of the recombinant plasmids for IF1 were confirmed by Fasmac sequencing service (Fasmac, Japan).

Cells of Escherichia coli C43 were transformed with the constructed plasmids and grown in LB medium containing 100 μg/mL carbenicillin at 37°C for 4 h as a preculture. The culture medium was then transferred to SB medium containing 100 μg/mL carbenicillin at 37°C. When the absorbance
of the culture was 0.6 at 600 nm (for ~4 h), isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM to induce protein expression. After 24 h of growth at 20°C, cells were harvested by centrifugation (7000×g, 8 min, 4°C). Subsequent procedures were performed at 4°C, except for the gel-filtration process. The harvested cells were suspended in buffer A [50 mM KPi (pH 7.5), 200 mM KCl, 10% Glycerol, and 25 mM Imidazole], disrupted by an ultrasound disintegrator, and subjected to ultracentrifugation (81,000×g, 20 min). The supernatant was applied to Ni-Sepharose FF resin (GE Healthcare) equilibrated in buffer A. After binding IF₁ to the resin, it was washed with 10 volumes of buffer A. IF₁ was eluted with elution buffer [50 mM KPi (pH 7.5), 200 mM KCl, 10% glycerol, and 500 mM imidazole].

To remove His-tag, TEV protease was used. The eluted fractions containing the proteins were concentrated with a centrifugal concentrator (3 kDa for IF₁₁-60(ΔmS), and 10 kDa for wild-type IF₁₁-60 and mutants; Centricon50; Millipore Corp.). The concentrated fractions were diluted 20-fold with TEV treatment buffer [20 mM KPi (pH 8.0), 50 mM NaCl 0.04% 2-mercaptoethanol, and 0.1 mg/mL TEV protease]. After treatment for 16 h at 4°C, the solution was concentrated with a centrifugal concentrator and diluted 30-fold with buffer A. The resultant solution was applied to a Ni-Sepharose FF resin. The flow-through and wash fractions were concentrated with a centrifugal concentrator after adding DTT at a final concentration of 5 mM. The resulting samples were further purified by passing
through a gel-filtration column (Superdex 75 for IF$_1^{1-60}$(ΔmS), and Superdex 200 for wild-type IF$_1^{1-60}$ and mutants; GE Healthcare) equilibrated with Gel-filtration buffer [20 mM HEPES-KOH (pH7.5), 100 mM KCl, and 10% glycerol]. If necessary, the fractions were concentrated with a centrifugal concentrator. The concentration of IF$_1$ was determined based on the absorbance at 280 nm. The purified samples were flash-frozen in liquid nitrogen and stored at -80°C before use. Their molecular masses were verified by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) analysis (Fig. S1). Further confirmation of the molecular masses for wild-type IF$_1^{1-60}$ and IF$_1^{1-60}$(ΔmS) was performed by MALDI-TOF/TOF mass spectrometry (Genomine. Inc., Korea) (Table. S1).

**Construct and purification of bMF$_1$**

bMF$_1$ was prepared as previously described (14), with slight modifications. We removed ATP from buffers A, B, and gel-filtration buffer for measurements of ATPase activity at low ATP conditions because the carryover ATP from the purified bMF$_1$ sample to the cuvette was not negligible under these conditions. The ATPase activity of bMF$_1$ purified without ATP was identical to that purified with ATP.

**Biochemical assay with NADH-coupled ATP-regenerating system**
As previously reported (14), the ATPase activity of \( bMF_1 \) was measured using a spectrometer at 25°C in HEPES-KOH (pH 7.5) containing KCl, MgCl\(_2\), and an ATP-regenerating system supplemented with NADH and lactate dehydrogenase. The ATPase reaction was initiated by adding purified \( bMF_1 \).

IF\(_1\) was added 180 s after the addition of \( bMF_1 \) to avoid ADP inhibition. The change in NADH absorbance was monitored for over 10 min. All data points were measured at least in triplicate. The apparent rate constants for IF\(_1\) inhibition (\( k_{\text{app inhibition}} \)) were measured from the exponential decay of ATPase activity after IF\(_1\) addition, using the following equation:

\[
y(t) - y_0 = V_\infty t + \frac{V_0 - V_\infty}{k_{\text{app inhibition}}} \{1 - \exp\left(-\frac{t}{k_{\text{app inhibition}}}\right)\} \tag{Eq. 1}
\]

where \( y(t) \) and \( y_0 \) are the absorbances at time points \( t \) and 0 after IF\(_1\) addition, respectively, and \( V_0 \) and \( V_\infty \) are the initial and final rates of reaction, respectively. For precise fitting, we have fitted the time course 60 s after IF\(_1\) injection to remove noise caused by solution injection. Examples of \( V_0 \) and \( V_\infty \), estimated in Fig. 2B at 1 mM ATP, were shown in Fig. S6.

**Modeling the two-step reaction of IF\(_1\) inhibition**

The kinetic scheme is as follows:

\[
F_1 + IF_1 \xrightleftharpoons[k_{\text{off}}]{k_{\text{on}}} F_1 \cdot IF_1 \xrightarrow{k_{\text{lock}}} F_1 \cdot IF_1^{\text{lock}} \tag{Scheme. 1}
\]

where \( k_{\text{on}} \) is the rate constant for IF\(_1\) binding to
$F_1$, $k_{off}$ is the rate constant for IF$_1$ release from F$_1$, and $k_{lock}$ is the rate constant for isomerization to the locked state. F$_1$ and $F_1 \cdot IF_1$ are active and $F_1 \cdot IF_1^{lock}$ is inactive. In the first step, IF$_1$ loosely binds to F$_1$, forming an intermediate state. Following this, IF$_1$ is irreversibly locked, forming a dead-end complex. For derivation of $k_{app}^{inhibition}$, see the supplemental text.
Results

Preparation of IF$_1$ protein

C-terminal residues of IF$_1^{1-60}$ in bovine IF$_1$ were fused with mScarlet, a bright monomeric red fluorescent protein with a short linker sequence, to enhance its expression in E. coli, as previously described (29). The fusion IF$_1$ is hereafter referred to as wild-type IF$_1^{1-60}$ or IF$_1^{1-60}$ for simplicity, unless mentioned otherwise. For comparison, we also purified IF$_1^{1-60(ΔmS)}$ lacking the linker sequence and the mScarlet domain. For the preparation of IF$_1^{1-60(E30A)}$ or IF$_1^{1-60(Y33A)}$, a single point mutation was introduced into the wild-type IF$_1^{1-60}$ plasmid. For purification with an Ni-Sepharose column, the His-tag and TEV recognition sequences were fused to the N-terminal region of the IF$_1$ proteins. After collecting the eluted fractions from the Ni-Sepharose column, the His-tag was cleaved with TEV-protease. The resultant products were identified by SDS-PAGE analysis (Fig. S1) and mass spectroscopy (Table. S1).

Time course of IF$_1$-mediated inhibition

ATP hydrolysis activity of bMF$_1$ was monitored using an ATP regenerating system. The decay of NADH absorbance at 340 nm represents ATP hydrolysis leading to NADH oxidation. Upon
injection, bMF₁ initially showed rapid catalysis, followed by slow deceleration to reach steady-state catalysis (Fig. 1B). The slow auto-inactivation is a typical feature of ADP-inhibition. Under the present conditions, bMF₁ activity almost reached steady state within 180 s (Fig. S2). Subsequently, IF₁ was injected at 180 s after F₁ injection. Since the time constant for ADP inhibition at 1 μM ATP was longer than 400 s, ADP inhibition didn’t reach an equilibrium state at the time of IF₁ injection. However, additional analysis of IF₁ inhibition including ADP inhibition (Supplemental text and Fig. S3) revealed that it has little impact on IF₁ kinetics, probably due to the slow and modest suppression at 1 μM ATP.

Fig. 2A shows the typical time courses of IF₁-mediated inhibition at 1 mM [ATP]. Immediately after the injection of IF₁₁-60, ATPase activity decreased, reaching almost zero activity. The time course of IF₁-mediated inhibition was well fitted with an exponential function (Eq. 1), giving the apparent rate constant of the inhibition, \( k_{\text{app, inhibition}} \). The inhibition rate increased with [IF₁₁-60], reaching a plateau when [IF₁₁-60] was over 0.65 μM. We also measured the time courses of IF₁-mediated inhibition to determine \( k_{\text{app, inhibition}} \) at all [ATP]s ranging from 100 nM to 1 mM. In Fig. 2B and Fig. S4, data points of \( k_{\text{app, inhibition}} \) are plotted against [IF₁₁-60] at all [ATP]s. At a given [ATP], \( k_{\text{app, inhibition}} \) always increases with [IF₁₁-60] and reaches a plateau, following a hyperbolic curve. Further, we tested the inhibitory capacity of a monomer IF₁ without mScarlet (IF₁₁-60(ΔmS)) and found that the result was almost identical to that of wild-type IF₁₁-60 (Fig. S5), suggesting that mScarlet in the C-
terminal region did not affect the kinetics of IF\textsubscript{1}-mediated inhibition. It should be noted that previous biochemical assay based on mutagenesis (29) showed monotonous enhancement of $k_{\text{app}}^{\text{inhibition}}$, whereas clear hyperbolic curve was observed in our assay. Such an apparent difference is attributable to low [IF\textsubscript{1}] employed in the assay (29), which enabled to visualize the limited region of the hyperbolic curve in the 2-step inhibition mechanism. Another possible reason for this discrepancy is the difference in experimental conditions: including pH, temperature, and chemicals.

The observed hyperbolic curves suggest a two-step model, where IF\textsubscript{1} reversibly associates with F\textsubscript{1}, and the resulting $F_1 \cdot IF_1$ complex isomerizes to the final locked state. Thus, we assume the following reaction scheme for IF\textsubscript{1}-mediated inhibition:

$$F_1 + IF_1 \xleftrightarrow[k_{\text{on}}]{k_{\text{off}}} F_1 \cdot IF_1 \xrightarrow{k_{\text{lock}}} F_1 \cdot IF_1^{\text{lock}}$$

(Scheme. 1)

where, $F_1 \cdot IF_1^{\text{lock}}$ represents the catalytically locked state of the $F_1 \cdot IF_1$ complex. $k_{\text{on}}$ and $k_{\text{off}}$ represent the rate constants of association and dissociation, respectively. $k_{\text{lock}}$ is the rate constant of isomerization to the locked state. This scheme calculates the apparent rate constant of IF\textsubscript{1} inhibition, $k_{\text{inhibition}}^{\text{app}}$, as follows:

$$k_{\text{inhibition}}^{\text{app}} = \frac{[IF_1]}{K_M^{IF_1} + [IF_1]} \cdot k_{\text{lock}}$$

(Eq.2)

The experimentally obtained data points at each [ATP] were well fitted with Eq. 2, giving $k_{\text{lock}}$ and $K_M^{IF_1}$. 

$$K_M^{IF_1} \equiv \frac{k_{\text{off}} + k_{\text{lock}}}{k_{\text{on}}}$$

(Eq.3)
[ATP] dependence of $k_{lock}$ and $K_{M}^{IF_1}$

Fig. 3A shows the plot of $k_{lock}$ against [ATP]. Although not well constant, $k_{lock}$ was around 0.02 s⁻¹ irrespective of [ATP]; this value is almost consistent with that estimated from previous studies (38, 39). $k_{lock}$ is evidently lower than the catalytic turnover rate, 1-200 s⁻¹, indicating that IF₁ transforms into the inhibitory locking state during the rotation of the γ subunit. Fig. 3B shows the plot of $K_{M}^{IF_1}$ against [ATP]. $K_{M}^{IF_1}$ was determined to be in the sub-μM to μM range, consistent with that of previous biochemical studies with isolated F₁ and sub-mitochondrial particles (31–34, 38). $K_{M}^{IF_1}$ reveals a clear [ATP] dependence, decreasing from 27 μM to 0.1 μM when [ATP] is over 10 μM. Considering constant $k_{lock}$ over [ATP], this means that the rate constant of IF₁ association to F₁ follows a hyperbolic increment with [ATP].

Here, we define the effective rate constant of IF₁ association to F₁ as follows:

$$k_{eff}^{on} \equiv \frac{k_{lock}}{K_{M}^{IF_1}}$$

(Eq. 4)

Fig. 3C shows the plot of $k_{eff}^{on}$ against [ATP]. As expected, $k_{eff}^{on}$ shows a typical hyperbolic saturation curve. Considering that IF₁ cannot bind ATP by itself, it is reasonable to attribute the ATP binding to F₁, c.f. IF₁ is predominantly associated with F₁ in the post-ATP-bound state. In light of the reaction scheme for rotation and catalysis, this means that IF₁ is not associated with F₁ in the binding dwell, but it preferentially associates with F₁ in the catalytic dwell.

With the assumption that IF₁ preferentially binds to F₁ in the catalytic dwell, we tested the
mutant bMF$_1$(βE188D). Previous studies have shown that Glu188 of bMF$_1$ or the corresponding glutamic residues of other F$_1$s are the most critical residues for catalysis (8, 16, 40). Mutagenic substitution of Glu188 with aspartic acid largely retarded bMF$_1$ catalysis, lengthening the catalytic dwell by 400 times (14). We investigated IF$_1$-mediated inhibition of bMF$_1$(βE188D) at 1 mM [ATP] (Fig. S7). Contrary to the expectation, bMF$_1$(βE188D) did not exhibit enhanced IF$_1$ inhibition rate compared with wild-type bMF$_1$ at saturating [ATP]s. The hyperbolic inhibition curve of bMF$_1$(βE188D) at 1 mM [ATP] was similar to that of wild-type bMF$_1$ at 100 nM [ATP], where the binding dwell is dominant (Fig. 2B and Fig. S4). Considering that F$_1$ mediates two reactions through the catalytic dwell, hydrolysis and presumably inorganic phosphate release, the catalytic state and the conformation of bMF$_1$(βE188D) at the catalytic dwell maybe different from that of the wild-type in some aspects.

**Mutation of Glu30 and Tyr33**

A previous comprehensive mutagenetic study identified E30 and Y33 as the most critical residues for the inhibitory function of IF$_1$ (30). Although the mutants created by substituting these residues with alanine suppressed the activity down to an undetectable level (29), we reinvestigated these mutants, IF$_1^{1-60}$(E30A) and IF$_1^{1-60}$(Y33A), in our experimental setup.
Experiments were conducted using 1 mM [ATP]. Fig. 4A shows the time course of its inhibition by IF$_{1-60}$(E30A) at 0.5, 1, and 3 μM concentrations, which are similar to the range for wild-type IF$_{1-60}$. ATPase activity showed slight decay and reached steady state with some activity remaining. Compared to wild-type, where the complete inhibition was observed (Fig. 2A), the inhibition by IF$_{1-60}$(E30A) was not irreversible. Based on the exponential fitting of the time courses in Eq. 1, $k_{app}^{inhibition}$ of IF$_{1-60}$(E30A) was determined (Fig. 4B, green) and found to be quite similar to that of wild-type IF$_{1-60}$ (Fig. 4B, gray) at each [IF$_{1-60}$(E30A)]. The resultant $k_{lock}$ and $k_{off}$ were almost consistent with those for the wild-type IF$_{1-60}$ (Table. S2). These results show that the E30A mutant also follows a two-step mechanism for inhibition. However, IF$_{1-60}$(E30A)-mediated inhibition is evidently less efficient, as seen in the partial inhibition of ATPase activity. Fig. 4C shows the final ATPase activity observed at the end of the measurement (360 s after IF$_1$ injection). While wild-type IF$_1$ suppresses ATPase activity down to almost zero at each [IF$_{1-60}$], IF$_{1-60}$(E30A) suppresses ATPase activity down to only 20-40% at [IF$_{1-60}$(E30A)]'s lower than 1 μM. Even at 3 μM [IF$_{1-60}$(E30A)], where $k_{app}^{inhibition}$ reached a plateau, a fraction of bMF$_1$ retained its activity.

The Y33A mutant, IF$_{1-60}$(Y33A), was also examined at 1 mM ATP. Fig. 4D shows the time course of its inhibition. This mutant required a significantly higher concentration of [IF$_{1-60}$(Y33A)], at least 10 μM [IF$_{1-60}$(Y33A)], to induce clear inhibition. However, in contrast to the E30A mutant,
the Y33A mutant suppressed ATPase activity to almost zero (Fig. 4F). These observations suggest that the Y33A mutant has high inhibitory activity, although the time scale for its association with \( F_1 \) was significantly longer than that for the wild-type IF\(_1^{1-60}\). Fig. 4E shows \( k_{\text{app inhibition}} \) of IF\(_1^{1-60}(\text{Y33A})\), which follows a hyperbolic curve consistent with the two-step model of IF\(_1\) inhibition. The plateau determined the \( k_{\text{lock}} \) of IF\(_1^{1-60}(\text{Y33A})\) to be 0.019 s\(^{-1}\), which is very similar to that of wild-type IF\(_1^{1-60}\). The significant difference was observed for \( K_M^{IF_1} \), which was 25 \( \mu \text{M} \) in case of IF\(_1^{1-60}(\text{Y33A})\), 160 times higher than that of wild-type IF\(_1^{1-60}\) (Table. S2).

Thus, the effect of the mutations contradicted each other. Although the E30A mutant had normal \( k_{\text{app inhibition}} \), it was evidently deficient in locking the catalysis of \( F_1 \). The Y33A mutant was quite slow for the association with \( F_1 \) and had lower \( k_{\text{app inhibition}} \) and higher \( K_M^{IF_1} \), although this mutant finally locked the catalysis almost completely.
Discussion

[ATP] dependence of $k_{\text{eff}}^{\text{on}}$

The present study showed that the effective binding rate of IF1 to bMF1, $k_{\text{eff}}^{\text{on}}$, evidently followed an [ATP]-dependent saturation curve (Fig. 3C). As IF1 itself cannot bind ATP, the [ATP] dependence of $k_{\text{eff}}^{\text{on}}$ can be attributed to bMF1. As IF1 inhibition requires catalytic turnover of bMF1, it is reasonable to assume that the [ATP] dependence is the result of the Michaelis-Menten kinetics of ATPase activity. Kinetic analysis of bMF1 rotation (14) shows that F1 principally has two conformational states: binding dwell and catalytic dwell. Here, we neglect the short dwell between the binding dwell and the catalytic dwell because its duration is significantly shorter than those of the other dwells. The duration of the binding dwell is inversely proportional to [ATP] ($\approx 1/(k_{\text{ATP}}^{\text{on}} [\text{ATP}])$), whereas that of the catalytic dwell is constant at approximately 0.3 ms, irrespective of [ATP] (14).

Thus, we consider the following two states of F1:

\[
F_1 (\text{binding dwell}) \xrightarrow{k_{\text{ATP}}^{\text{on}} [\text{ATP}]} k_{\text{ATP}} \xrightarrow{k_{\text{cat}}^{\text{on}}} F_1 (\text{catalytic dwell}) \#(\text{Scheme. 2})
\]

where, $k_{\text{ATP}}^{\text{on}}$ represents the rate of the catalytic dwell (~2100 s$^{-1}$) and $k_{\text{ATP}}^{\text{on}} [\text{ATP}]$ is the rate of ATP binding to bMF1. The simplest assumption to explain the [ATP] dependence of $k_{\text{eff}}^{\text{on}}$ is that IF1 preferentially associates with F1 in the catalytic dwell. Here, we calculate the duty ratio of the catalytic dwell in the overall
reaction time \( (R_c) \) as follows:

\[
R_c = \frac{[ATP]}{K_m^{ATP} + [ATP]} \quad \text{(#Eq.5)}
\]

where, \( K_m^{ATP} \) is defined as \( k_{cat}^{ATP} / k_{on}^{ATP} \) from the Michaelis-Menten fitting of the rotation speed, 77 \( \mu \)M (14). Along with Eq. 4 and Eq. 5, we re-define \( k_{on} \) and \( k_{on}^{eff} \) as follows:

\[
k_{on} = R_c \cdot k_{on}' \quad \text{(Eq.6)}
\]

\[
k_{on}^{eff} = \frac{k_{lock}}{k_{lock} + k_{off}} \cdot k_{on} = \frac{k_{lock}}{k_{lock} + k_{off}} \cdot R_c \cdot k_{on}' \quad \text{(Eq.7)}
\]

where, \( k_{on}' \) is the genuine rate constant of IF\(_1\) binding to F\(_1\) in the catalytic dwell. In Fig. 3C, the calculated \( R_c \) (orange) is plotted with the experimental data points of \( k_{on}^{eff} \) (gray) after normalization. The experimental data points were fitted to determine \( k_{on}' \) and \( K_m^{ATP} \). Since we haven’t estimated \( k_{off} \) from our experimental data, we simply assume \( k_{off} \) as 0.0017 \( \text{s}^{-1} \), which was previously estimated (29). The resultant \( k_{on} \) was determined to be \( 1.5 \times 10^5 \text{ M}^{-1}\text{s}^{-1} \), in good agreement with previous studies that determined \( k_{on}' \) in the order of \( 10^4 \text{ to } 10^6 \text{ M}^{-1}\text{s}^{-1} \) \( (29, 31, 32, 34) \). However, \( K_m^{ATP} \) was determined to be 9 \( \mu \)M, which was obviously lower than the expected concentration of 77 \( \mu \)M. One of the possible explanations for this is the difference in experimental conditions: the rotation assay that determined \( K_m \) to be 77 \( \mu \)M selectively analyzed actively rotating particles, whereas this study was based on a solution experiment where the value was averaged over molecules including those in the ADP-inhibited form. However, since the determined \( K_m \) from ATPase measurement was 218 \( \mu \)M (14), we don’t have convincing interpretations at this moment. Future single-molecule analysis of IF\(_1\) inhibition is required to
Proposed IF\textsubscript{1} inhibition scheme and correlation with crystal structures

The present study observed hyperbolic curves at different [ATP]s that allows us to discuss the IF\textsubscript{1} inhibition mechanism based on the rotary catalysis of bMF\textsubscript{1}. We also carefully examined the mutant IF\textsubscript{1}s with a mutation at critical points: Glu30 and Tyr33. These mutants significantly affect the IF\textsubscript{1} inhibition at different processes. Taking the present results into account, together with those of previous single-molecule analyses (14) and structural studies (30), we propose the following IF\textsubscript{1}-mediated inhibition mechanism (Fig. 5), where the role of Glu30 and Tyr33 in the two-step inhibition by IF\textsubscript{1} was clarified as well as the catalytic state of bMF\textsubscript{1}. Initially, IF\textsubscript{1} weakly associates with F\textsubscript{1} in the catalytic dwell. This weakly bound state corresponds to the intermediate state in the two-step model, as represented \(F_1 \cdot IF_1\) in Scheme 1, which undergoes reversible association and dissociation.

We assume that this state represents IF\textsubscript{1} bound to the \(\alpha_E\beta_E\) site in the crystal structure of the bMF\textsubscript{1}-(IF\textsubscript{1}\textsuperscript{1-60})\textsubscript{3} complex (30), where only a small part of the N-terminal helix is folded and associates with \(\beta_E\) via a few interactions at distal points to the \(\gamma\) subunit. In this state, a large part of the N-terminal helix remains unfolded and IF\textsubscript{1} principally does not interfere with \(\gamma\) rotation and catalysis. Tyr33 of IF\textsubscript{1} interacts with Lys401 of \(\beta_E\) in its crystal structure. The present study showed that Y33A mutation
of IF₁ slows the association step of IF₁, in consistent with the crystal structure. Therefore, it is reasonable that the Y33A mutation largely suppresses the formation of the initial F₁-IF₁ complex.

After the formation of the initial F₁-IF₁ complex, IF₁ slowly transforms its conformation to the fully folded state, inserting the N-terminal helix deeply into the αβ interface, as seen in IF₁ at the α₀ββ₀ site in its crystal structure. This state completely locks the rotation and catalysis of F₁ (F₁ · I

\[ F₁^{\text{lock}} \]) in Scheme 1). In the crystal structure, Glu30 of IF₁ forms a salt bridge with Arg408 of β₀DP or β₀TP but not with Arg408 of β₁E, indicating that Glu30 is involved in the isomerization of IF₁ to the fully stretched state. Consistent with this, the present study shows that the Ala mutant of Glu30 significantly destabilizes the locked state, without affecting formation of the initial F₁-IF₁ complex.

Notably, the mean duration for this isomerization from the initial F₁-IF₁ complex to the final locked state is 50 s ( = \( 1/k_{\text{lock}} \)), which is significantly longer than the mean time for rotation (5–1,000 ms). This suggests that F₁ drives thousands of γ rotation with a hanging IF₁. In other words, IF₁ almost always fails to isomerize to the locking state in each catalysis or each turn.

However, this contradicts the observation that IF₁ preferentially associates with F₁ in the catalytic dwell to form an initial complex. The contention that F₁ can drive many turns while associated with IF₁ indicates that F₁ has some affinity to IF₁ in any state besides the catalytic dwell. Although we do not have clear explanations for this contradiction, simplification of the reaction scheme may be a
possible cause. Nevertheless, the crystal structure suggests that IF₁ can have several conformations, and our model assumes only two states, neglecting intermediate states. Another possible reason for the contradiction is that some minor states were neglected through ensemble averaging and curve fitting of the time courses of IF₁ inhibition. Considering the intrinsic heterogeneity of the F₁ states among molecules, different experimental approaches that can assess molecular heterogeneity are required to resolve this problem and to elucidate the molecular mechanism of IF₁-mediated inhibition.
Acknowledgements

We thank M. Hara (University of Tokyo) for technical support, Chun-Biu Li (Stockholm University) for data analysis, and all members of the Noji laboratory for their valuable comments.

Funding and additional information

This work was supported in part by Grant-in-Aid for Scientific Research on Innovation Areas (JP18H04817, JP19H05380) from the Japan Society for the Promotion of Science (to H.U.).

Conflicts of interest: The authors declare no competing interest.
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Fig. 1. Experimental concept and procedure.

(A) Schematic image of inhibition by IF$_1$. IF$_1$ loosely associates with the $\alpha_E$ and $\beta_E$ subunits. Then, IF$_1$ is deeply inserted into the $\alpha_{DP}\beta_{DP}$ interface (PDB: 2JDI and 4TT3). The initial association and the following isomerization are represented by three rate constants, $k_{on}$, $k_{off}$, and $k_{lock}$. (b) Time course of 340 nm NADH absorbance at 1 mM ATP and 1 $\mu$M IF$_1^{1-60}$. At 180 s after F$_1$ addition, IF$_1^{1-60}$ was added to the reaction mixture and ATPase activity decreased. To see this figure in color, go online.

Fig. 2. Kinetic analysis of IF$_1$ inhibition in bMF$_1$.

(A) Time course of IF$_1$ inhibition at 1 mM ATP. Color represents different IF$_1^{1-60}$ concentrations; (Red) 1 $\mu$M, (Blue) 0.65 $\mu$M, (Green) 0.3 $\mu$M, and (Orange) 0.1 $\mu$M. The final concentration of bMF$_1$ was 10 nM. (B) Determined $k_{app}^{inhibition}$ plotted against [IF$_1^{1-60}]$. The mean value and SD for each data point in (B) are shown with circles and error bars, respectively (n=3 for each measurement). Solid line represents the fitting curve of Eq. 2. For wider [IF$_1^{1-60}$] range of wild-type bMF$_1$ at 100 nM ATP and bMF$_1$(βE188D) at 1 mM ATP, see Fig. S4. To see this figure in color, go online.
Fig. 3 Fitted parameters derived from Fig. 2B and Fig. S4.

(A) $k_{\text{lock}}$ and (B) $K_{M}^{IF_1}$. In (A) and (B), the circles and error bars in each data point represent the fitted parameter and fitting error determined in Fig. 2B. (C) The effective binding constant $k_{\text{eff}} = \frac{k_{\text{lock}}}{K_{M}^{IF_1}}$. The gray line represents the fitting curve of Eq. 5 and the orange line represents the duty ratio of catalytic dwell against overall reaction time. The circles and error bars in each data point represent the mean value and the SD calculated from Fig. 3A and 3B. In Fig. 3A-3C, gray and blue points represent the results for $bMF_1$(wild-type) at 0.1-1000 μM and $bMF_1$(βE188D) at 1 mM ATP, respectively. To see this figure in color, go online.

Fig. 4 Analysis of (A-C) IF$_1^{1-60}$(E30A) and (D-F) IF$_1^{1-60}$(Y33A) at 1 mM ATP.

(A), (D) Time course of IF$_1$ inhibition for (A) IF$_1^{1-60}$(E30A) and (D) IF$_1^{1-60}$(Y33A). The final concentration of $bMF_1$ was 10 nM. (B), (E) Determined $k_{\text{app, inhibition}}$ plotted against [IF$_1^{1-60}$]s. Solid line represents the fitting curve of Eq. 2. (C), (F) Relative ATPase activity at the end of the measurement. The mean value and SD for each data point are shown with circles and error bars, respectively (n=3 for each measurement). To see this figure in color, go online.
Fig. 5 The possible IF₁ inhibition scheme.

Each circle represents the catalytic state of the β subunit. The asterisks following “ATP” represent the catalytically active state to undergo hydrolysis of a bound ATP. All nucleotide binding states of bMF₁ in this figure represent catalytic dwell, where bMF₁ executes cleavage of ATP. To see this figure in color, go online.
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