The ε subunit, a small subunit located in the F1 domain of ATP synthase and comprising two distinct domains, an N-terminal β-sandwich structure and a C-terminal α-helical region, serves as an intrinsic inhibitor of ATP hydrolysis activity. This inhibitory function is especially important in photosynthetic organisms as the enzyme cannot synthesize ATP in the dark, but may catalyse futile ATP hydrolysis reactions. To understand the structure–function relationship of this subunit in F1, from photosynthetic organisms, we solved the NMR structure of the ε subunit obtained from the thermophilic cyanobacterium Thermosynechococcus elongatus BP-1, and examined the flexibility of the C-terminal domains using molecular dynamics simulations. In addition, we revealed the significance of the C-terminal α-helical region of the ε subunit in determining the binding affinity to the complex based on the assessment of the inhibition of ATPase activity by the cyanobacterial ε subunit and the chimaeric subunits composed of the N-terminal domain from the cyanobacterium and the C-terminal domain from spinach. The differences observed in the structural and biochemical properties of chloroplast and bacterial ε subunits explains the distinctive characteristics of the ε subunits in the ATPase complex of the photosynthetic organism.

Key words: cyanobacterium, ε subunit, F1-ATPase, Thermosynechococcus elongatus.

INTRODUCTION

The FoF1-ATP synthase catalyses ATP synthesis driven by an electrochemical proton gradient formed across an energy-transducing membrane [1–4]. F1 is the aqueous portion, composed of subunits α, β, γ, δ and ε, displaying a stoichiometry of αβ3γδ1εi [5]; F1 serves as a proton channel. The F1 moiety shows ATP hydrolysis activity that is coupled to rotation of the central γ subunit in the αβ3 ring [6,7]. Previous studies show clearly that the hydrolysis of one ATP molecule induces a 120° rotation of γ [8], and this 120° step is separated further into 80° substeps for ATP binding and 40° for ATP hydrolysis and release of the product, phosphate [9]. Recently, ADP release at 0°, 120° and 240° positions following two successive ATP hydrolysis reactions at the other two catalytic sites has been shown, implying that γ requires 240° turns to complete the ATP hydrolysis reaction at one catalytic site [10–12].

The ε subunit is shown to act as an endogenous inhibitor of chloroplast and bacterial F1-ATPases [13–16], and this inhibition is frequently called ε-inhibition. The molecular architecture of the ε subunit has been the subject of extensive studies, particularly in bacterial F1; both the crystal structure and the solution structure of the isolated ε subunits from Escherichia coli F1 (EF1) [17,18] and the thermophilic Bacillus PS3 F1 (TF1) [19] have been published. The basic architecture of this subunit, which is composed of two distinct domains, an N-terminal β-sandwich domain (NTD) and a C-terminal α-helical domain (CTD), is well conserved in these structures. The CTD is thought to be responsible for the inhibitory effect of ε by interaction with the γ subunit and the catalytic β subunits [20]. The CTD is composed of two α-helices, which are folded into a hairpin configuration (so-called retracted state) in both the crystal and the solution structures. However, a different structure of the ε subunit was observed in the enzyme complex. When ε of EF1 was co-crystallized with a truncated form of the γ subunit, ε showed an extended structure, in which the CTD predominantly interacts with the coiled-coil structure of the γ subunit [21]. Interestingly, inhibition of ATP hydrolysis activity of TF1 by the ε subunit became weaker during the reaction [16]. The conformational change of the CTD from the extended to the retracted form was thought to be the cause underlying the decrease in the extent of inhibition during the reaction [22]. This conformational change of the ε subunit in EF1, EF1, and in TF1 was then resolved by cross-linking experiments [23,24]. In addition, FRET (fluorescence resonance energy transfer) observation showed a real-time mutual transition of the CTD between the retracted state and the extended state in TF1 during catalysis [25]. These reports suggest that the CTD adopts the extended conformation governed by the membrane potential, and/or the concentrations of both ATP and ADP around the enzyme [22–25], and inhibits the ATPase
activity. ATP binding to both the monomer TF1-ε [26] and ε in the γ–ε subunit of spinach CF1 has been studied. In addition, it has been shown that both the isolated ε subunits from *Bacillus subtilis* F1 [28] and EF1 can bind ATP with lower affinity than TF1-ε [19]. Sequence analysis revealed that most of the bacterial ε subunits have an ATP-binding site within the well-conserved sequence motif, (I/L)DXXRA, located at the start of the CTD sequence. NMR analysis revealed that ATP binding to the ε subunit stabilizes the retracted conformation, suggesting that the TF1-ε subunit is a sensor for ATP concentrations in the cytosol and thereby controls the function of F, F1 via a conformational change of the CTD that is caused by ATP binding and release. This regulation is thought to be important in order to maintain the cellular ATP level [29]. ATP-dependent conformational change of the ε subunit [25] and the change of the extent of inhibition dependent on ATP concentrations [30] have been reported in TF1-ε.

In contrast, the ε subunit of the chloroplast-type F1 (CF1) is known as a stable intrinsic inhibitor of the ATPase activity that is independent of ATP concentration [15]. Although the conformational change of the CTD in CF1-ε was shown by chemical modification [31] and by a change of the accessibility of a specific antibody against the CTD [32], the conformational change of the CTD is considered to be controlled differently from those in the bacterial ε subunits because of the lack of the ATP-binding motif (see Supplementary Figure S1A at http://www.BiochemJ.org/bj/425/bj4250085add.htm). Instead, CTD changes its conformation by the formation of the membrane potential due to illumination of the thylakoid membranes [32]. The higher-plant CF1-ATPase has an additional regulatory mechanism, which is caused by a redox change of two cysteine residues located in the γ subunit of CF1 [33–35]. In the light, the reducing equivalents produced by photosynthetic electron transport reduce thioredoxin, which in turn reduces the disulphide bond formed within the CF1-γ subunit, yielding the active enzyme. Consequently the membrane potential required for initiation of the ATP formation becomes small [36]. In contrast, two cysteine residues on ε form the disulphide bond, and consequently CF1 becomes less active in the dark. The redox change of these cysteine residues may interfere directly with both rotation of the γ subunit and the conformational change of ε [37,38]. However, the lack of information on the structure of the CF1-γ and CF1-ε subunits has limited the understanding of the enzyme’s regulatory mechanism to date. We have previously successfully prepared a heterologous expression system in *E. coli* for both the α,β,γ complex and the ε subunit of the thermostable cyanobacterium *Thermosynechococcus elongatus* BP-1, and analysed the interaction between the complex and ε at the single-molecule level [39]. In that study, we successfully assigned the stop position of the γ subunit in the complex by ε-inhibition. In addition, we found that the biochemical properties of both the cyanobacterial ATPase and the ε subunit (cyano-ε) are very similar to those of CF1, and therefore these cyanobacterial proteins are considered to be useful tools for CF1 research at the molecular level.

In the present study, we have successfully solved the solution structures of both the isolated cyanobacterial ε subunit and the chimaeric one composed of the NTD from *T. elongatus* and the CTD from spinach CF1. Moreover, in order to understand the characteristic inhibitory function of the ε subunit of CF1, we prepared chimaeric ε subunits in which the CTD was replaced by the related sequences of EF1 and TF1. Assessment of the inhibition by these chimaeric ε subunits revealed the significance of CTD in determining the binding affinity of the ε subunit to the complex. In the present paper, we report on an interesting specific feature of CTD of the CF1-type ε subunit compared with those of the bacterial ε subunits.

**EXPERIMENTAL**

**Materials**

TEMPO [N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)] maleimide was purchased from Sigma–Aldrich Japan. 15NH4Cl and [U-13C]glucose were from Cambridge Isotope Laboratories. Other chemicals were of the highest grade commercially available.

**Construction of expression plasmids for chimaeric ε**

We prepared the chimaeric ε subunits combining the NTD (Met1–Asp381) from *T. elongatus* BP-1 ε subunit and the CTD from spinach CF1-ε (Pro99–Ser131), EF1-ε (Glu91–Met139) and TF1-ε (Val89–Lys133). To this end, a DNA fragment containing the coding region for the NTD of the *T. elongatus* ε subunit was amplified by PCR from the *T. elongatus* ε subunit expression plasmid [39] using the primers 5′-CCCGCGCAATTATAAGACACTCACTATAGGGAG-3′ and 5′-GGCGACGCGCGGGAACAAATCTGCTTGGCCGCCC-3′. The restriction site for Clal is underlined. Since the 5′-terminus of the NTD coding region contains an Ndel restriction site, the fragment was treated with the restriction enzymes NdeI and Clal to yield the desired DNA fragment (Ndel/Clal fragment). The coding region for the CTD of CF1-ε, EF1-ε and TF1-ε were amplified using the primers 5′-GGCGACGCGATCAGTCCCACAGAGAAGG-3′ (the Clal restriction site is underlined) and 5′-CGCAAGGGGTGTGTTGC-10-GAAGTTTACAAAGCTTCCCGCCGGG-3′ (the HindIII restriction site is underlined) for CF1-ε, 5′-GGCGACGCGATCAGTCCCAGCCGCGG-3′ (the HindIII restriction site is underlined) and 5′-CCCAAAAGCGATGTAAAAGCTTCCCGGAGG-3′ (the HindIII restriction site is underlined) for EF1-ε, and 5′-GGCTGAACGGGCGGAGGACATCGAT-3′ (the HindIII restriction site is underlined) and 5′-CGGTAAACGGGCGGAGGACATCGATGTC-3′ (the Clal restriction site is underlined) for TF1-ε. In the CTD of spinach CF1-ε, Lys109 was replaced by alanine in advance. This mutation was effective in preventing degradation of the expressed recombinant protein and did not affect the inhibitory effect on ATPase activity. Hence this mutant CF1-ε-(K109A) was used as CF1-ε in the present study. To obtain the chimaeric ε subunits, the Ndel/Clal fragment for the NTD of *T. elongatus* ε and the Clal/HindIII fragments for the CTD of CF1-ε, EF1-ε or TF1-ε were ligated directly into pET23a. The resulting chimaeric subunits were designated as cyano-εCS, cyano-εCE and cyano-εCT respectively (see Supplementary Figure S1).

**Overexpression and purification of cyano-ε and the chimaeric ε subunits**

The genes for the cyano-ε, CF1-ε and the chimaeric ε subunits harbouring in pET23a were expressed in *E. coli* strain BL21 (DE3). Expression was induced by adding 1 mM IPTG (isopropyl β-D-thiogalactoside) and the expressed ε subunit proteins were then purified from the cell lysates by the method described in [40] and pooled and solid ammonium sulfate was added to 70% saturation. The purity was confirmed by SDS/15% (w/v) PAGE. Within the expressed proteins, cyano-εCT was expressed as inclusion bodies and was purified and refolded as described in [40]. Since the other three ε subunits (cyano-ε, cyano-εCS and cyano-εCE) were expressed and purified as soluble proteins, these subunits were denatured once using 8 M urea and refolded before the ε-inhibition experiments to set up the same experimental
conditions as those for cyano-ε-CS. The inhibition properties of ε [39] both for the affinity and the extent of inhibition were in no way affected by this denaturation process.

NMR measurements and structure determination

Uniformly 15N-, and 13C/-15N-labelled cyano-ε, CF1-ε, and cyano-ε-CS were produced by cultivating the transformed cells in M9 minimal medium containing 4 g of [U-13C]glucose and 0.5 g of 15NH4Cl per litre. Expression and purification of the ε subunits were performed as described above. NMR spectra were obtained by using Bruker DRX500 and DRX600 NMR spectrometers at 303 K. All proteins were dissolved at 1 mM in 50 mM potassium phosphate buffer (pH 6.8) containing 10 mM NaCl, 1 mM NaN3 and 10% 2H2O. 1H–15N-HSQC (heteronuclear single quantum coherence) spectra were obtained with a data size of 256 (15N) × 2048 (1H) complex points. Sequential assignments of the backbone resonances of the cyano-ε and cyano-ε-CS subunits were performed using HNCACB, CBCA(CO)NH, HNCO, HN(CO)CA and HBHA(CO)NH spectra. Side-chain signals were assigned by H(CCO)NH and C(CO)NH spectra. All NMR data were processed with NMRPipe and NMRDraw [41], and analysed with SPARKY3, a program developed by T. D. Goddard and D. G. Kneller, University of California, San Francisco (http://www.cgl.ucsf.edu/home/sparky/).

Distance and backbone torsion angle restraints were acquired by two-dimensional NOESY. 15N-edited NOESY and three-dimensional 13C-edited NOESY spectra (100 ms mixing time), and TALOS analysis [42]. The restraints for the backbone dihedral angles, φ and ψ, were applied in the forms of average § 2 S.D. and § 3 S.D. for Good and New categories respectively. The structures were calculated with CYANA 1.0.6 software [43] by means of molecular dynamics in a torsion angle space, with 40000 steps. The best 20 of 100 calculated structures were analysed with MOLMOL [44] and PROCHECK-NMR software [45].

TEMPO modification of the ε subunit

For TEMPO modification of cyano-ε and cyano-ε-CS, cysteine-substituted mutants at position 46 for cyano-ε and at 46 and 79 for cyano-ε-CS were generated. Uniformly 15N-labelled mutant cyano-ε-(T46C), cyano-ε-CS-(T46C) and cyano-ε-CS-(N79C) subunits were dissolved at 1 mM in 50 mM potassium phosphate buffer (pH 6.8), containing 1 mM EDTA and 1 mM DTT (dithiothreitol), and reacted with 5-fold molar excess of TEMPO-maleimide for 1 h at room temperature (25 °C). The mixture was then subjected to a gel-filtration column (Superdex 75HR GE) equilibrated with 50 mM potassium phosphate buffer (pH 6.8) to remove excess reagents. The conversion of intensity loss of cross-peak to distances was calculated using the following equation:

$$\text{In}(I_w/I_n) = t(K/r^b)[4\tau_c + 3\tau_e \times (1 + \alpha_{n\tau_c}^2)]^{-1}$$

where $I_w$ and $I_n$ are the peak intensities of resonances with and without spin label respectively, $t$ is the total time (9.0 ms) during the INEPT (Insensitive nuclei enhanced by polarization transfer). $K$ is a constant related to spin properties of the system ((1.23 × 10^-32) cm² s⁻¹), $r$ is the distance between the nitroxide and a site giving rise to a cross-peak of interest, $\tau_c$ is the effective correlation time for tumbling of the protein (27 ns), and $\alpha_{n\tau_c}$ is the precession frequency for the amide proton (600 MHz).

Molecular dynamics simulations

All simulations were performed at 298 K using MARBLE [46]. The CHARMM22 potential [47] and the TIP3P model [48] were used for the proteins and water molecules respectively. The periodic boundary condition was applied with the particle mesh Ewald method [49]. The symplectic integrator for rigid bodies was used with a time step of 2 fs, in which water and CH₃, NH, (x = 1, 2 and 3), SH and OH groups were treated as rigid bodies. The initial structures for the simulations of cyano-ε and cyano-ε-CS were generated from the solved structures, and those for EF-ε and TF-ε were generated from crystal structures (PDB accession codes 1AQ7 [17] and 2ESY [19] respectively). All simulations were performed under the NPT condition [46] with $T$ = 298.15 K and $P$ = 101.325 kPa. After the equilibration run, in which positions of all atoms except solvent atoms were constrained, the 20-ns product run was performed for each ε subunit without any constraints. The calculations of the RMSFs (root mean square fluctuations) of the Cα atom (Cα-RMSF) were performed based on superposition using the Cα best fits for NTD of each ε-subunit.

Measurement of ATP hydrolysis activity of the $\alpha\beta\gamma\delta$ subcomplex and estimation of binding affinity of the chimaeric ε subunits to the $\alpha\beta\gamma\delta$ subcomplex

ATP hydrolysis activity was measured using the recombinant $\alpha\beta\gamma\delta$ subcomplex obtained from T. elongatus F, [39] in the presence of an ATP-regenerating system [50] in 50 mM Hepes/KOH (pH 8.0), 100 mM KCl, 2.5 mM MgCl₂, 5 mM ATP, 50 μg/ml pyruvate kinase, 50 μg/ml lactate dehydrogenase, 2 mM phosphoenolpyruvate and 0.2 mM NADH. The assay was carried out at 40°C. The rate of ATP hydrolysis after addition of the enzyme and the extent of ε-inhibition were determined by monitoring the decrease in NADH absorption at 340 nm. The proportion of the complex-bound ε subunit was estimated from the extent of the inhibition of the ATPase activity of the $\alpha\beta\gamma\delta$ subcomplex as described in [39].

RESULTS

Overall structure of the cyano-ε

In order to gain insight into the structure-function relationship of the ε subunits, we first determined the solution structures of the wild-type cyano-ε subunit. Figure 1(A) shows the two-dimensional 1H–15N-HSQC spectrum of uniformly 15N-labelled cyano-ε. The resonance dispersion was well resolved. The backbone chemical shift of amide 1H and 15N resonances were assigned by means of the multi-dimensional double- and triple-resonance NMR spectroscopies. All 1H and 15N resonances were assigned, except for those of proline, N-terminal methionine and SH and OH groups were treated as rigid bodies. The solution structure of cyano-ε was then calculated using the software CYANA based on the data on distance, torsion angle and hydrogen-bond restraints listed in Table 1. Superposition of the final 20 structures, which exhibited the best target functions of 100 calculated structures, is presented in Figure 2(A). The overall structure of cyano-ε was found to be composed of two domains, the NTD (Met¹–Glu⁸⁵) and the CTD (Leu⁹⁶–Ile¹²⁸), connected by a short linker (Arg¹⁶³–Asp¹⁸⁸). The NTD is composed of two $\beta$-sheets that are packed against each other. Each of the sheets consist of an assembly of five $\beta$-strands ($\beta$1, Met¹–Ile⁵; $\beta$2,
Nitroxide modification of cyano-ε-(T46C) caused complete quenching of seven amide signals at Gln117–Arg124 on the CTD (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/425/bj4250085add.htm). The intensity change of cross-peaks by spin labelling can be used to estimate the distance between the nitroxide free radical and the protons giving rise to particular cross-peaks [51]. The distance, which was estimated using completely disappeared cross-peaks, was approx. 14 Å (see the Experimental section), and this value was used for the structural calculation as a distance constraint, which is the upper limit between nitroxide free radical and each amide of undetectable residues. The average RMSDs of the final 20 overall structures were 0.88 Å for the backbone and 1.32 Å for all heavy atoms.

Overall structure of the chimaeric cyano-εCS

Determination of the solution structure of the spinach CF1-ε subunit was also attempted. However, potentially due to aggregation, the resolution of the 1H–15N-HSQC spectrum of CF1-ε was poor and the data could not be obtained (Figure 1B).
Figure 2  Overviews of the cyano-ε and cyano-εCS structures

(A) Stereo view of the overlaid 20 NMR solution structures of cyano-ε with the lowest target function of CYANA. The backbone heavy atoms are superimposed. (B and C) Ribbon drawings of the representative structure of cyano-ε (B) and cyano-εCS (C).

Figure 3  Comparison of the secondary structures of the CTD of various ε subunits

Sequence homology alignment of the CTD in the four ε subunits, whose structures have been determined, was obtained using ClustalW. The secondary-structure elements are indicated under each of the sequences. The secondary-structure elements of E. coli (EF1) and PS3 (TF1) are constructed based on the solution (PDB codes 1BSN for EF1 and 2E5T for TF1) and crystal structures (PDB codes 1AQT for EF1 and 2E5Y for TF1), and indicated as S and C respectively. The residues corresponding to the loop region observed in both the solution and crystal structures are shown in grey. The corresponding numbers of the loop region are represented on each sequence. The numbers of residues constructing the α-helix are indicated in parentheses on each of the secondary-structure elements. The residues belonging to the ATP-binding motif [I(L)DXXRA] in EF1 and TF1 are identified by asterisks. Spiol, spinach (Spinacia oleracea).
Figure 4  Intrinsic flexibility of various ε subunits in the molecular dynamics simulations

The C°-RMSF calculated based on superposition using the C°-atom best fits for the NTD of each ε subunit are mapped on to the structures of cyano-ε (A), cyano-εCS (B), EF1-ε (C) and TF1-ε (D). The C°-RMSF is shown on the right. Green dotted lines represent stable interactions between interdomain pairs of residues contacting each other for 98% of the time of the simulations. The residue contact was defined when at least a pair of non-hydrogen atoms of the two residues was within 4.5 Å of each other. Residues involving interdomain NOEs are shown in stick representation coloured grey, orange, yellow and cyan. Each colour indicates a pair of interdomain NOEs.

Chimaeric ε subunits combining the NTD from T. elongatus BP-1 F1 and the CTD from spinach CF1 were prepared instead. The chimaeric subunit obtained was designated cyano-εCS and was used for the structure analysis. There appears to be no remarkable difference between the structure of the NTD of the bacterial ε and that of the cyanobacterial ε, whereas the CTD shows a large variety at the amino acid sequence level and was expected to be involved in the function of this subunit via its conformational change. The 1H–15N-HSQC spectrum of cyano-εCS was obtained with the same quality as that of cyano-ε (Figure 1C), and the solution structure was calculated successfully with CYANA using the data shown in Table 1. The CTD structure of cyano-εCS was found to be well ordered (Table 1), being composed of two α-helices (α1, Pro89–Arg104; α2, Ala109–Asn130) similar to cyano-ε or bacterial ε. The arrangement of the two domains was then determined by distance constraints from 12 interdomain NOEs and nitroxide spin labelling, by the same technique applied to cyano-ε. In comparison with the HSQC spectra of cyano-ε, the chemical shifts of Asn79 and Leu77 of cyano-εCS were significantly perturbed compared with other residues in the NTD (see Supplementary Figure S3 at http://www.BiochemJ.org/bj/425/bj4250085add.htm). In order to carry out nitroxide spin labelling at this position, the N79C mutant of cyano-εCS was then constructed. Nitroxide modification of cyano-εCS-(N79C) caused complete quenching of NMR signals between Glu114 and Arg123 within the CTD (see Supplementary Figure S4 at http://www.BiochemJ.org/bj/425/bj4250085add.htm). Although the nitroxide spin labelling at Thr46 was also examined, no quenching of NMR signals was observed in the same region (see Supplementary Figure S4). Consequently the CTD configuration of cyano-εCS was determined to be different from those observed for the bacterial ε and cyano-ε (Figure 2C), in which the second α-helix of CTD is placed at the opposite side of the γ contact surface (see Figure 2B). Obviously, this structure does not reflect the structure of the ε subunit in the complex, however, since the position of the CTD cannot be achieved because of structural hindrance by the contact region of the γ subunit when the ε subunit binds to γ.

Mobility of the CTD of the ε subunits

As ε-inhibition takes place in the extended conformation of CTD [22,23], and the inhibition by the chloroplast-type ε is more stable than that by TF1-ε, the differences in the CTD α-helix structures, the length of both the loop region and two α-helices, and the mobility of the CTD seem to be critical for their function. The intrinsic flexibility of the ε subunit molecules was therefore examined using 20-ns molecular dynamics simulations based on the solved structures, and was compared with that of EF1-ε [17] and TF1-ε [19]. The C°-RMSFs of the ε subunit show that the CTD of cyano-ε is somewhat more flexible compared...
with the bacterial ε subunit, although the observed architecture of the NTD and the CTD is very similar (Figure 4). Several stable interactions (green dotted lines) were obtained in the TF1-ε (Figure 4C) and EF1-ε (Figure 4D) structures, whereas no stable interaction was determined in the cyano-ε (Figure 4A) and only one was found to occur between the CTD and the loop region of cyano-εCS (Figure 4B). Consistent with these simulations, the NMR experiments showed that NOEs between NTD and CTD of cyano-ε were only localized between β6 (Thr46, Gly47 and Val48) and α2 (Glu126, Ala127, Ala129 and Arg130). This result was well reflected in the flexibility obtained by NMR relaxation analysis, in which the small heteronuclear NOEs values were observed in relatively wide parts containing the first helix end towards the loop and around the C-terminal end. The T2 values show a similar tendency, suggesting that these regions are more flexible in terms of the fluctuations in the pico–nano-second time range than other regions of cyano-ε (see Supplementary Figure S5 at http://www.BiochemJ.org/bj/425/bj4250085add.htm). In addition, the suggested high flexibility of the loop region of cyano-ε is in agreement with the fact that resonance between Lys112 and Ala114, which might be induced by a conformational change or solvent exchange of this region, was undetectable. This flexibility is supposed to make interaction within two α-helices in the CTD and that between the NTD and CTD weaker. In the case of the cyano-εCS molecule, three stable interactions between the NTD and CTD, β7 (Met6), and α2 (Leu117), β10 (Glu2) and α2 (Arg2), and α10 (Arg8) and α2 (Glu127), were observed in the molecular dynamics simulations (green dotted lines in Figure 4B), and therefore the CTD of cyano-εCS in the solution structure might be less mobile than that of cyano-ε. Consistent with these simulation results, flexible regions were only observed in loop regions and in the C-terminal end, except for Ala109 and Leu117 which were also found in the NMR relaxation analysis (see Supplementary Figure S5). In cyano-εCS, interdomain NOEs between the NTD and CTD, β7 (Met6) and α2 (Leu117), β10 (Glu2) and α2 (Arg2), the loop connecting the NTD to the CTD (Thr86) and α2 (Arg12), and the turn between β1 and β2 (Pro89 and Asp125) and α2 (Thr125 and Arg125), were observed and found to be consistent with the results of the molecular dynamics simulation (Figure 4B).

Significance of the CTD for the inhibitory function of the ε subunit

In order to determine whether the variety in the CTD region of the ε subunits at both the structural and amino acid sequence levels and the differences in their mobility affects the inhibitory function of this subunit, chimaeric ε subunits combining the NTD from T. elongatus BP-1 F1 and the CTDs from EF1 and TF1 were prepared and designated as cyano-εCS and cyano-εCT respectively (see Supplementary Figure S1). The extent of ε-inhibition of the ATPase activity of the T. elongatus F1 α1β2γ subcomplex using these chimaeric ε subunits was then measured (Figure 5). Figure 5(A) shows a typical time course of ε-induced inhibition. The decrease in activity following the addition of ε occurred within a few minutes and the curve obtained was fitted to a single exponential function. Since complete inhibition of the enzyme activity caused by the ε binding had already been confirmed by single-molecule experiments [39], the gradual decrease in activity observed in this study must indicate binding of the ε subunit to the complex. Thus the binding rate constant, kobs, was calculated from these curves. On the basis of the observed linear relation of kobs against the ε concentrations (Figures 5B and 5C), kobs values were determined as shown in Table 2. Although the extrapolated intercepts of Figures 5(B) and 5(C) give the theoretical kobs values, they were deemed unsuitable for calculation of Kd values owing to large experimental fluctuations. Kd values obtained from the inhibition of steady-state ATPase activity by the ε subunits shown in Figure 6 were applied instead, allowing determination of the koff values as shown in Table 2. The Kd values of these ε subunits used for these calculations were determined on the basis of the

### Table 2 Rate constants of the ε-binding obtained from ε-inhibition

<table>
<thead>
<tr>
<th>Subunit</th>
<th>kobs (M−1 s−1)</th>
<th>koff (s−1)</th>
<th>Kd (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyano-ε</td>
<td>7.2 × 10^5</td>
<td>8.6 × 10^4</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Cyano-εCS</td>
<td>3.8 × 10^5</td>
<td>8.7 × 10^4</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td>Cyano-εCT</td>
<td>2.0 × 10^5</td>
<td>4.9 × 10^4</td>
<td>24.6 ± 2.2</td>
</tr>
<tr>
<td>Cyano-εCT</td>
<td>1.4 × 10^5</td>
<td>3.9 × 10^4</td>
<td>13.6 ± 1.9</td>
</tr>
</tbody>
</table>

Kd values were obtained as an average for three independent experiments shown in Figure 6.
extent of inhibition in the presence of various concentrations of the ε subunits (Figure 5A). Thus, $K_d$ values of 24.6 ± 2.2 nM for cyano-$\varepsilon_{\text{CS}}$ and 34.6 ± 1.9 nM for cyano-$\varepsilon_{\text{CT}}$ were obtained by curve fitting using the equation described in [39] (Figure 6). In contrast, the affinities of cyano-ε and cyano-$\varepsilon_{\text{CS}}$ were much higher, and the enzyme activity was mostly inhibited in the presence of equimolar concentrations of ε. The inhibition assay with 1 nM enzyme was therefore examined and $K_d$ values of 1.2 ± 0.2 nM for cyano-ε and 2.3 ± 0.6 nM for cyano-$\varepsilon_{\text{CS}}$ were obtained. These values were comparable with the reported $K_a$ for EF-ε [52]. In the case of cyano-$\varepsilon_{\text{CT}}$ and cyano-$\varepsilon_{\text{CS}}$, the extents of inhibition of ATPase activity of the $\alpha_3\beta_3\gamma_3$ subcomplex at high concentrations of ε (1–3 μM) were almost the same as those by cyano-ε and cyano-$\varepsilon_{\text{CS}}$, suggesting that this extrinsic CTD can also adopt a similar conformation for inhibition. This implies that, in addition to the interaction between the CTD and the $\beta_{\text{DELSEED}}$ region [20], the interaction between the coiled-coil structure of the γ subunit and the CTD is important for inhibition. The CTD must therefore also play a critical role in determining the binding affinity of the ε subunit to the $\alpha_3\beta_3\gamma_3$ subcomplex.

**DISCUSSION**

The significance of the CTD for the inhibitory function of the ε subunit has been studied in detail previously using TF-ATPase [22]. Mutation studies suggest that the intensity of the inhibitory effect is closely related to the positive charges on the CTD and its partner, the negatively charged cluster located at the so-called DELSEED region on the β subunit [20]. In addition, an ATP-dependent conformational change of CTD was reported, and this change was attributed to the recovery of the ATPase from the inhibited state [26]. Moreover, recent crystallization studies have solved ATP binding on the CTD of TF-ε [19]. In contrast, the strong permanent inhibition by the ε subunit of the chloroplast ATPase was not recovered even in the presence of high concentrations of ATP [39]. Detailed information on the structure of the chloroplast ε subunit would help to resolve the basis of the inhibition of the CF1-type ε subunit, which seems to be different from that of TF1-ε, but which may be similar to EF1-ε. In the present study, the NMR structures of the cyano-ε and the CTD of spinach ε has been solved using the chimaeric subunit. The basic architecture of cyano-ε comprising two domains, the NTD and the CTD, was as conserved as that of the bacterial ε subunit. When cyano-ε and bacterial-ε NTDs are superimposed, the fit is very good. In contrast, a significant difference in the CTD was observed between CF1-type and bacterial ε (Figures 2B and 2C). Although all reported structures of the CTD are composed of two α-helices connected by the short loop region and these two α-helices are found in a retracted state, the length of the two α-helices and that of the loop connecting two helices showed interesting variations (Figure 3). The loop region of the CF1-type ε consists of four to five amino acids, which is much shorter than the bacterial ε subunit (six to nine amino acids). In addition, the length of the first α-helix of cyano-ε is much longer than those of the other ε subunits, whereas those of the second α-helix are longer in spinach CF1 and EF1. The way in which these variations are linked to differences in the extent of inhibition remains unclear.

The observed difference in the resonance signals between cyano-ε and cyano-$\varepsilon_{\text{CS}}$ might be attributed both to the different structure of the loop region and the difference in their retracted structures. However, the observed solution structure of cyano-$\varepsilon_{\text{CS}}$ is unlikely to reflect that occurring in the complex, and unlikely to be sufficiently stable, since the observed position of the CTD in the solution structure must obstruct the interaction between the ε subunit and the γ subunit, and cyano-$\varepsilon_{\text{CS}}$ could inhibit the ATPase activity of the $\alpha_3\beta_3\gamma_3$ complex almost with the same affinity and to the same extent as cyano-ε (Figure 6). In addition, another factor which may affect conformation of the ε subunit is likely to be the structure of the γ subunit. The higher-plant CF1 γ has a 40-amino-acid insertion region, including two regulatory cysteine residues, that is not found in non-photosynthetic bacteria.
the partially truncated cyano-intramolecular structure is reflected in their specific features: CTD as mentioned (Figures 4C and 4D). The stability of their ε subunit co-crystallized with 5 mM ATP (Figure 6) . Although cyano-γ does not have these two regulatory cysteine residues, it still possesses a 30-amino-acid insertion, which may help in stabilizing the extended inhibitory state when the cyano-ε subunit is in the context of the full cyano-F₁ [56] . In addition, deletion of this insertion from the cynobacterial γ subunit made the αβγ complex less sensitive to the ε subunit [39] . Thus the extended-state CTD may be induced by the γ structure and easily contact with the coiled-coil structure of the γ subunit or the β DELSEED region or both when they are attached to the complex. Indeed, cyano-ε CT may cause complete inhibition of the ATPase activity of the cynobacterial complex, although this subunit maintains all of the amino acid residues involved in ATP binding, suggesting that this subunit may not adopt a retracted conformation even in the presence of 5 mM ATP (Figure 6).

In contrast, the retracted structures of EF₁-ε and TF₁-ε were calculated to be rather stable, through formation of several stable interactions between the NTD and the CTD and those in the CTD as mentioned (Figures 4C and 4D). The stability of their intramolecular structure is reflected in their specific features: cyano-ε CE and cyano-ε CT showed lower kₘₐₚ values and a lower affinity to the complex compared with those for cyano-ε and cyano-ε CS, although they finally adopt the extended conformation and completely inhibit ATPase activity upon interaction with the complex (Figure 6). To date, there are only two different ε structures in the complex; the retracted structure of the mitochondrial δ subunit in the whole F₁ complex [57] and the partially extended structure of the ε subunit co-crystalized with the partially truncated γ subunit of EF₁ [21] . Further structural information of the ε subunit in the various F₁ complexes will be instrumental in aiding our understanding of the intrinsic inhibitory function of this subunit in detail.

AUTHOR CONTRIBUTION
Hiromasa Yagi, Hiroki Konno and Toru Hisabori defined the experimental strategy and wrote the article. Tomoe Fuse-Murakami, Atsuko Isu and Hiroki Konno performed the biochemical analysis. Hideko Akutsu planned the NMR experiments, and Hiromasa Yagi solved the NMR structure. Tomotaka Oroguchi and Mitsuori Ikeguchi carried out the molecular dynamics calculation.

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SUPPLEMENTARY ONLINE DATA

Structural and functional analysis of the intrinsic inhibitor subunit ε of F1-ATPase from photosynthetic organisms

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Figure S1 Preparation of the chimaeric ε subunits

(A) Amino acid sequences of the CTD of the chimaeric ε subunits. Residues 1–88 are derived from the ε subunit of T. elongatus BP-1, residues 89–131 (89–134 by CF1 ε numbering) from CF1 ε for cyano-εCS (green), residues 89–132 (90–133 by EF1 ε numbering) from EF1 ε for cyano-εCE (blue), residues 89–136 (91–138 by TF1 ε numbering) from TF1 ε for cyano-εCT (red) respectively. The ATP-biding motif on CTD of TF1-ε is highlighted in yellow. (B) The purity of the chimaera ε subunits was analysed by SDS/15% PAGE. Each lane contained 6 µg of protein. Molecular masses are indicated in kDa.

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The atomic co-ordinates and structure factors for the ε subunit of the F1-ATPase from Thermosynechococcus elongatus BP-1 and the ε subunit chimaera combining the N-terminal β-sandwich domain from T. elongatus F1 and the C-terminal α-helical domain from spinach chloroplast F1 have been deposited in the Protein Data Bank under accession codes 2RO6 and 2RO7 respectively.

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Figure S2  Effects of TEMPO modification of cyano-ε-γ

(A) 1H–15N-HSQC spectrum of cyano-ε-(T46C). Residues which are affected by the nitroxide spin label are indicated. (B) The same region of spectrum modified by TEMPO-maleimide. (C) Mapping of the residues on the solution structure of cyano-ε-γ affected by the nitroxide spin label. The position of spin label residue (Thr46) is shown in yellow. Red indicates the undetectable residues (Gln117–Arg124) by broadening.

Figure S3  Average chemical shift difference ($\Delta \delta_{\text{ave}}$) of 1H–15N-HSQC spectra between cyano-ε-γ-γ and cyano-ε-γ-γCS in the NTD

Absence of a bar for a residue indicates that no chemical shift was detected.
Figure S4  Effects of TEMPO modification of the chimaeric ε

(A) $^1$H–$^{15}$N-HSQC spectra of cyano–εCS-(N79C) (upper) and cyano–εCS-(T46C) (lower) chimaeric ε. Residues which are affected by the nitroxide spin label are indicated. (B) The same region of spectra modified by TEMPO-maleimide. (C) Mapping of the residues on the solution structure of chimaeric ε affected by the nitroxide spin label. Asp$^{79}$ and Thr$^{46}$ spin-labelled positions are shown in magenta and yellow. Red indicates the undetectable residues (Glu$^{114}$–Arg$^{123}$) by broadening.
Figure S5  Relaxation parameters of the amide $^{15}\text{N}$ spin at each residue of cyano-$\varepsilon$ and cyano-$\varepsilon_{\text{CS}}$

(A) Longitudinal relaxation, $T_1$; (B) transverse relaxation, $T_2$; and (C) heteronuclear $\{^1\text{H}\} - ^{15}\text{N}$ steady-state NOE values recorded at 600 MHz. $T_2$ and NOE values with asterisks at 134 of cyano-$\varepsilon_{\text{CS}}$ are 635 ms and $-0.82$ respectively. $T_1$ was deduced from the data with relaxation delays of 5, 250, 500, 750, 1000, 1250, 1500, 1750, 2000, 2250 and 2500 ms, and $T_2$ from the data with relaxation delays of 7.2, 36, 72, 108, 144, 180, 216, 252, 288, 324 and 360 ms. In the heteronuclear NOE experiments, $^1\text{H}$ saturation for 5.0 s during the relaxation delay was applied.