Review

ATP synthase: from single molecule to human bioenergetics

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Abstract: ATP synthase (F₀F₁) consists of an ATP-driven motor (F₁) and a H⁺-driven motor (F₀), which rotate in opposite directions. F₀F₁ reconstituted into a lipid membrane is capable of ATP synthesis driven by H⁺ flux. As the basic structures of F₁ (αβγδε) and F₀ (αβειδο) are ubiquitous, stable thermophilic F₀F₁ (TEF₁) has been used to elucidate molecular mechanisms, while human F₁F₀ (HF₁Fo) has been used to study biomedical significance. Among F₁s, only thermophilic F₁ (TF₁) can be analyzed simultaneously by reconstitution, crystallography, mutagenesis and nanotechnology for torque-driven ATP synthesis using elastic coupling mechanisms. In contrast to the single operon of TEF₁F₁, HF₁Fo is encoded by both nuclear DNA with introns and mitochondrial DNA. The regulatory mechanism, tissue specificity and physiopathology of HF₁F₁ were elucidated by proteomics, RNA interference, cytoplasts and transgenic mice. The ATP synthesized daily by HF₁F₁ is in the order of tens of kilograms, and is primarily controlled by the brain in response to fluctuations in activity.

Keywords: F₀F₁, molecular motor, mitochondria, omics, cytoplasts, bioenergetics

Introduction

All human activity depends on ATP, which is primarily synthesized via mitochondrial oxidative phosphorylation (oxphos). By analogy with glycolytic ATP synthesis, there have been many futile attempts to isolate hypothetical high-energy intermediates, such as phosphoenolpyruvate, that tightly couple respiratory energy to ATP synthesis. In 1961, Mitchell proposed the chemiosmotic hypothesis, which states that in oxphos, the respiratory energy is coupled to an imaginary anisotropic “ATPase system” located in an ion-impermeable membrane (Fig. 1 of Ref. 1) via H⁺/OH⁻ flux driven by the electrochemical activity ([H⁺]Left/H⁺]Right = 10⁷ × [ATP]/[ADP]) across the membrane.1 At the same time, soluble ATPase, known as coupling factor 1 (F₁), was purified from mitochondria in Racker’s laboratory.2 When F₁ was bound to F₀F₁-deficient mitochondrial membrane, respiratory energy was coupled to ATP synthesis.2 The author then isolated the entire ATP synthase, later called F₀F₁ (Fig. 1, right), and reconstituted F₀F₁ into liposomes capable of converting energy from ATP hydrolysis to that for H⁺ flux driven by the electrochemical potential of protons across the membrane (ΔµH⁺), where ΔµH⁺ = FΔψ − 2.3RTΔpH.3 The H⁺ flux through

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F$_{o}$F$_{1}$ liposomes was demonstrated first by a pH meter (Fig. 6 of Ref. 3). After the primary chemiosmotic hypothesis$^{1}$ was thus partially established,$^{3}$ the subunit-subunit interactions in F$_{o}$F$_{1}$ during ATP synthesis became the next research target. Boyer proposed a hypothetical two-subunit model of conformational energy transfer, and finally proposed the rotational hypothesis in 1981,$^{4}$ based on our report on thermophilic F$_{1}$ (TF$_{1}$) subunits with ubiquitous ADP or ATP, respectively.$^{6,7}$ The rotational hypothesis$^{4}$ assumes that on ATP synthesis, the eccentric central stalk (γε) connected to the c$_{10}$ ring is rotated against the αβ$_{3}$ hexamer in the clockwise direction, as viewed from the F$_{1}$ side (Fig. 1, right). This rotation induces cyclic conformational changes of β in the order of β$_{E}$→β$_{D}$→β$_{T}$→β$_{E}$ so as to change the affinity for nucleotides, and finally release ATP to return to β$_{E}$ (Fig. 1, middle and upper right).$^{4,7}$ In fact, Walker’s X-ray crystallography of most of αβ$_{3}$γ of BF$_{1}$ visualized the distinct conformations of β with different nucleotide occupancies (β$_{E}$, β$_{D}$ and β$_{T}$).$^{3}$

The concept of F$_{o}$F$_{1}$ as an H$^{+}$-driven rotor (γ→c$_{10}$) and stator (δ→β$_{2}$), rotating with a torque of 42 pN nm, was predicted in 1996 (Fig. 1, right).$^{10}$ The most convincing evidence of the rotational hypothesis was the direct observation of the rotation of an actin filament attached to γ against the fixed αβ$_{3}$ hexamer by Noji$^{11}$ in 1997, using the TF$_{1}$ gene.$^{12}$ Briefly, the ATP-driven F$_{1}$ motor rotates clockwise (Fig. 1, upper left) and the H$^{+}$-driven F$_{o}$ motor rotates in the anticlockwise direction as viewed from the F$_{1}$ side (Fig. 1, lower left).$^{7,10,11}$ Preliminary experiments on “the 120° rotation of the c subunit oligomer” have been reported,$^{13}$ but this was not sensitive to F$_{o}$ inhibitor and may represent the γ.
In the F1 portion of FoF1, rotation in the F1 portion of FoF1 revealed that the ring of c subunits contains 10 protomers, rather than the widely anticipated 12 (4H+ per 120° rotation), and tightly connected to the c10-ring.

Although crystal of EFo is not available, exact experiments on TFoF1 confirmed the c10-ring structure (Fig. 1, right).15) By using single-molecule FRET measuring the change in the distance between the subunits a and c during the rotation (see section 6.1), a 36° sequential stepping mode of the c10-ring rotation in FoF1 was confirmed.16) ATP is synthesized at the clefts between α, β, and γ subunits of TF1,31)32)33) (Fig. 1).7),17) In fact, the γδε complex was dissociated from BF1 by guanidine treatment as a stable heterodimer (Papageorgiou, S., and Solaini, G., 2004, PubMed abstract). So called minor subunits (e, f, g and A6L) of HFo are unlikely to have a role directly in ATP synthesis, but they appear to influence oligomeric state of HFoF1.21),25) Supramolecular structures of HFoF1 include HFoF1 dimer30) and quintuple yeast deletion YF1 mutant (ΔαβΔγΔδΔε) is complemented by genes encoding BF1,18) the major structure of eukaryotic F0F1 is apparently universal.19)20)21)22)23)24)25) Thus, X-ray crystallography data for F0F1 subunits from different species were taken from RCSB Protein Data Bank (http://www.rcsb.org/pdb/results/results.do?outformat) and assembled according to sequence data for F0F1.21) No high-resolution structural data are available for subunit a and the hinge region of subunit b. ATP synthase contains both PIC and ANC.31) PIC: Phosphate carrier; ANC: Adenine nucleotide carrier.
and ATP synthasome$^{31}$ composed of phosphate carrier (PIC), adenine nucleotide carrier (ANC) and HFoF1 (Fig. 2). Moreover, there are tissue differences in HF1.27) The complex gene structure,21),28) specific regulation systems,21),28) expression and alternative splicing27),32) of HFoF1 and related regulatory genes were elucidated by recent cytoplast technology,22),33) transgenic mice,32) transcriptomics21),32) and proteomics.31),32) Based on the knowledge from these extensive studies,7),17),21) human energetics in physiological activities21) and diseases21),23) have been analyzed.

In this article, mechanistic studies of FoF1, including a reconstitution study and crystallography, will be reviewed, and then, more complex mitochondrial cytobiology and human biomedical studies will be described, because mitochondrial structure, neuro-hormonal control, tissue-specific activity and disease are not found in bacteria. Historical evaluations of contributions made by scientists in mechanistic studies are summarized in excellent reviews (Refs. 4 and 17, and references therein).

1. Isolation of ATP synthase (FoF1) by membrane biology

FoF1 is a membrane protein of oxidative phosphorylation. In the 1960s, membrane biology was in its infancy, and the many attempts to purify ATP-synthesizing membrane proteins from mitochondria had been unsuccessful. Long before Fleischer’s group extracted phospholipids from the mitochondrial membrane with aqueous-acetone (10% water) and restored electron transport activity by adding back phospholipid micelles in 1962,34) Kakiuchi succeeded in a similar experiment in 1926.35) Okunuki36) succeeded in preparing several cytochromes. Green’s group prepared electron transport components from mitochondria.37) However, even after phospholipids were added back and electron transport activity was restored,34)–37 liposomes capable of \( \Delta \mu H^+ \)-driven oxphos, as predicted by Mitchell,1) were not reconstituted.

Membrane proteins were classified into extrinsic and intrinsic proteins.3) Extrinsic proteins, such as cytochrome c$^{36}$ and F1$^{3,3}$ or components of F1, including OSCP$^{3,25}$ and F0$^{3,25}$ (Figs. 1 and 2), are easily detached from the biomembrane by treatment with ultrasonic irradiation, chelating agents, and chaotropic anions (KI, KCN).3 and can be purified as soluble proteins in the water phase by chromatography and ammonium sulfate fractionation. However, intrinsic proteins including cytochrome oxidase$^{36,37}$ and F0$^3$ are hydrophobic and embedded in the lipid bilayer, and require proper detergents for solubilization$^{38}$ (Fig. 3, right). In 1966, detailed phase diagrams of phospholipid cholate system were reported,38) and these were useful for solubilizing intrinsic proteins and reconstituting functional biomembrane.3,3,40) The detergent concentration needed to solubilize F0,F1 is near its critical micelle concentration.3)
The $F_1$ reported by Racker’s laboratory$^2$ was not sensitive to an energy transfer inhibitor of oxphos, oligomycin, but became oligomycin sensitive when bound to $F_1$-depleted mitochondrial membrane.$^{41}$ The oligomycin sensitivity conferring factor ($F_o$, $H^+$-driven motor, Fig. 1) was characterized as an intrinsic membrane protein, and by using cholate, oligomycin-sensitive ATPase, later designated $F_oF_1$, was isolated (Fig. 3).$^3,42$ $F_oF_1$ was reconstituted from $F_o$ and $F_1$.$^42$ The electron microscopic images of $BF_oF_1$ revealed spherical $^3H$-acetyl-$BF_1$ attached to membrane-embedded $BF_o$.$^43$ In combined morphological-biochemical studies to identify the in situ structure of $BF_oF_1$,$^49$ radiolabeled $BF_1$ was added to $BF_o$ membrane, and then the structure, radioactivity and ATPase activity were removed in parallel.$^{45}$ However, mitochondrial $F_1$s, including $BF_1$ and $HF_1$, are unstable and reconstitution of $F_1$ from each isolated subunit was unsuccessful, even with chaperones. Thus, $TF_1$,$^7,44$ and $TF_oF_1$,$^7,45$ were purified from thermophilic bacillus PS3, and stable $TF_oF_1$ was reconstituted from $TF_1$ and $TF_o$ (Fig. 1).$^7,44,45$ Recent proteomics using mild detergent extraction of $TF_1$ and $TF_o$ ($PIC+ANC$)$^{31}$ (Fig. 2) (see section 9).

2. Reconstitution of $F_oF_1$ membrane capable of ATP synthesis by proton flux

$F_oF_1$ converts energy of $\Delta \mu H^+$ driven $H^+$ flux into ATP synthesis. Incorporation of membrane proteins into the lipid bilayer is essential in activity studies in membrane biology. The most difficult step was the reconstitution of liposomes containing active membrane proteins capable of producing $\Delta \mu H^+$.$^3,39,40$ The use of removable detergents, including cholate, was essential in the preparation of $BF_oF_1$ (Fig. 3).$^3,39,40$ and after cholate extraction, Triton-X100 was used in the chromatography to purify $TF_oF_1$.$^{45}$ The removal of $^{14}C$-cholate during dialysis was estimated by radioactivity.$^3,40$ and tight closure of the liposome membrane was estimated by radioactivity of enclosed $^{14}C$-inulin and confirmed by electron microscopy of enclosed ferritin.$^3,40$ Electron microscopy showed liposomal membranes studded with closely neighboring $F_1$ (Fig. 3, lower right).$^3,43$ As several extrinsic proteins in oxphos, including $F_1$, OSCP and $F_6$, were partially lost during the extraction and dialysis of $F_oF_1$, the best activity was attained by adding these after dialysis.$^3,39$ These liposomes showed $H^+$ translocation on addition of ATP,$^3,40$ ATP-Pi exchange reactions,$^3,39,46$ or $H^+$-driven ATP synthesis (Fig. 1, right).$^{46}$ Thus, $F_oF_1$-liposome was shown to be the anisotropic “ATPase system” imagined by Mitchell.$^1$ ATP synthesis was sensitive to the combination of valinomycin ($K^+$ ionophore) and nigericin ($H^+-K^+$ exchange ionophore), which collapsed $\Delta \mu H^+$. $^{3,39,46}$

More stable $TF_oF_1$-liposomes synthesized ATP from ADP and Pi with energy from proton flux driven by $\Delta \mu H^+$ formed by $\Delta \rho H$ and $\Delta \psi$ across their membranes.$^{46}$ Using chloroplasts, ATP synthesis from ADP and Pi was demonstrated by applying $\Delta \rho H$ using acid–base transition$^{37}$ and by imposing $\Delta \psi$ using an electric pulse.$^{48}$ However, chloroplasts contain an electron transport system and other components that are energized by either $\Delta \rho H$ or $\Delta \psi$. Thus, net ATP synthesis by applying $\Delta \rho H$ (acid–base transition) or $\Delta \psi$ (external electric pulse) to purified $TF_oF_1$ reconstituted in liposome$^{46}$ was the most convincing evidence of chemiosmotic theory.$^1$

The electrical potential between the inside and outside of liposomes formed by $K^+$ diffusion in the presence of valinomycin was calculated as $\Delta \psi = RT/F \times \ln([K^+]_{\text{out}}/[K^+]_{\text{in}})$. Maximal net ATP synthesis from ADP and Pi was achieved by incubating vesicles in malonate at pH 5.5 with valinomycin, and then rapidly transferring them to a solution of pH 8.4 and 150 mM $K^+$. To synthesize ATP, the minimal $\Delta \mu H^+ = (\Delta \psi - 60\Delta \rho H$ atm, 30°C) of 210 mV and optimal $\Delta \mu H^+$ of 290 mV was required.$^{46}$ The $H^+$-conducting activity of $TF_o$-liposome through $TF_o$ was proportional to the imposed $\Delta \mu H^+$ (6H$^+$/sec/103 mV at pH 8.0).$^{49}$ The pH profile of the rate revealed that a proton, not a hydroxyl ion, was the true substrate.$^{49}$ Because of the kinetic equivalency between $\Delta \psi$ and $\Delta \rho H$ as driving forces of ATP synthesis,$^{46}$ $\Delta \psi$ is expected to replace $\Delta \rho H$. In fact, $TF_oF_1$-liposomes irradiated with external electric pulses (760 V/cm, 30 ms, rectangular) catalyzed net ATP synthesis.$^{50}$ The amount of ATP synthesized increased with the number, voltage and duration of electric pulses.$^{50}$ The net synthesis of ATP by application of $\Delta \mu H^+$ across the $TF_oF_1$-liposomes$^{46,50}$ firmly established the chemiosmotic hypothesis.$^1$ To directly measure $\Delta \psi$ with electrodes on both sides of the membrane, $TF_oF_1$ was reconstituted into planar phospholipid bilayers, and the magnitude of the electric current generated upon addition of ATP was shown to follow simple Michaelis–Menten type kinetics, and the Km was found to be 0.14 mM.$^{51}$ There was no apparent
dependence of Km on $\Delta \Psi$. This observation indicates that $\Delta \mu H^+$ does not directly affect Km to release the ATP formed on TF$_o$F$_1$, and opens the way for conformational energy transfer in ATP synthesis.

Since the success of the F$_o$F$_1$-liposome, the reconstitution method has been used to analyze the activity of intrinsic proteins including channels, receptors and membrane proteins. Using the liposome method, the important outer membrane diffusion channel known as VDAC, (voltage-dependent anion channel) in mitochondrial transport was isolated in 1980 (see sections 9 and 10).

3. Reconstitution of F$_1$ subunit complexes capable of ATP synthesis by torque

3.1. Core components of F$_o$ are a, b, and c subunits and those of F$_1$ are $\alpha$, $\beta$, $\gamma$, $\delta$ and $\varepsilon$. The structure of F$_o$F$_1$ is shown in Figs. 1 and 2. Complete reconstitution of F$_1$ (370 kDa), after complete denaturation of all subunits into their primary structures with sodium dodecylsulfate and urea and refolding into tertiary structure, from subunits $\alpha$ (55 kDa), $\beta$ (52 kDa), $\gamma$ (32 kDa), $\delta$ (20 kDa) and $\varepsilon$ (14 kDa) was possible only in TF$_1$ (Fig. 3). The intermediate core subunit complexes, $\alpha_1$$\beta_1$ dimer, and $\alpha_3$$\beta_3$ hexamer, were also only obtained in TF$_1$, although the sequence homologies of core subunits were conserved in HF$_1$ (371 kDa). Enzymology revealed that $\alpha_3$$\beta_3$ hexamer was an oligomer, while $\alpha_1$$\beta_1$ dimer was a protomer. Owing to the stability of TF$_1$, the subcomplexes with chemically modified and mutated subunits were useful for nanotechnology.

TF$_o$ (148 kDa) is composed of a (30 kDa), b (17 kDa) and c (8 kDa) in a stoichiometric ratio of 1:2:10 (Figs. 1 and 3). HF$_o$ and BF$_o$ contain, in addition to the common subunits a (also called ATP6; 25 kDa), b (25 kDa), and c (8 kDa), minor subunits d (19 kDa), e (8 kDa), f (10 kDa), g (11 kDa), F$_6$ (9 kDa) and A6L (also called ATP8; 8 kDa) in a stoichiometric ratio of 1:2:10 (Figs. 1 and 3). The single stalk BF$_o$ obtained by urea-cholate treatment of BF$_o$F$_1$ was reconstituted with externally added $3^\text{H}$-acetyl-F$_1$ and the additional peripheral stalk components OSCP and F$_6$ were demonstrated in 1971. Isolated BF$_o$ subunits other than OSCP and F$_6$ are unstable, and their reconstitution has been unsuccessful without presequence and organizing machinery, even with chaperones. These BF$_o$ subunits were only identified on gel electrophoresis and X-ray crystallography. F$_o$F$_1$ was seen as a sphere ($\alpha_3$$\beta_3$ hexamer portion, diameter 12 mm x height 10 nm) connected by two stalks (central and peripheral) to a basal piece (subunits a and c$_{10}$ ring) (Fig. 3, lower right).

3.2. Protomeric, oligomeric and rotational ATPases: $\alpha_3$$\beta_3$ dimer, $\alpha_3$$\beta_3$ hexamer and $\alpha_3$$\beta_3$$\gamma$ heptamer. The isolated $\alpha$ and $\beta$ subunits of TF$_1$ both have AT(D)P-Mg binding activity accompanied by conformational changes without ATPase activity (Fig. 3, left). The open structure of $\beta_3$ and closed structures of $\beta_3$ and $\beta_1$ in the presence of ligands were confirmed in the isolated thermophilic $\beta$ using $^3$H-NMR. Both thermophilic $\alpha$ and $\beta$ subunits were reconstituted to form an active $\alpha_1$$\beta_1$ dimer by forming a catalytic $\alpha_3$ interface. Three $\alpha_1$$\beta_1$ dimers were reconstituted to form an allosterically active $\alpha_3$$\beta_3$ hexamer (Fig. 3, upper middle). Both the high catalytic activity and formation of F$_1$-bound ATP from ADP + Pi depend on the $\alpha_3$$\beta_3$$\gamma$ structure with rotational ATPase. There are six potential nucleotide-binding sites on F$_1$ and $\alpha_3$$\beta_3$$\gamma$ three catalytic sites on $\beta$ and three noncatalytic sites on $\alpha_1$ as confirmed by X-ray crystallography.

Depending on the occupancy of catalytic sites with increasing ATP concentration ([ATP]), there are three types of ATPase activities of F$_1$: uni-site, bi-site and tri-site. Uni-site catalysis is measured at sub-stoichiometric ATP concentrations ([ATP] < [F$_1$]). Uni-site activity is very low, and the apparent K$_{\text{M,ATP}}$ is less than 20 mM. The ATPase activity of the $\alpha_1$$\beta_1$ dimer of TF$_1$ showed typical Michaelis-Menten kinetics with only one K$_{\text{M,ATP}}$ value of 70 µM, and a V$_{\text{max}}$ value of 0.1 unit/mg, without the cooperative characteristics of a protomer. In contrast, ATPase activity of the $\alpha_3$$\beta_3$ hexamer showed the cooperative characteristics of an oligomer. The apparent K$_{\text{M,ATP}}$ of oligomeric ATPase of $\alpha_3$$\beta_3$ at 25°C were about 150 µM (bi-site) and 490 µM (tri-site). K$_{\text{M,ATP}}$ of rotational ATPase of TF$_1$ (Fig. 3, right) were about 80 µM (bi-site) and 490 µM (tri-site). The $\gamma$-containing complexes $\alpha_3$$\beta_3$$\gamma$, $\alpha_3$$\beta_3$$\gamma$$\delta$ and $\alpha_3$$\beta_3$$\gamma$$\varepsilon$, show common kinetic properties. The $\alpha_3$$\beta_3$ hexamer was inhibited by only one mole of $^3$H]-3'-O-(4-benzoyl) benzoyl-ADP per hexamer, similarly to both BF$_1$ and TF$_1$. Thus, the presence of only one inhibited-β in the hexamer blocked multi-site steady-state ATPase activity. This single-bit inactivation and cooperativity is an inherent property of the symmetrical $\alpha_3$$\beta_3$, but is not the due to the inhibition of rotation by TF$_1$ or $\alpha_3$$\beta_3$$\gamma$.

3.3. Rotation of the $\gamma$ subunit in $\alpha_3$$\beta_3$ hexamer of TF$_1$: One mole ATP hydrolysis at
one β subunit drives 120° rotation of γ subunit in a concerted manner. The rotational hypothesis of F₁Fₒ⁴ was also proposed by Oosawa as the “loose coupling mechanism of rotational proton ATPase” based on analogy with the H⁺-driven flagella motor in 1986.⁶¹) The rotation of the γ subunit axis in the cylinder of the α₃β₃ hexamer in the FₒF₁ motor with the torque of 42 pN nm was predicted by many lines of evidence.¹⁰) The rotation of γ was directly demonstrated in single-molecule studies using α₃β₃γ from TF₁.¹³) Rotational motion was visualized by attaching a fluorescently labeled actin filament (1–4 µm) to γS107C of artificially induced mutant γ subunit with the biotin-streptavidine bridge. The α₃β₃ hexamer was immobilized on a glass surface of Ni-nitrilotriacetate by artificially attaching decapoly-histidine to β.¹¹) and the ATP-driven rotation of the γ subunit was found to be anticlockwise when F₁ was observed from the Fₒ side (Fig. 1, upper left).¹¹),¹⁷)

The work performed by the rotating γ in a fixed α₃β₃ is the frictional torque times angle of rotation. The hydrodynamic frictional drag coefficient (ξ) of the actin filament for the propeller rotation is given by ξ = (π/3)ηL²/ln(L/2r) − 0.447, where, η (10⁻³ N s m⁻²) is the viscosity of the medium, L, the length of the actin filament (1–4 µm) and r, the radius of the filament (5 nm).¹¹) The observed rates of filament rotation at 2 mM ATP are 7, 1, and 0.1 revolutions per second, when the lengths of f-actin are 1, 2 and 4 µm, respectively.¹¹) The frictional torque ξω was about 40 pN nm, where ω is the angular velocity.¹¹) Hydrolysis of one ATP molecule drives a 120° rotation of the γ subunit relative to the cylinder of the α₃β₃ hexamer, and therefore, hydrolysis of three ATP molecules is required for one complete 360° revolution.⁵⁵),⁶²) In order to analyze rapid rotation by reducing the viscosity resistance of long actin filament, fluorescent gold beads (40 nm) were attached to γ. At nanomolar ATP concentrations, βE waits until the next ATP molecule is bound, and the duration of the pause depends on the ATP concentration (ATP-waiting dwell time).⁶²) ATP binding to βE is the power step that drives the 80° rotation of γ.⁵⁵) This rotation leads to simultaneous release of ADP from the catalytic site of βF, and hydrolytic cleavage of ATP into ADP and Pi at βT after a pause (catalytic dwell time), and a 40° rotation occurs to complete the 120° rotation.⁶²)

Using mutant β (E190D) of TF₁, in the same rotational experiments, the catalytic activity of each β subunit was shown to be coordinated with the other two β subunits to drive rotation of the βE, βF, and βT cycle.⁶³) Hybrid F₁ containing one or two mutations with altered catalytic kinetics rotates in an asymmetric stepwise fashion with different dwell times. Analysis of the rotation revealed that for any given β subunit, the subunit binds ATP at 0°, cleaves ATP at approximately 200° and carries out a third catalytic event at approximately 320°. This demonstrates the concerted nature of the F₁ complex activity, where all three β subunits participate to drive each 120° rotation of the γ subunit with a 120° phase difference.⁶³)

3.4. Torque-driven ATP synthesis by TF₁. ATP is synthesized by mechanical energy applied on the γ subunit without proton flux. ATP synthesis driven by mechanical energy (Fig. 1) was directly shown by attaching a magnetic bead (diameter = 700 nm, biotinylated) to the γ subunit of α₃β₃γ of mutant TF₁ (C193α, H197β, and S107Cγ), E210Cγ) on a glass surface, and rotating the bead using electrical magnets.⁶⁴) After the ATP-driven rotation of the beads was confirmed, the magnet was turned on and several bursts of hundreds of revolutions at 10 Hz were imposed.⁶⁴) Anticlockwise forced rotation of the γ subunit by the magnetic beads resulted in the appearance of ATP in the medium, as detected by counting the photons emitted from the luciferase–luciferin reaction with a camera (Hamamatsu Photonics).⁶⁴) This shows that torque working at one particular point (γ) on a protein complex can influence a chemical reaction occurring at physically remote catalytic sites (β), driving the reaction far from equilibrium.⁶⁴)

4. Genes for TF₂F₁ and HF₂F₁: Single operon vs. nuclear and mitochondrial genes

Detailed genetic analysis and site-directed mutagenesis have been reported by Futai using E. coli F₁Fₒ (EF₁), as E. coli genetics are well understood.¹⁸),⁶⁵) The catalytic, structural and regulatory significance of an amino acid residue in EF₁ was elucidated by site-directed mutagenesis.⁶⁵) However, many crucial experiments, including the planar F₁Fₒ bilayer and torque-driven ATP synthesis,⁶⁴) have not been successful to date with EF₁Fₒ, due to its fragility. Thus, a special sequencing method for thermophilic genes was developed.⁶⁵) The structure of the TF₁Fₒ operon (number of amino acid residues)¹²),¹⁹) I(127)-a(210)-c(72)-b(163)-δ(163)-α(502)-γ(286)-β(473)-ε(132), was similar to that of the EF₁Fₒ operon.¹⁸),¹⁹)

Amino acid residues in the different α, β, and γ subunits from TF₁,¹²),¹⁹) HF₁,¹³),¹⁷),¹⁸) BF₁,²⁴) and
EF1 are aligned and expressed in the format α10, which refers to residue #10 in the α subunit. The residue numbers of amino acid sequences in the α and β subunits of TF1 are shown in Fig. 4 (dots indicate every tenth residue). Primary structures are homologous, with 59% sequence identity between thermophilic α/human α and 68% between thermophilic β/human β. The primary structure of the TF1 β subunit showed homology with 270 residues which are identical in the β subunits from HF1, CF1, and EF1. The homologies of the amino acid sequence between BF1 and YF1 were 73%, 79% and 40%, respectively, for the α, β and γ subunits. As these YF1 subunits were functionally complemented with corresponding BF1 subunits, the essential structure is conserved among YF1, BF1 and HF1 (sequence is nearly identical to that of BF1, but there were polymorphisms in HF1). Residues forming reverse turns (Gly and Pro) were highly conserved among the β subunits. Conserved residues (green and blue letters in Fig. 4) among TF1, HF1 and EF1 are closely related to catalytic and regulatory functions. The observed substitutions in the thermophilic subunit increased its propensities to form secondary structures, and its external polarity to form tertiary structure.

Fig. 4. Aligned amino acid sequences and secondary structure elements of α and β subunits in TF1. Solid black lines indicate folds, and these were classified into α-helices (A–H, 1–8) and β-sheets (a–f, 0–8). The labels for folds are provided only for the β subunit, except for the three C-terminal α-helices in the α subunit. Dots indicate every tenth residue. I–XI: areas of α/β contact. Red: catalytic contact areas of β. Pink: catalytic contact areas of α. Blue: non-catalytic contact areas of β. Green: non-catalytic contact areas of α. Colored bars indicate contact residues in T/F1. Sequences are divided by red asterisks (*) to indicate the three domains.
experiments on torque-driven ATP synthesis. Species-specific residues (black letters in Fig. 4) may have phylogenetic components, including thermophilic loops (-ARNENEV-) (Fig. 4, first line I′) that render TF1 stable. Since determining the nucleotide sequence of TFoF1, numerous rotating ATP synthases of thermophilic F-type or V-type (vacuolar ATPase) have been sequenced and characterized.

In contrast to the single operon TFoF1, the gene structure of HFoF1 is highly complex; most subunits are encoded by nuclear DNA, with signal peptides to target this protein to the mitochondrial inner membrane, but subunits a and A6L of HFo are encoded by mitochondrial DNA. The complete sequence of the 16,569-base pair human mitochondrial DNA contains genes for 12S and 16S rRNAs, 22 tRNAs, ATPase subunits 6 (corresponding to the a subunit of HFo) and 8 (corresponds to A6L of HFo), and 11 other protein coding genes.

5. Crystallographic analysis of FoF1: Detailed structure of H+-driven and ATP-driven motors

5.1. Crystallography of Fo. The c subunits form a ring around a central pore. The numbers of the c subunit in the Fo ring differ depending on the species: in CFo, it is 14, while that for YFo and TFo is 10. The conserved carboxylates E61 of CFo (corresponds to E56 of TFo) are involved in proton transport, are 1.06–1.08 nm apart in the c-ring, the peripheral stalk, central stalk and the stator (Fig. 2).

X-ray crystallography (0.325 nm resolution) of the complex of EF1 was also recently reported (Ducan, T.M., personal communication, 2010). The peripheral stalk consists of a continuous curved α-helix about 16 nm in length in the single b-subunit, augmented by the predominantly α-helical d and F6 (Fig. 2, right).

5.2. Crystallography of FoF1. The c subunits form a ring around a central pore. The most detailed structural information for amino acid residues in a protein is obtained by crystallographic analysis. In 1977, a two-dimensional crystal of TF1 showed the pseudo-hexagonal structure of αββγ. Three-dimensional crystals of TF1 (Fig. 5B) and αββγ hexamer (Fig. 5C) were obtained using dye-ligand chromatography columns. The high resolution power of the Photon Factory synchrotron (for TF1, 0.32 nm resolution at Tsukuba) revealed the detailed structure of αββγ (Fig. 5D), the c-ring, the peripheral stalk, central stalk and the stator (Fig. 2). X-ray crystallography (0.325 nm resolution) of the αββγ complex of EF1 was also recently reported (Ducan, T.M., personal communication, 2010). The peripheral stalk consists of a continuous curved α-helix about 16 nm in length in the single b-subunit, augmented by the predominantly α-helical d and F6 (Fig. 2, right).
show increased hydrophobicity and reduced hydrogen bonding.72) Upon deprotonation, the conformation of E61 is changed to another c subunit and becomes fully exposed to the periphery of the ring.72) Reprotonation of E61 by a conserved R in the adjacent a subunit returns the E61 to its initial conformation.72) Genetically modified TF₂F₁₈s, each containing a c subunit dimer (c₂) to a dodecamer (c₁₂), were prepared by genetic cross-linking.15) Among these, TF₂F₁₈s containing c₂, c₅, or c₁₀ showed ATP-synthesis and other activities, but those containing c₉, c₁₁ or c₁₂ did not. Thus, the c-ring of functional TF₂F₁ is a decamer (c₁₀).15) When TF₁ was removed from the modified TF₂F₁₈s, TF₂₈s containing only c₂, c₅ or c₁₀ worked as proton channels.15) In fact, a 36° step size of proton-driven c₁₀-ring in FoF₁ was determined at 0.28-nm resolution by Walker group.9) In the structure of BF₁ crystallized in the presence of ligand (AMP-PNP, ADP, Pi = 50:1:0, and Mg²⁺), the three catalytic β subunits differed in conformation and in bound nucleotide. There were four unhydrolyzable ATP analogue (AMP-PNP) molecules, three in equal three α subunits, one in β (β₁) and one ADP in β (β₀); the remaining β was empty (β₀).9)

The ATP-binding site of β₁ is surrounded by the residues shown in Fig. 5A (black numbers for BF₁) are identical to those of HF₁, red numbers for TF₁).9,71) In the P-loop of β₁ of TF₁, the essential K164 forms hydrogen bonds with the phosphate of the nucleotide, and the oxygen of T165 coordinates with Mg²⁺, while in the GER-loop, E190 interacts with water (Fig. 5A).9,71) The catalytic activity of β requires α that supplies thermophilic α-R365 and thermophilic α-S336 to the ATP-binding site at the αβ interface (Fig. 5A).9,71,74) The positive charge of α-R365 stabilizes the phosphatase of ADP, and R256, R191 and K164 of thermophilic β interact with the negative charges of the phosphates of ADP (Fig. 5A). The cross-linking of thermophilic ΔY341 with azido-ATP71,74) predicted a hydrophobic interaction between the adenine ring, and Y341, F414 and F420 (Fig. 5A).9,71)

5.4. Basic structure of αββ₁ is rendered asymmetric by addition of γ and/or nucleotides. The basic structure of F₁ is a symmetrical αββ₁ hexamer that is composed of three pairs of alternating αE and β₁ (Fig. 5D).71) However, the asymmetry induced by introduction of γ and/or ADP to αββ₁ hexamer is critical in the mechanism of ATP synthesis.9) The nucleotide-free β₁ in both the F₁ crystal9) and solution56) has an open structure (Fig. 6) that is essentially identical to β₁ in the nucleotide-free αββ₁ hexamer.71) Both β₁ and β₂, as well as α₁, assume closed structures (Fig. 6, direction of the open arrow).9) Interconversion of the open-close conformational states of β is achieved by addition of γTF₁ to the isolated β₁₁₁ of TF₁.9,71) However, nucleotide-free YF₁ contained β₁ and β₁₁ as those of nucleotide-bound BF₁.75) This suggests that βγ interactions at the three contact points (Fig. 6, middle, γ₁–3), including interaction of Arg residue at position 75 (γ₁R75) with β₁E395 in the DELSEED sequence of BF₁ to change the mutual conformation,10,14) are as important as nucleotide occupancy in converting open β₁ to the closed β₁₁.75) As genes for α, β and γ of YF₁, in the α-β₁γ deleted mutant yeast are complemented with those of BF₁,8) the functional residues are essentially equal between YF₁ and B(H)F₁. Occupancy of the catalytic site by ATP or ADP can be mimicked by convenient BeF₃-ADP complexes that bind to the catalytic sites of β₁₁₁ and β₁₀.70) The structure is representative of an intermediate in the reaction pathways.70) The conformational change of β induced by γ-rotation is essential for ATP synthesis (ATP release from the catalytic site), while that induced by ATP-binding to β is necessary to elicit torque on γ.71,74)

5.5. Three domains in α and β: β barrel, nucleotide-binding and α-helical bundle domains. The overall molecular structure of α and β can be divided into three domains9,71,75): an N-terminal β barrel (Fig. 6, top N to *), a central nucleotide-binding domain (Fig. 6, middle * to **), and a C-terminal α-helical bundle (Fig. 6, bottom ** to C).7) The locations of amino acid residues in the α-helices (Fig. 4, solid black lines A–H, 1–8) and β-sheets (Fig. 4, solid black lines a–f, 0–8)71,74) are compared in the three-dimensional structure of thermophilic β (Fig. 6). As the amino acid sequence of BF₁ is nearly identical to that of HF₁ (99% homology in β),19,21) the following discussion on BF₁
is also applicable to HF1. Superposition of the overall crystallographic structures of thermophilic βE and bovine βE or bovine α revealed that the folding of these structures is very similar.9,74) The β barrel domains (Fig. 4, To 21–94, T/β1–82) contain six β strands (Fig. 6, plate form arrows, βα–βα).74) The nucleotide-binding domains (T α95–371, T/β83–354) consist of nine-stranded β-sheets surrounded by eight α-helices (Fig. 6, αA to αH) and a small antiparallel β-sheet. The C-terminal α-helical bundle domain of α (thermophilic α375–502) contains six helices (Fig. 4, α1, 2, 4, 6–8), while that of β (thermophilic β355–473) consists of six α-helices (Fig. 4, α1–6). Thus, the largest difference between α and β is found in the C-terminal region.9,74) and the DELSD(ED) sequence localized in this region was shown to be the most important β interface.10)

5.6. Catalytic and non-catalytic αβ interfaces and conformational change. The crystal structure of BF19) and α3β1 of TF174) indicates that, in general, the conserved residues lie on the αβ interfaces, as shown by detailed homology search among TF1, HF1, CF1 and EF1.19) There are two types of αβ interface that contain either a catalytic site or non-catalytic site.9,74,75) The area where pairs of residues connecting the αβ interface are located is defined as contact area. Contact residue pairs within a limit of 0.40 nm across the αβ interfaces in BF1 and the α3β3 hexamer of TF1 were analyzed by a computerized atom search using the CCP4 Suite: Program Contact.74) The contact areas composed of homologous residue pairs found in both TF1 and BF1 were defined as homological contact areas. The contact areas found only in one species, such as the thermophilic loop of TF1 (I in Fig. 4) were defined as species specific contact areas. These areas are expressed as primary structure in Fig. 4. The contact areas are located in both β and α at catalytic (red and pink bars in Fig. 4), and non-catalytic (blue and green bars in Fig. 4) interfaces. There are seven catalytic (red I–III, V–VIII in Figs. 4 and 6) and non-catalytic (blue I–VII in Figs. 4 and 6) contact areas on the open β form (βP). The number of contact areas on closed β (βP and βT) increased to 11 (red I–XI in Fig. 6) and 9 (blue I–IX in Fig. 6), respectively, in the catalytic and non-catalytic interfaces. The barrel domain harbors the universal contact areas I and II (Fig. 6, upper), and the common electrostatic bond in II is thermophilic βR72–thermophilic αE67 (=human βR71–human αE67).9,74) At the catalytic nucleotide-binding domain, areas III, V, VI, VII and VIII are universally detected (Fig. 4). However, in TF1, the P-loop contact area IV is latent, in contrast to that area in thermophilic αE (Fig. 6). Human αR373 interacts with oxygen in the β and γ-P of ATP bound at IV.9) In V, the common electrostatic bonds are thermophilic βR193–αD339. In VI and VII of βT and βP, human αF299–βM222 and human αS344–βR260, respectively, interact. However, we identified no direct contact in the α-helical bundle domain in βE of TF1.74) In the catalytic αβ interface of human βE, the contact areas (17.6 nm²) are
homologous to those of thermophilic β, while the areas in human βD (30.3 nm²) and human βT (22.0 nm²) are increased to 11 and 10, respectively. This is caused by the 30° upward motion of the C-terminal domains.

5.7. Catalytic sites. The catalytic α/β interface is located on the left side of β in the α3β3 hexamer (Fig. 5D), and the structure of catalytic domain (Fig. 5A) is strictly conserved among species. The catalytic domain accommodates the P-loop located between sheet 3 and helix B (T/β163–178) and the conserved thermophilic E190 (=human E188) in the GER loop localized between β-sheet 4 and N-terminal end of α-helix C (Fig. 4, GER, and Fig. 6, middle). As predicted by X-ray crystallography of AMPPNP-BF1, NMR analysis revealed that thermophilic βR191 (Fig. 5A, upper left) forms a hydrogen bond with the γ-phosphate of ATP. 

6. Nanotechnological analysis of TF<sub>o</sub>F<sub>1</sub> by single-molecule imaging: Dynamic movement of F<sub>a</sub>F<sub>1</sub>

X-ray crystallography of F<sub>1</sub> and F<sub>a</sub>F<sub>1</sub> is a static snapshot of inhibited ATPase crystallized in the presence of AMPPNP or BeF<sub>2</sub> or in the absence of nucleotides. These crystals do not represent the transient movement of subunits of TF<sub>1</sub> during γ-rotation, or activity of TF<sub>a</sub>F<sub>1</sub> in a liposome. Thus, the dynamics of rotating TF<sub>f</sub> or ATP synthesis in F<sub>a</sub>F<sub>1</sub>-lipoosomes must be measured by using TF<sub>1</sub> containing mutant β subunits, and also using modalities such as fluorometry or NMR.

The efficiency of florescence resonance energy transfer (FRET) between a donor and an acceptor fluorophore depends on their distance. If two fluorophores are bound to appropriate amino acid residues in rotor and stator subunits, relative subunit movements can be observed in real time by confocal microscopy.

6.1. Nanomotor movement analysis by single-molecule FRET. In a single TF<sub>1</sub> molecule fixed on a glass surface, a donor fluorophore (Cy3) was bound to one of the three βs and an acceptor fluorophore (Cy5) was bound to the protruding portion of γ, and single pair (Cy3-Cy5) FRET was performed to estimate the waiting conformation during ATP hydrolysis. As Cy3- and Cy5-maleimide are bound to cysteine residues, site-directed mutagenesis [α(C193S), β(S205C) and γ(S107C)] was performed to bind Cy5 to β, and to bind Cy3 to γ, and to prevent binding of Cy3 and Cy5 to α (residue numbers of α and β are indicated in Fig. 4). The sole cysteine in a mutant subcomplex of TF<sub>f</sub>, α(C193S)/β(His-10 tag at N terminus) γ(S107C), was labeled with Cy5-maleimide. The (Cy5-γ)TF<sub>f</sub> was incubated with Cy3-β(S205C) at 1:10 at 45°C for 2 days, and the free β subunit was removed on a size exclusion column. The energy of the laser beam (532 nm) on Cy3 was transferred to Cy5 and emitted light (670 nm) when the Cy3-Cy5 distance was small, while only Cy3 light (570 nm) was emitted when the Cy3-Cy5 distance was great. FRET yield changed cyclically as γ rotated and the Cy3-Cy5 distances were estimated during the conformation change.

The distance between the two dyes changed continuously as 5.7, 7.9, and 7.9 nm during rotation at low ATP concentrations, and the conformational change corresponded to the ATP-waiting state of TF<sub>f</sub>.
The relative subunit movement during ATP synthesis has also been measured by FRET between two fluorophores bound to a stator subunit (b-subunit) and a rotor subunit (γ- or ε- or c-subunit) (Fig. 1). The labeled FoF1 was reconstituted in the liposome (one FoF1 per liposome) and ΔρH\textsuperscript{+} was applied, so that F₁F₁Δρ carried out \textit{H}\textsuperscript{+}-driven ATP synthesis.\textsuperscript{[46]} Analysis of the time course of FRET efficiency in the F₁F₁-liposome showed the rotation of \(γ\) and \(ε\)-subunit relative to b-subunit in 120° steps,\textsuperscript{[80]} and that of the c₁₀-ring in 36° steps.\textsuperscript{[16]} The \(β\) motions through an attached fluorophore, concomitantly with the 80° and 40° substep rotations of \(γ\) in the same single molecules, showed the sequence of conformations that each \(β\) undergoes in three-step bending, an approximately 20° counterclockwise turn followed by two approximately 20° clockwise turns, occurring in synchronization with two substep rotations of \(γ\).\textsuperscript{[80]} The results indicate that most previous crystal structures mimic the conformational set of three \(β\) in the catalytic dwells,\textsuperscript{[80]} while the previously described set of \(β_0\, β_0\) and \(β_7\) was revealed in the ATP-waiting dwells.\textsuperscript{[80]} These fluorescent studies thus bridge the gap between the chemical and mechanical steps in F₁F₁. Starting from the ATP-waiting dwell (0°), the 80° and 40° substeps of \(γ\) rotation are induced by ATP binding and ADP release, and ATP hydrolysis and Pi release, respectively.\textsuperscript{[62],[79]–[81]}

### 6.2. H\textsuperscript{+}/ATP ratio and elastic power transmission in F₁F₁

One of the unsolved questions in the mechanistic study is the analog–digital conversion of energy in F₁F₁. The electrochemical energy of the \(H^+\) current\textsuperscript{[14]} through F₁F₁ in liposomes\textsuperscript{[3]} and planar bilayers,\textsuperscript{[51]} and the electric,\textsuperscript{[48],[50]} magnetic\textsuperscript{[84]} and mechanical energy of the rotation\textsuperscript{[11],[62]} are all analog quantities. The numbers of ATP molecules synthesized and protons transported are digital quantities. Thus, the elastic power transmission in F₁F₁ analog/digital conversion during the \(γ\)-rotation was predicted in 1996.\textsuperscript{[10]} Oosawa also proposed a loose coupling mechanism in which the number of protons necessary for the synthesis of one ATP is not an integer but varies depending on the environmental conditions.\textsuperscript{[81]} As one proton is translocated by one \(360°\) of the c₁₀-ring, the inevitable consequence is noninteger ratios of rotation step sizes for F₁F₁ (120°/36°) and for \(H^+/\text{ATP}\) (10:3).\textsuperscript{[14],[15],[74]} This step-mismatch necessitates elastic twisting of F₁F₁ during rotation and elementary events in catalysis.\textsuperscript{[7],[74]} The \(H^+/\text{ATP}\) ratio in F₁F₁ addresses this analog/digital problem. F₁\(β\)s with c₁₀ ring are present in organisms that maintain Δ\(μ\)H\textsuperscript{+} mainly in the form of Δ\(ψ\).\textsuperscript{[14],[15]} whereas F₁\(β\)s with c₁₀ ring are mostly found in species with Δ\(μ\)H\textsuperscript{+} existing predominantly in the form of Δ\(ψ\).\textsuperscript{[72]} H\textsuperscript{+}/ATP ratios of 4.7 and 3.3 are thus expected for CF₁F₁ (with 14\(c\)/3\(β\))\textsuperscript{[72]} and E(T)F₁F₁ (with 10\(c\)/3\(β\))\textsuperscript{[14],[16]} respectively. To confirm the effects of c/β subunit ratios on H\textsuperscript{+}/ATP ratio, pH of the internal phase of the reconstituted F₁F₁-liposomes was equilibrated with acidic medium.\textsuperscript{[46],[47]} Then, an acid-base transition\textsuperscript{[85]} was induced by adding alkaline medium to the liposomes to produce ΔpH across the membrane, and the initial rate of ATP synthesis was measured with luciferase.\textsuperscript{[83]} From the shift in the equilibrium ΔpH as a function of Q (=[ATP]/([ADP][Pi])\textsuperscript{[7]}), the standard Gibbs free energy for phosphorylation, ΔG\textsubscript{p0}′, and the H\textsuperscript{+}/ATP ratio were determined.\textsuperscript{[82]} The results were as follows: ΔG\textsubscript{p0}′ = 38 ± 3 kJ/mol and H\textsuperscript{+}/ATP = 4.0 ± 0.2 for CF₁F₁; and H\textsuperscript{+}/ATP = 4.0 ± 0.3 for EF₁F₁. This indicates that the thermodynamic H\textsuperscript{+}/ATP ratio is the same and that it differs from the subunit c/β stoichiometric ratio.\textsuperscript{82} However, in order to estimate actual energetics, the very low turnover rates (<1 ATP/s) in this experiment\textsuperscript{[82]} need to be examined under different physiological conditions (>100 ATP/s).

The site of analog–digital conversion by elasticity\textsuperscript{7} was estimated by direct measurement of the torsional stiffness.\textsuperscript{83} Most parts of F₁, particularly the central γ shaft in F₁, and the long eccentric bearing had high stiffness (torsional stiffness \(κ > 750 \text{ pN nm}^{−1}\)).\textsuperscript{[83]} One domain of the rotor, namely, where the globular portions of γ and ε contact the c-ring, was more compliant (κ congruent with 68 pN nm).\textsuperscript{[83]} The \(γ\)-induced or nucleotide-dependent open–close conversion of conformation is an inherent property of an isolated \(β\) and energy and signals are transferred through α/β interfaces.\textsuperscript{7,[11],[74]} Rotation of the central shaft γ in α₂β₂ hexamer is assumed to be driven by domain motions of the \(β\)s. These \(β\) motions were directly observed through an attached fluorophore by FRET\textsuperscript{79}[80] and NMR,\textsuperscript{56}.

The mechanisms underlying the open(\(β_3\))–close(\(β_7\)) motion were investigated for TF₁ in solution, using mutagenesis and NMR.\textsuperscript{56} The hydrogen bond networks involving side chains of K164 (162 for human)  is shown in parentheses), T165(163), R191(189), D252(256), D311(315) and R333(337) in the catalytic region (Fig. 5A, red text for TF₁ and black text for HF₁).\textsuperscript{[74]} They are significantly different for the ligand-bound and free \(β\) subunits. The role of each amino acid residue was examined by
A (alanine) substitution. The chemical shift perturbation of backbone amide signals of the segmentally labeled mutant indicated stepwise propagation of the open/close conversion on ligand binding. Upon ATP binding, the open/close conformation change regulated by hydrogen-bond switching from K164/D252 to T165/D252 (Fig. 5A, right upper, red test) would take place in the thermophilic subunit because ATP-binding is the major driving force for the first 80° rotation. The resulting closing motion of the hinge (HGG) between α-helix B and β-sheet 4 (Fig. 6, right) generates the torque of γ rotation through the DELSD(E)ED contact point (Fig. 6, bottom). Although the time scale of atomic fluctuations is in the order of tens of nanoseconds, molecular simulation will solve the detailed movements of residues in αβ during the γ rotation (order of milliseconds) in the future.

7. Biogenesis of human FoF1: Regulated expression, splicing, import and assembly

The biogenesis of HFoF1 is an intricate process, starting from transcription, splicing of nuclear encoded subunits, translation of mitochondrial DNA-encoded subunits and nuclear DNA-encoded precursor peptides for all F1 subunits, followed by targeting, importing and processing of nuclear DNA-encoded precursor peptides in the mitochondrial matrix. Precursor importing requires both "7 H⁺ to drive translocation and specific carrier proteins in the outer and inner mitochondrial membrane, as well as general chaperones present in the cytosol and mitochondrial matrix. Processing of the presequence of precursor requires a specific protease, and after the removal of the presequence, nuclear-encoded subunits are assembled into HFoF1 with two mtDNA-encoded Fo subunits (a and A6L). Targeting the presequence of three isoforms of subunit c liberated after proteolysis is required for the assembly of cytochrome oxidase.

As both the amino acid and nucleotide sequences of subunits in HFoF1 are available in internet databases, only physiologically important points...
in the biogenesis of HFoF1 will be reviewed here.\textsuperscript{21,28,80,89} In contrast to prokaryotic FoF1, including TFoF1,\textsuperscript{7} proteomics have revealed a large universe of pseudogene products,\textsuperscript{21,84} splice variants (muscle-type F1\textsubscript{α}) (Fig. 7),\textsuperscript{27,32,90} post-translational modifications (phosphorylated HFoF1, etc.),\textsuperscript{91} dimeric HFoF1,\textsuperscript{30} a supramolecular complex called ATP synthasome (Fig. 2),\textsuperscript{31} and ectopic HFoF1 (Fig. 10, right).\textsuperscript{92}

7.1. Expression of HFoF1 genes. The gene structure of the HF1\textsubscript{α} subunit is 14 kbp in length and contains 12 exons interrupted by 11 introns.\textsuperscript{21,84} Primer extension and S1 mapping analysis showed the presence of multiple transcription initiation sites in the HF1\textsubscript{α} gene.\textsuperscript{84} The 5′-flanking region of the HF1\textsubscript{α} gene has an unconserved GC-rich region, including several binding motifs of transcriptional factors, such as Sp1, AP-2 and GCF. The basal promoter activity was located near the GC-rich region. Comparison of the 5′-upstream region of the HF1\textsubscript{α} gene with those of the genes for BF1\textsubscript{α}, HF1\textsubscript{β}\textsuperscript{85} and HF1\textsubscript{γ}\textsuperscript{27} indicated three common sequences (CS1,
CS2 and CS3) in the regulatory regions, suggesting that putative cis-elements coordinate the expression of the three subunit genes for HF1. The enhancer activities derived from 5′-deletion mutants of a HF1-CAT (chloramphenicol acetyltransferase) chimeric gene were different in cell lines from four different human tissues, thus suggesting the existence of cell type-specific gene regulation. The HF1 gene is 14 kbp in length and contains 10 exons, with the first exon corresponding to the non-coding region and most of the presequence, which targets this protein to the mitochondria. Eight Alu repeating sequences including inverted repeats were found in the 5′-upstream region and introns. An S1 nuclease protection experiment revealed two initiation sites for transcription. Three CCAAT boxes were found between the two initiation sites, and two GC boxes were located in the 5′-upstream region. Promoter activity was estimated by the CAT method and an enhancing structure for transcription was detected between nucleotides -400 and -1100 in the upstream region. The system coordinating expression of nuclear-coded mitochondrial proteins was investigated by examining the 5′-flanking region of the HF1/β gene. In one of the enhancing regions, a consensus sequence was found for the genes of other mitochondrial proteins, such as those for cytochrome c1 and the pyruvate dehydrogenase α-subunit. The characteristics of this enhancing element were examined by introducing a synthetic oligonucleotide element into the CAT plasmid with a deleted enhancing element. The resulting plasmid showed full recovery of promoter activity, and this activity was independent of the orientation or location of the insert. Therefore, this enhancer may be common to the nuclear genes of some mitochondrial proteins involved in energy transduction.

The functions of subunits in mitochondrial F1,F0 were confirmed by expression of genes in deletion mutants, and their functional complementation. The genes encoding BF1 subunits (except for those of δ) were expressed in a quintuple yeast YF1 deletion mutant (ΔαΔβΔγΔδΔε) after introduction of a chimeric BF1 subunit gene construct that uses the YF1 transcriptional promoter and termination sites, as well as the presequence for YF1. Expression of the α-, β-, γ-, or ε-subunit of BF1 complemented the corresponding individual mutations in YF1. All BF1 subunits (with chimeric δ) expressed in yeast produced an F1 that was purified to a specific activity of about half of that of original BF1. These results indicate that the molecular machinery required for the targeting, proteolysis and assembly of the mitochondrial F1,F0 is conserved from yeast to humans (Fig. 9). Similarly, bovine OSCP and some
F_o components have been functionally complemented (quoted in Ref. 8). In one functional study, the gene for γ subunit (atp-3) was partially blocked by RNA interference (RNAi) in Caenorhabditis elegans, and ATP levels decreased from 15 mmol/mg protein (control) down to 4 mmol/mg, and the behavior extended the lifespan. However, tissue differentiation is absent in yeast, alternate splicing must be studied in human cells.27,32

7.2. Tissue-specific splicing of HF_1γ: Analysis using transgenic mice and minigenes. Mammalian F_oF_1 is characterized by tissue-specific expression of the F_1 gene, which was analyzed using transgenic mice32 and minigenes in cultured human cells.90 The muscle-specific isoform of HF_1γ was generated by alternative splicing, and exon 9 was found to be lacking in skeletal muscle and heart tissue (Fig. 7, A).27 Using transgenic mice,27,90 the alternative splicing of exon 9 was shown to require de novo protein synthesis of a cis-acting element on the spliced exon of HF_1γ gene. An HF_1γ wild-type minigene, containing the full-length gene from exons 8 to 10, and two mutants were prepared; one mutant involved a pyrimidine-rich substitution on exon 9, whereas the other was a purine-rich substitution (abbreviated as HF_1γ Pu-del and HF_1γ Pu-rich mutants, respectively).90 Pu-del inhibited exon inclusion, indicating that a Pu-del mutation disrupts an exonic splicing enhancer. On the other hand, Pu-rich blocked muscle-specific exon exclusion.90

Transgenic mice bearing both mutant minigenes were then analyzed for their splicing patterns in tissues.90 Based on an analysis of HF_1γ Pu-del minigene transgenic mice, the purine nucleotide in this element was shown to be necessary for exon inclusion in non-muscle tissue. In contrast, analysis of HF_1γ Pu-rich minigene mice revealed that the HF_1γ Pu-rich mutant exon had been excluded from heart and skeletal muscles in these transgenic mice, despite the fact that mutation of the exon inhibited muscle-specific exon exclusion in myotubes at early embryonic stages.32 These results suggest that the splicing regulatory mechanism underlying HF_1γ pre-mRNA differs between myotubes and myofibers during myogenesis and cardiogenesis.32

A detailed mutational analysis of exon 9 (Fig. 7, B) revealed a purine-rich exonic splicing enhancer (ESE) element (5′-AAUGAAAAA-3′) functioning ubiquitously, with the exception of muscle tissue. An exonic negative regulatory element responsible for muscle-specific exclusion of exon 9 was discovered using both in vitro and in vivo splicing systems.32,90

Mutation analyses on the HF_1γ Ex8-9 minigene using a supplementation assay demonstrated that the muscle-specific negative regulatory element is positioned in the middle region of exon 9, immediately downstream from ESE. Detailed mutation analyses identified a muscle-specific exonic splicing silencer (MS-ESS) (5′-AGUUCCA-3′) responsible for exclusion of exon 9 in vivo and in vitro (Fig. 7, C).90 This element was shown to cause exon 9 skipping of in vivo splicing systems.90 Although there are three variants of the C subunit88 and several alternate splice variants in the human mitochondrial fusogenic proteins (mitofusin 1, 2),87 the γ subunit of HF_1 is the only well-characterized variant in F_oF_1.86,90

8. Mitochondrial cytology of HF_oF_1: cytoplasts lack nDNA and ρo cells lack mtDNA

HF_oF_1 is encoded by both mitochondrial DNA (mtDNA)22,26,33 and nuclear DNA (nDNA) (Fig. 9).21 In order to analyze the roles played by mtDNA and nDNA, mtDNA-less cells (ρo cells)22,33 and nDNA-less cells (cytoplasts) were developed (Fig. 8).87 Using ethidium bromide, mtDNA was removed and the resulting ρo cells became strictly dependent on glycolysis to compensate for the oxphos that supplies ATP.33 Thus, a glucose medium is essential for ρo cells.22 Cytoplasts are enucleated cells that contain mitochondria (Fig. 8, upper left), with examples being enucleated oocytes,87 synaptosomes94 and platelets.94 Since DNA sequence of an individual differs from each other owing to the genetic polymorphism of mtDNA,21 personal collection of mtDNA is essential to elucidate mitochondrial diseases. Human mitochondria with intact mtDNA have been directly isolated from postmortem platelets.94 Expression of nDNA-encoded HF_oF_1 subunits was not affected in ρo cells,87 while that of mtDNA-encoded HF_o subunits (F_0a and A6L) was lost in cytoplasts, as nDNA-encoded mitochondrial transcription factor A (Tfam)95 was lacking (Fig. 9).87 The ρo cells have no respiratory chain, because of the loss of mtDNA-encoded subunits of cytochromes and NADH dehydrogenase.26,87 Despite the absence of oxphos, ρo cells require mitochondrial compartments with a sufficient ∆ψH+ for energy driven transport of matrix components.87 The essential ∆ψH+ of ρo cells is maintained by the electrogenic exchange of ATP 4− for ADP 3− by ANC.94 To energize the inner membrane, α/β (ATPase active)53 in the matrix of ρo cells regenerates ADP from translocated ATP.94
The term heteroplasmy refers to cells that contain a mixture of mtDNAs with different sequences (Fig. 8, upper right), whereas homozygosity means that 100% of their mtDNA has an identical sequence (Fig. 8, upper left).22) Cybrids were formed by cytoplast fusion with $\rho^0$ cells using polyethylene glycol (PEG) (Fig. 8, bottom).87,94) The majority of pathogenic mtDNA mutations are heteroplasmic, with mutated and wild-type mtDNA coexisting in the same cell (Fig. 8, upper right).86,87) Owing to the absence of protecting histones, mtDNA is highly susceptible to mutations that result in heteroplasmy. Mutations in the tRNA gene of mtDNA often block translation and cause complete deletion of mtDNA-encoded proteins (sym mutation), including HFo subunits.57)

During development, cell division unevenly distributes heteroplasmic mtDNA into daughter cells and eventually segregates homoplasmic cells with wild-type and sym− mutant mtDNA (Fig. 8, bottom right). This stochastic segregation of the sym− mutation results in the sym− mutant concentrated tissues and causes mitochondrial diseases, including mitochondrial diabetes.87) The homoplasmic sym− cells lack oxphos and depend on glycolysis. Under the influence of polymorphisms in mtDNA and nDNA, a vicious circle of reactive oxygen species will damage cells. However, mitochondrial transfer from wild-type homoplasmic cytoplasts by fusion to form cybrids will normalize the diseased cells87) and sym− mutation will be alleviated by mitochondrial fusion.87,97) We analyzed heteroplasmy and polymorphisms related to diabetes and its complementation by fusogenic proteins.87) Mitochondria in human cells are visualized as a network or as filaments that undergo continuous changes in shape and in localization within the cells. Mitochondrial fusion proteins including mitofusins87) and OPA198) that may work as natural modulators have been implicated in the regulation of mitochondrial biogenesis encoded by both nDNA and mtDNA (Fig. 9). A number of transcriptional factors that are common to the expression of these gene products. As Tfam encoded by nDNA is essential for both the initiation of transcription and the replication of mtDNA, we cloned and sequenced the human Tfam gene.95) There were sequences in the 5′-upstream regulatory region of Tfam common to those in HFoβ.95) In the absence of mtDNA-coded Fα subunits, expression of other FαF1 subunits was not affected, but most nDNA-coded subunits other than α and β of FαF1 could not be assembled.87,96) For in vivo analysis of this regulation, transmitochondrial mice carrying various proportions of deletion mutant mtDNA ($\Delta$mtDNA) were generated by introducing $\Delta$mtDNA from cultured cells into the fertilized eggs of mice.139) The great advantage of transmitochondrial...
drial mice is that they share exactly the same nDNA background and their genetic variations are restricted to the proportions of pathogenic mtDNA.100)

Transcription factors in the expression of HFoF1 include PPARγ coactivator 1α (PGC-1α), in cooperation with several factors, such as peroxisome proliferator-activated receptor (PPAR), nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2), or the specificity protein 1 (Sp1), a ubiquitous transcription factor known to regulate the constitutive expression of the catalytic activity protein 1 (IF 1) (Fig. 2).78) However, mitochondrial transcription is directed by a small number of nucleus-encoded factors, including Tbam.95) Expression of these factors is coordinated with that of nuclear respiratory proteins through the action of PGC-1 family coactivators.101)

Using these cytological methods (Fig. 8), transcriptomics and proteomics (Fig. 9), human bioenergetics at both cell level (Fig. 10) and in vivo levels were elucidated.

9. Supramolecular structure of HFoF1: Dimeric HFoF1, ATP synthasome and ecto-HFoF1

Mitochondrial FoF1, including HFoF1, is typically isolated as a monomeric complex that contains 16 protein subunits21) and the natural inhibitor protein (IF1) (Fig. 2).78) However, mitochondrial FoF1 was isolated in dimeric and higher oligomeric states using digitonin for one step mild solubilization followed by blue native (BN) or clear native (CN) electrophoresis.102) Recent developments in proteomics have revealed HFoF1 in its natural supramolecular state.30),31),102) Single bands in the gel can be analyzed by proteomics approaches including immunoprecipitation,103) and mass spectrometry to identify the amino acid sequence of the components.103) Electron microscopy of these oligomeric mitochondrial FoF1 particles was reported in 1972 (Fig. 2C in Ref. 3). Dimeric and trimeric FoF1 were purified from mammalian mitochondria in five different tissues by BN electrophoresis and CN electrophoresis,30),102) and these were active, thus suggesting that oligomeric FoF1 is constitutive in mitochondria. Using BN electrophoresis, two membrane proteins (6.8 kDa proteolipid and diabetes-associated protein) that had previously been removed during purification were shown to be stoichiometrically associated with FoF1,30) and this may provide insight for further functional investigations.30)

In situ mitochondrial $\Delta fH^+$ was directly estimated by rhodamine 123, which is accumulated in mitochondria depending on $\Delta fH^+$.103) The futile ATP hydrolytic activity of HFoF1 during ischemia that lowers $\Delta fH^+$, is prevented by IF1.78) Bovine IF1 is an o-helical dimer and residues 1–37 of IF1 open the catalytic interface between $\alpha_D$–$\beta_D$.78) Atomic force microscopy images show how these FoF1 molecules form dimers with a characteristic 15-nm distance between the axes of their rotors through stereo-specific interactions of the membrane-embedded portions of their stators.104) A different interaction surface is responsible for the formation of rows of oligomers, suggesting the role of subunits e and g of HFoF1 in dimerization.104) Some dimers have a different morphology, with a 10-nm stalk-to-stalk distance, in line with FoF1S8, which are thus accessible to IF1.104) Dimeric or polymeric HFoF1 is related to morphology of cristae104) under the influence of OPA1.98)

A channel protein, porin, is now known as VDAC (voltage-dependent anion channel) and is the most abundant protein in the mitochondrial outer membrane.52) VDAC helps ATP/ADP exchange by forming a complex with ANC (ANC–VDAC complex).103) Finally, a supramolecular structure called ATP synthasome composed of FoF1, ANC, PIC and perhaps VDAC was isolated and characterized (Fig. 2).31) Parallel immuno-electron microscopic studies revealed the presence of PIC and ANC located non-centrally in the basepiece, and other studies indicated an ATP synthase/PIC/ANC stoichiometry near 1:1:1 (Fig. 2).31) Collectively, these findings support a mechanism in which the entry of the substrates ADP and Pi into mitochondria, the synthesis of ATP on FoF1, and the release and exit of ATP are localized in a supramolecular structure in a highly coordinated system.

9.1. Ectopic HFoF1: plasma membrane localization in lipid rafts. HFoF1 is located not only on the mitochondrial inner membrane, but also on the cell surface. Extracellular ATP synthesized by the ectopic HFoF1 is not an energy source but a regulator for various cellular responses that are initiated by purinergic receptors (P2X and P2Y) and signaling processes and are terminated by breakdown of ATP by ectonucleotidases (Fig. 10, right). By using $^3$H-ADP, net $^3$H-ATP synthesis by cell surface HFoF1 was confirmed (to rule out ATP + AMP synthesis by adenylate kinase). ATP synthesis was inhibited by membrane-impermeable HFoF1-specific inhibitors (angiotstatin and picatannol) and anti-HF1 anti-

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body. Immunoprecipitation indicated that ectopic HFoF1 and a surface protein of endothelial cells, caveolin-1, are physically associated. Adipocyte ectopic HFoF1 may contain Fo, as it is inhibited by oligomycin and influenced by a proton conductor (uncoupler) (quoted in Ref. 92). HFoF1 is selectively localized in lipid rafts with other mitochondrial proteins. Lipid rafts are detergent-resistant membrane microdomains enriched in cholesterol and caveolin-1. Intracellular traffic may translocate HFoF1 containing α, β, γ, δ, d, F6 and OSCP from mitochondria to lipid rafts.

The ectopic HFoF1 has been implicated in numerous activities, including the mediation of intracellular pH, cellular response to antiangiogenic agents, and cholesterol homeostasis as a receptor for apolipoprotein A-I. HFoF1 is expressed on the surface of endothelial cells, where it binds angiostatin, regulates surface ATP levels, and modulates endothelial cell proliferation and differentiation via purinergic receptor (Fig. 10, right). However, inter-organellar traffic is critical for plasma membrane is inside negative. These ectopic FoF1 activities have never been reported in prokaryotic FOF1, although F0F1-like V-type ATPase is widely distributed in prokaryotic and mammalian membrane structures to transport ions. In mammalian tissues, some proteins involved in energy metabolism may exert entirely different functions; cytochrome c, for example, is an electron carrier but also serves as the central signal protein in apoptosis.

10. In vivo ATP synthesis: FoF1 in human bioenergetics and diseases

The function and survival of all organisms is dependent on the dynamic control of energy metabolism. Energy demand is matched to ATP supply by FoF1 and glycolysis (Fig. 10, bottom). The increase in ADP + Pi produced by ATP consumption results in the instant ΔµH+–driven ATP synthesis by FoF1 and increases in electron transport activity to compensate ΔµH+ (respiratory control). The increase in ADP is amplified as AMP increase by myokinase reaction (2ADP = ATP + AMP). If ATP synthesis by FoF1 is not enough, especially when oxygen supply is limited by high metabolic equivalents (METs >5), increased AMP/ATP ratio activates phosphofructokinase to compensate ATP by glycolysis. Although the regulatory mechanism of TFoF1 is basically ubiquitous, TFoF1 is specialized for human activity. One of the characteristics of TFoF1 among other F0F1s is genetic polymorphisms during evolution, particularly in mtDNA, that cause ethnic and interindividual differences in physical activity, aging and disease susceptibility. Voluntary will of the brain triggers muscle contraction and other organ specific activities that consume about 50 kg of ATP per day, but METs change from 0.9 to 15 in a normal adult (Fig. 10, right). Direct measurement of in vivo HFoF1 activity and AMP/ATP ratio is possible by using 31P magnetic resonance spectroscopy revealed that ATP synthase flux correlated with O2 uptake (METs) and insulin sensitivity. The increase in METs is mainly caused by actomyosin contraction (Fig. 10, right), and muscle-specific γ subunit splice variants of HFoF1 are seen during myogenesis and cardiogenesis (Fig. 7). Lactic acidemia is present not only in individuals undergoing high MET exercise, but also in the majority of patients with...
mitochondrial disorders, including impaired HF$_o$, and cells isolated from such patients, similarly to rhabdomyosarcoma cells, require glucose medium (Fig. 8). Mitochondrial disorders can be due to defects in nDNA directly affecting the assembly or function of HF$_o$F$_1$ and respiratory chain, defects in mtDNA affecting HF$_o$ and the respiratory chain or nDNA influencing mtDNA structure and viability.

The regulatory mechanism of HF$_o$F$_1$ including four independent inhibitory sites are more complex than those of TF$_o$F$_1$. AMP-activated protein kinase (AMPK) activates both oxphos and glycolysis, functioning as a ‘fuel gauge’ to monitor AMP/ATP ratio (Fig. 10, left). ATP-sensitive K$^+$ channels both in the plasma membrane and in mitochondria also monitors ATP levels to regulate cellular activities. Increases in ATP concentration close the K$^+$ channel, and the resulting depolarization opens L-type Ca$^{2+}$ channels (Fig. 10, bottom). Increased intracellular Ca$^{2+}$ activates many metabolic processes and proteins for mitochondrial dynamics and secretion. For example, in the β-cells of mitochondrial myopathy, ATP-sensitive K$^+$ channels are not closed and defective Ca$^{2+}$-dependent insulin secretion results in mitochondrial diabetes.

Mitochondrial ATP-sensitive K$^+$ channels regulate energy transfer through their regulation of intermembrane space volume and are accordingly essential for the inotropic response during periods of high METs. Although the target residues in HF$_o$F$_1$ and their signal routes have not yet been determined, mitochondrial ATP-sensitive K$^+$ channels are closely related to kinases including protein kinase C.$^*$ In fact, detailed proteomics have revealed, for example, phosphorylation of α576, βT213, βS259, γY44 or γY52, and acetylation of αK132, βK133, γK79 in mammalian F$_o$F$_1$. The monomeric form of HF$_o$F$_1$ contains a phosphorylated γ (γY52) that is not present in the dimeric form.

In contrast to bacteria, ATP synthesis by HF$_o$F$_1$ requires exchange of Pi + ADP and ATP between cytosol and mitochondria by PIC and ANC, which are organized as the ATP synthasome (Fig. 2). VDAC$^{52}$ forming a complex with ANC also plays an important role in cytoplasm-mitochondrial communication.$^{103}$ As mammalian cells are about 1000 times as large as bacteria, and a mitochondrion is as large as a bacterium, mitochondrial dynamics are essential to distribute synthesized ATP (Fig. 10, upper left). The concept of mitochondrial dynamics includes the movement of mitochondria along the cytoskeleton, the regulation of mitochondrial morphology and distribution, and connectivity mediated by tethering and fusion/fission events.$^{114}$ The relevance of these events in HF$_o$F$_1$ activity has been unraveled after the identification of mitofusins$^{87}$ and OPA1.$^{98}$ Subjects with diabetes showed reduced expression (by 26%) of mitofusin 2 and a 39% reduction in the α-subunit of F$_o$F$_1$. Chronic exercise led to increases in VDAC, and the α-subunit of F$_o$F$_1$ in muscle from control subjects and from those with diabetes.$^{115}$ Acute exercise caused a 4-fold increase in PGC-1α expression in muscle from control subjects, but not in those with diabetes.$^{115}$

10.1. Inhibition of HF$_o$F$_1$ activity and diseases related to bioenergetics. In the energy metabolism of whole human body,$^{114,116}$ if Δ$\mu$H$^+$ is too low, ATP production by HF$_o$F$_1$ cannot meet demand (mitochondrial diseases)$^{22,87}$ and if it is too high, reactive oxygen species (ROS) are produced.$^{23,89,116}$ Oxphos is linked to disease through a lack of energy, excess ROS production, or a combination of both.$^{89,116}$ and diseases caused by mitochondrial dysfunction include diabetes, cancer, neurodegenerative disorders and ischemia-reperfusion injury.$^{23,89,116}$

Because of its complex structure, HF$_o$F$_1$ is inhibited by over 250 natural and synthetic inhibitors.$^{117}$ In the absence of torque-driving energy, HF$_o$F$_1$ switches from an ATP synthase to an ATP hydrolase, and this occurs during myocardial ischemia. The degree of ATP inefficiently hydrolyzed during ischemia may be as high as 50–90%.$^{118}$ At the start of F$_o$F$_1$ study, oligomycin was shown to inhibit F$_o$.$^{3,39,42}$ and this portion of F$_o$F$_1$ was therefore designated ‘oligomycin sensitivity conferring factor’. Oligomycin cannot be used to treat myocardial ischemia, as it would reduce ATP synthesis in normal tissue.$^9$ Only when cellular pH is decreased below 6.8 under ischemia, IF$_1$.$^{78}$ inhibits ATPase at the αβ interface of HF$_o$.$^{78}$ The restoration of Δ$\mu$H$^+$ favoring ATP synthesis displaces IF$_1$ from the αβ interface. However, IF$_1$ does not completely block hydrolase activity. BMS-199264 selectively inhibits ATPase activity during ischemia while having no effect on ATP synthesis, and enhances the recovery of contractile function following reperfusion.$^{118}$ IF$_1$, ectopic HF$_o$F$_1$ and the opener of mitochondrial ATP-sensitive K$^+$ channel$^{113}$ protect cardiomyocytes from ischemic/reperfusion damage.

As HF$_o$F$_1$ supplies most ATP needed for human activity, further study will provide useful insight for
emergency medicine, mitochondrial cardiomyopathy, and obesity-related chronic diseases.

Conclusions

The excellent work to date on ATP synthases (F,F1) has been reviewed based on data obtained in studies on TF,F1 and HFoF1. The chemiosmotic theory was firmly established by ΔμH+–driven ATP synthesis in TF,F1 liposomes (Figs. 1 and 3), and the rotational theory was established by crucial observations of γ–rotation and ATP synthesis on externally added torque to rotate γ in TF1 (Fig. 1). TF1 is the only F1 sufficiency stable to be consistently analyzed by reconstitution, crystallography, mutagenesis, and nanotechnology for torque-driven ATP synthesis.

Crystallographic analysis using BF1 (Figs. 2 and 5) and TF1 (Figs. 4–6) directed mutagenesis using EF,F1 site-directed mutagenesis using EF,F1 and TF,F1 (Figs. 5 and 6) dynamic nanotechnology have contributed to elucidating elastic and loose energy coupling. Based on the fundamental mechanism of ATP synthesis in TF,F1, functional complementation of TF,F1-deleted yeast with BF1 genes, human bioenergetics was developed by research on HF,F1 using plasmids, transgenic mice (Fig. 1), the only F1 sufficient to be analyzed by reconstitution, crystallography, mutagenesis, and nanotechnology for torque-driven ATP synthesis.

Crystallographic analysis using BF1 and TF1, mitochondrial genetics (Fig. 2), mitochondrial biogenesis (Fig. 9), the complex regulation of HF,F1 has been shown to be essential for daily human activity that is triggered by the brain (Fig. 10), thus human bioenergetics is also applicable to emergency medicine and obesity/diabetes and mitochondrial diseases.

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