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# REVIEW

# Structure and Function of $H^+$-ATPase

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## Abstract

(1) Extensive studies on proton-translocating ATPase ($H^+$-ATPase) revealed that $H^+$-ATPase is an energy transforming device universally distributed in membranes of almost all kinds of cells. (2) Crystallization of the catalytic portion ($F_1$) of $H^+$-ATPase showed that $F_1$ is a hexagonal molecule with a central hole. The diameter of $F_1$ is about 90 Å and its molecular weight is about 380,000. (3) Use of thermophilic $F_1$ permits the complete reconstitution of $F_1$ from its five subunits ($\alpha$, $\beta$, $\gamma$, $\delta$, and $\epsilon$) and demonstration of the gate function of the $\gamma\delta\epsilon$-complex, the catalytic function of $\beta$ (supported by $\alpha$ and $\gamma$), and the $H^+$-translocating functions of all five subunits. (4) Studies using purified thermostable $F_0$ showed that $F_0$ is an $H^+$-channel portion of $H^+$-ATPase. The direct measurement of $H^+$-flux through $F_0$, sequencing of DCCD-binding protein, and isolation of $F_1$-binding protein are described. (5) The subunit stoichiometry of $F_1$ may be $\alpha_3\beta_3\gamma\delta\epsilon$. (6) Reconstitution of stable $H^+$-ATPase-liposomes revealed that ATP is directly synthesized by the flow of $H^+$ driven by an electrochemical potential gradient and that $H^+$ is translocated by ATP hydrolysis. This rules out functions for all the hypothetical components that do not belong to $H^+$-ATPase in $H^+$-driven ATP synthesis. The roles of conformation change and other phenomena in ATP synthesis are also discussed.



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## Introduction

$H^+$-ATPase ($F_0$-$F_1$) has been found in all eukaryotic and prokaryotic cells examined except erythrocytes.[1] This wide distribution is because $H^+$-translocation through biomembranes is essential not only for oxidative and photosynthetic phosphorylation (1–5), but also for translocation of ions and substrates (6), movement of flagella (7), and other activities, such as transfer of information (7).

$H^+$-ATPase was first extracted from mitochondria (8) and called oligomycin-sensitive ATPase. It is composed of a soluble ATPase portion ($F_1$) (9) and an insoluble portion ($F_0$) (10). Combination of $F_0$ with $F_1$ renders the latter sensitive to energy transfer inhibitors, such as DCCD and oligomycin (8, 10). In the chemiosmotic hypothesis, $H^+$-ATPase is postulated to be responsible for ATP synthesis using energy of $H^+$-flow through the membranes of mitochondria and chloroplasts (2). Support in favor of this hypothesis is provided by demonstration that oligomycin-sensitive ATPase, reconstituted into liposomes, mediates accumulation of $H^+$ at the expense of ATP hydrolysis (11).

The subject of this review is important recent developments (12) that were not described in the reviews that appeared before 1977 (1–7). These recent developments are: the structural analysis of crystalline $F_1$ (13–15), complete reconstitution of $F_1$ from its purified individual subunits (16, 17), and net synthesis of ATP with pure $H^+$-ATPase reconstituted into liposomes loaded with an artificial electrochemical gradient of $H^+$ (18, 19).

## Universal Distribution of $F_1$ and Its Properties

An ATPase [EC 3.6.1.3] identified with the coupling factor 1 ($F_1$) of oxidative phosphorylation was first purified from mitochondria (9, 20). Later similar ATPases capable of oxidative phosphorylation (3, 5), photophosphorylation (4, 21), and ion translocation of (7) were isolated from $H^+$-translocating biomembranes of eukaryotic and prokaryotic cells (Table I). These ATPases are identical, with the 90-Å particles distributed over the

[1] Abbreviations: 9AA, 9-aminoacridine; AMPPNP, $\beta,\gamma$-imido-adenosine-5′-triphosphate; ANS, 8-anilinonaphthalene-1-sulfonate; $CF_1$, chloroplast $F_1$; DCCD, N,N′-dicyclohexylcarbodiimide; $F_1$, catalytic portion of $H^+$-ATPase (coupling factor 1); $F_0$, $H^+$-channel portion of $H^+$-ATPase; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; $H^+$-ATPase, proton translocating adenosine triphosphatase; OSCP, oligomycin sensitivity conferring protein; $TF_1$, thermophilic $F_1$ ($F_1$ obtained from thermophilic bacterium PS3); $TF_0$, thermophilic $F_0$.

**Table I.** Universal Distribution of $F_1$-like ATPase in $H^+$-Translocating Biomembranes. Molecular Weights of Oligomeric $F_1$ and Subunits of $F_1$ ($10^3$ daltons)

| | Eukaryotic cells | | | | | Prokaryotic cells | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Biomembrane | Mitochondria | Mitochondria | Chromaffin granule | Chloroplasts | Mitochondria | Chromatophores | Plasma membrane (fac. anerobe) | Plasma membrane (aerobe) | Plasma membrane (anaerobe) | Plasma membrane (thermophile) |
| Source | Ox heart | Rat liver | Ox adrenal medulla | Spinach leaves | Yeast *Saccharomyses cervisiae* | *Rhodospirillum rubrum* | *Escherichia coli* | *Micrococcus luteus* (*lysodeikticus*) | *Streptococcus faecalis* | Thermophilic bacterium PS3 |
| Molecular weight of $F_1$ | 354 (28), 390 (29) | 384 (30) | 400 (31) | 365 (79),[a] 380 (29) | 400 (32) | 350 (33) | 390 (34,38) | 349 (35) | 385 (36) | 380 (37) |
| Reference | (28) | (30) | (31) | (7) | (32) | (33) | (39) | (35) | (25) | (26) |
| Subunit $\alpha$ (1 or A) | 54 | 62 | 51 | 59 | 58.5 | 54 | 56 | 52.5 | 51 | 56 |
| Subunit $\beta$ (2 or B) | 50 | 57 | 50 | 56 | 55 | 50 | 54 | 47 | 47 | 53 |
| Subunit $\gamma$ (3 or C) | 33 | 36 | 28 | 37 | 34[b] | 32 | 32 | 41.5 | 37 (nectin) | 32 |
| Subunit $\delta$ (4 or D) | 17.3 | 12 | — | 17.5 | 10 | 13 | 13[c] | 28.5 | — | 15.5 |
| Subunit $\epsilon$ (5 or E) | 11 | 7.5 | — | 13 | 8.6 | 7.5 | 11.5 | — | — | 11 |

[a]Value obtained by low-speed centrifugation, that by high-speed centrifugation was 325,000 (79).
[b]There is another band of $42 \times 10^3$ daltons.
[c]Data from Ref. 40 in which no subunit $\epsilon$ was reported.

surface of the inner mitochondrial membrane and other membranes. These particles were first suggested to represent "elementary particles" of electron transport (22), but later using $^3$H-acetyl-$F_1$ they were identified as $F_1$ molecules (23).

All these ATPases have similar molecular weights of 3.5–4.0 × $10^5$ and can be resolved into five similar subunit polypeptides, three large ($\alpha$, $\beta$, and $\gamma$) and two small ($\delta$ and $\epsilon$, with the exceptions shown in Table I). $F_1$ is not affected by inhibitors of energy transfer that inhibit the original membrane-bound $H^+$-ATPase. The specificity of mitochondrial $F_1$ (3, 9, 24) for nucleotides is in the order ATP > ITP > GTP > UTP > CTP, and $F_1$ of chloroplasts (3) and bacteria (25, 26) shows greater activity with purine nucleoside triphosphates than with pyrimidine nucleoside triphosphates. Divalent cations, such as $Mg^{2+}$, $Co^{2+}$, $Mn^{2+}$, and $Ca^{2+}$, are usually required (3, 25) for ATPase activity. The $K_m$ of all $F_1$ preparations for ATP is usually of the order of 0.3 to 0.8 mM, and the $K_d$ for the tightly bound nucleotides is about 1 $\mu$M (3, 24, 25, 27). The $K_i$ value for ADP is also in the range of 0.2 to 1.0 mM (3), and most $F_1$ preparations are inhibited by azide and the ATP analogue AMPPNP.

The oligomeric structure of $F_1$ is easily dissociated, except in the very stable thermophilic $F_1$ ($TF_1$) (26), which will be discussed in a later section. These $F_1$s are all water-soluble after detachment from the membrane. Purification of $F_1$s usually involves gel filtration and ion-exchange chromatography (3, 4, 24–27), and $TF_1$ and $F_1$ can both be crystallized using ammonium sulfate (13–15, 26).

There have been more than a hundred reports on $F_1$ or $F_1$-like ATPases, and readers interested in the historical and comparative aspects of this subject should consult recent views on mitochondrial $F_1$ (3, 24, 27), chloroplast $F_1$ (4, 24), and bacterial $F_1$ (25–27). As shown in Table I (24–40), $F_1$-like ATPases have been found in all eukaryotic and prokaryotic cells tested, where $H^+$-translocation is essential for many kinds of energy-transforming processes (1, 2, 6, 7).

The molecular properties of these $F_1$s are similar (3–5, 24–40), but their functions may be different. The roles of $F_1$ in oxidative (1–3, 5, 24–27, 32, 34, 37, 38) and photosynthetic (4, 21, 33) processes have been well established. There are also $F_1$-like ATPases in anaerobic bacteria, such as *Streptococcus faecalis* (Table I) (25, 36) and *Clostridium pasteurianum* (41, 42). The molecular and kinetic properties of these $F_1$s from anaerobic bacteria are so similar to those of other $F_1$s that the $H^+$-ATPase of the phylogenically old and strictly anaerobic *C. pasteurianum* has been supposed to have been reversed from an ATPase to an ATP-synthetase ($H^+$-ATPase) of aerobic or photosynthetic organisms during evolution (42). It was shown that cations are translocated at the expense of the electrochemical potential difference of $H^+$

($\Delta\tilde{\mu}H^+$) (2, 41). The $\Delta\tilde{\mu}H^+$ was also shown to support translocation of amino acids catalyzed by a purified amino acid carrier, which was reconstituted into proteoliposomes (43).

$F_1$-like ATPase was purified from membranes of chromaffin granules of the adrenal medulla (Table I) (31). The physiological role of this ATPase may be to form $\Delta\tilde{\mu}H^+$ that causes uptake of catecholamines by the granules. There are many other $H^+$-translocating ATPases, such as lysosomal ATPase (44), HCl-secreting ATPase (45, 46), and $H^+$-ATPase of the plasma membranes of fungi (7). However, there is still little evidence to show that these are $F_0 \cdot F_1$ type $H^+$-ATPases.

Although not listed in Table I, $F_1$-like ATPases have also been found in the chloroplasts of *Euglena gracilis* (47), the plasma membranes of *Alkaligenes faecalis* (48), *Bacillus megaterium* (49), and *Bacillus stearothermophilus* (50) and in other biomembranes (3–5, 7, 25). The morphological, molecular, kinetic, and chemical properties of these ATPases are essentially similar to those of the enzymes listed in Table I.

## Comparison of $H^+$-ATPases ($F_0 \cdot F_1$)

Since the isolation of $H^+$-ATPase from the inner mitochondrial membrane and its reconstitution from $F_0$ and $F_1$ (8, 10), there have been many reports on this enzyme from various sources (26, 51–60) (Table II). All biomembranes that contain $F_1$ may also contain $F_0$, which binds $F_1$ and serves as an $H^+$-channel (1, 5, 12). However, owing to the difficulties in purifying insoluble membrane components, there is still no agreement on the composition of the $F_0$ moiety of $H^+$-ATPase. Moreover, different names have been given to $H^+$-ATPases studied in different laboratories: $F_0 \cdot F_1$ (1, 2, 8, 10, 26), oligomycin-sensitive ATPase (3, 51, 54), DCCD-sensitive ATPase (53), ATPase complex (5), Complex V (55), etc. However, even in crude preparations from ox heart mitochondria, Complex V and oligomycin-sensitive ATPase were shown to be identical (13 polypeptides) (55), and the $F_0 \cdot F_1$ fraction was also shown to be the same polypeptide assembly (56). Further purification of $F_0 \cdot F_1$ by density gradient centrifugation resulted in a preparation called fraction 9 (Table II) which still resembled Complex V (56).

Only mitochondrial $H^+$-ATPase is oligomycin-sensitive, but DCCD-sensitivity is rather universal. DCCD is bound covalently to a glutamyl residue of the DCCD-binding protein of $F_0$ (61, 62) and specifically blocks $H^+$-translocation through $F_0$ (63). Although $^{14}C$-rutamycin (an oligomycin analogue) is bound to $F_0$, it is easily released (8), and the binding ability is lost by a point mutation of the DCCD-binding protein (61, 62). Thus, the name oligomycin-sensitive ATPase is unsuitable.

**Table II.** Energy-Transforming Activity and Subunits of $H^+$-ATPase[a]

| Source: | Ox heart | Ox heart | Yeast | Thermophilic bacterium PS3 |
|---|---|---|---|---|
| Preparation: | Complex V | $F_0 \cdot F_1$ (fraction 9) | Oligomycin-sensitive ATPase | Thermophilic $F_0 \cdot F_1$ |
| $^{32}$Pi-ATP exchange: | (+) | (+) | (−) | (+) |
| Net ATP synthesis by acid–base transition: | (−) | (−) | (−) | (++) |
| Molecular weights of subunits ($10^3$ daltons): | 83 | 80 | — | — |
| | 68 | 70 | — | — |
| | 53 (α) | 56 (α) | 58 (α) | 56 (α) |
| | 50 (β) | 54 (β) | 54 (β) | 53 (β) |
| | 47 | 52 | — | — |
| | 44 | — | — | — |
| | 33 (γ) | 34 (γ) | 38.5 (γ) | 32 (γ) |
| | 31 | 32 | 31 (δ) | — |
| | — | — | 29 | — |
| | 24 | 23 | 22 | — |
| | 22.5 | 19 (OSCP) | 18.5 (OSCP) | 19 (OSCP) |
| | 15 (δ) | 14 (δ) | 12 (ε) | 15.5 (δ) |
| | 13 | 11 | 12 | 13.5 (FBP) |
| | 8–9 (ε) (DBP) | 8 (ε) (DBP?) | 7.5 (DBP) | 11 (ε) |
| | — | — | — | 7.3 (DBP) |
| References | (55) | (56) | (54) | (26) |

[a]Notation: OSCP, oligomycin sensitivity conferring protein; FBP, $F_1$-binding protein; DBP, DCCD-binding protein.

The name "complex" is also improper, because $F_1$ itself is a complex oligomer. Moreover, the names Complex I to IV (22) are used for components of the electron transport chain, but Complex V (55) is not.

Since $H^+$-translocation by these ATPase preparations has been demonstrated in reconstituted liposomes (11), and this property is also common to mitochondria (1, 2, 5, 7, 60), chloroplasts (4, 5), bacteria (7, 26), and even strict anaerobic bacteria (41, 42), these enzymes may be called $H^+$-ATPase, as in the case of $Na^+$, $K^+$, and $Ca^{2+}$-ATPase.

As shown in Table II, there are 3 to 10 subunits in the $F_0$ moiety of $H^+$-ATPase preparations. Since only 3 subunits are detected in thermophilic $F_0$ ($TF_0$) (53) and the energy-transforming activity, such as net ATP synthesis driven by an artificially imposed $\Delta\bar{\mu}H^+$, can be demonstrated (18), these 3 subunits are sufficient for activity. These subunits were isolated as follows: $H^+$-ATPase was treated with urea to remove $F_1$, as in the case of heart $F_0 \cdot F_1$ (10, 23) or yeast oligomycin-sensitive ATPase (using NaBr instead of urea) (54). Then the remaining $F_0$ portion was extracted with alkali (10) or treated with CM-cellulose to remove a basic protein called oligomycin sensitivity conferring protein (OSCP), which has a molecular weight of 18,000 to 22,000 (64). Then the remaining insoluble portion was dissolved in sodium dodecylsulfate and separated on a column of Sephacryl into $^{14}C$-DCCD-binding protein (DBP) and $F_1$-binding protein (FBP) (65). $H^+$-translocation and $^{32}Pi$-ATP exchange reaction were reconstituted with $F_0$ containing only DBP and FBP in the presence of $TF_1$ (65).

The heart enzyme contained many polypeptides (Table II). It is unlikely that any factor or protein besides those present in the energy-transforming $H^+$-ATPase participates in ATP synthesis by $H^+$-translocation. Six coupling factors have been reported: $F_1$ is the catalytic portion of $H^+$-ATPase; $F_3$, $F_4$, and $F_5$ are crude OSCP preparations (64); and $F_6$ may be part of $F_0$ (66). $F_2$, which is required to restore the activities of silicotungstate-treated submitochondrial particles (67), does not appear to be a component of $H^+$-ATPase. However, there may be many kinds of channels and gates which are removed by the drastic treatment involved in membrane extraction, and these channels may have to be sealed by factors unrelated to $H^+$-ATPase and thus these factors may restore the formation of $\Delta\bar{\mu}H^+$ during electron transport. The components of $H^+$-ATPase may be tested on pure $H^+$-ATPase liposomes (18) and by reconstitution of $H^+$-ATPase from its purified subunits.

## Crystallographic Analysis and Subunit Structure of $F_1$

There are many hypotheses of the structure of $F_1$. These are based on electron microscopic images (3, 36, 68), cross-linking reactions (69), aurovertin binding (70), immunological reactions (4), and chemical analyses (71–76). These hypotheses may be classified into two main groups: hexagonal (Fig. 1A) and tetragonal (Fig. 1B) hypotheses. There are some variations in the structures proposed, depending on the arrangement of $\alpha$ and $\beta$ subunits, and the numbers and topologies of the $\gamma$, $\delta$, and $\epsilon$ subunits. Since the binding of $F_1$ to $F_0$ requires some minor subunits, all or some of these are supposed to constitute a stalk connecting $F_1$ to $F_0$ (Fig. 1C) (72). A tetragonal model (Fig. 1D) has been proposed from cross-linking experiments (69), because $\gamma\epsilon$, $\gamma\epsilon_2$,

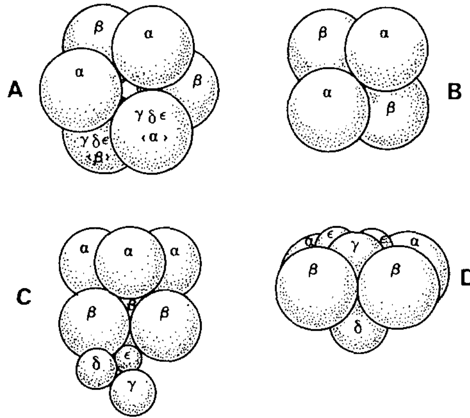


**Fig. 1.** Hexagonal and tetragonal models of the $F_1$ molecule. A, Hexagonal model; B, tetragonal model; C, hexagonal $\alpha_3\beta_3$ plus $\gamma\delta\epsilon$ (72); D, tetragonal $\alpha_2\beta_2$ plus $\gamma\delta\epsilon_2$ (69).

$\alpha\delta$, and $\beta\delta$ aggregates were formed. These hypotheses have been summarized in recent reviews (3–5, 24–27, 73, 74).

Recent image reconstruction studies on the two-dimensional crystal of $TF_1$ (26) clearly showed a pseudohexagonal structure (Fig. 2) (12, 14). The optical density on the film of the crystal was digitized by a computer-linked microdensitometer equipped with a moving stage scanner. The resulting digitized image was Fourier transformed numerically. The translationally filtered image was produced by numerical Fourier synthesis by combining only the Fourier components that were approximately consistent with the translational symmetry of the crystal. The resulting digital image was then displayed as a line-printer output and contours were drawn around peaks of density (Fig. 2B). The spacing along the *a*-axis and *b*-axis is 90 Å and every $TF_1$ molecule has a low-density region near its center (14).

It is interesting to compare these results with those on three-dimensional crystals of $F_1$ obtained from ox heart (13) and rat liver (15). The molecular weight of $F_1$ is about 390,000, and it is very difficult to observe molecular structure by X-ray crystallography. In contrast to the conclusion, deduced by electron microscopy (23, 32, 74) and hydrodynamic measurements (28–38), that $F_1$ is a sphere of 90-Å diameter, at least one dimension of the $F_1$ molecule was calculated to be no more than about 60 Å (13). A unit cell with the dimensions $a = b = 158 \pm 10$ Å, $\gamma = 90°$, was seen in the electron micrograph of the three-dimensional crystal and approximately 3.5 molecules of $F_1$ were present in the unit cell (13).

X-ray precession photographs showed that the crystals were rhombohedral, space group R32 ($D_3^7$ $N^0155$), with hexagonal cell dimensions $a = 148$ Å, $c = 368$ Å. The molecular weight of the asymmetric unit of the crystals

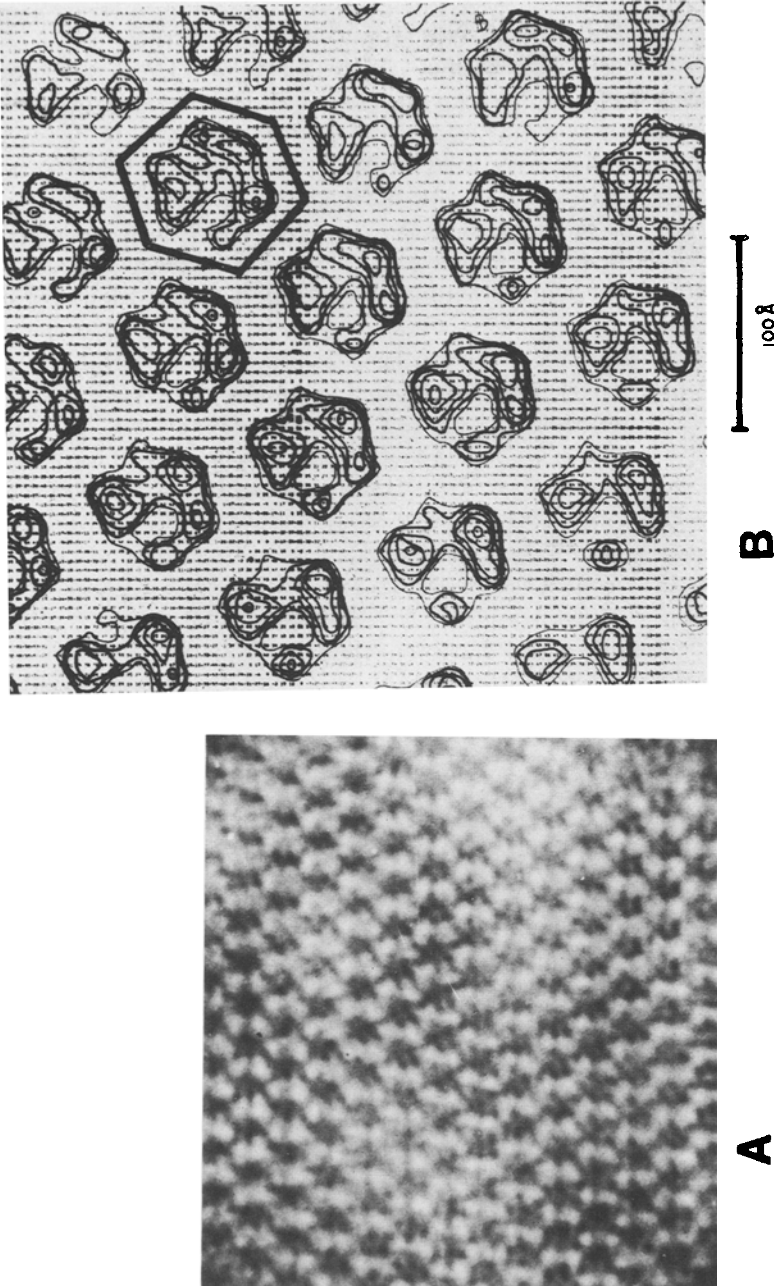


**Fig. 2.** Structure of crystalline thermophilic $F_1$ ($TF_1$) molecules. A, Optically filtered image of a two-dimensional crystal of the $TF_1$ (Y. Kagawa, M. Yoshida, and T. Wakabayashi, unpublished); B, computerized image reconstruction of $TF_1$. Translationally filtered image (14).

was 190,000 or about half the molecular weight (384,000) of the rat liver enzyme. This indicates that the crystallographic twofold axes of symmetry coincide with a molecular symmetry axis (15). The low molecular weights of $F_1$ that support tetragonal models [280,000 for ox heart $F_1$ (77, 78), 325,000 for chloroplast $F_1$ (79), and 296,000 for *Escherichia coli* $F_1$ (80)] seem to be incorrect being due to dissociation of the $F_1$ molecule during centrifugation. Results of recent reinvestigation with hardly dissociating $TF_1$ (380,000), and with ox heart $F_1$ (390,000) and chloroplast $F_1$ (380,000) in methanol water to prevent their dissociation, favored the hexagonal model (29). A much larger value (370,000) was reported for *E. coli* $F_1$ (81). Low-speed centrifugation gives an accurate molecular weight for $F_1$, but most of the values in Table I except those in Refs. 29 and 79 were obtained by high-speed centrifugation (10, 589–13, 410 rpm).

The stoichiometry of subunits in $F_1$ is controversial (3–5, 82–90) (Table III). This kind of controversy would not occur if the molecular weights of both the oligomer and its subunits were precisely determined. Owing to the fact that mesophilic $F_1$ dissociates, values for the molecular weight of $F_1$ have been underestimated (77–80). Those of $\alpha$ and $\beta$ have been overestimated (28, 29).

The two chief models are $\alpha_2\beta_2$ and $\alpha_3\beta_3$, and their molecular weights were calculated as described in Table III. The largest value expected in the $\alpha_2\beta_2$ group is for $\alpha_2\beta_2\gamma_2\delta_2\epsilon_2$ and this is only 340,000 (28, 39). However, values of 390,000, 380,000, and 380,000 were obtained for mitochondrial, chloroplast, and bacterial $F_1$, respectively (29, 89). These values supported the $\alpha_3\beta_3\gamma\delta\epsilon$ model. The idea that $F_1$ contains nonprotein material (8%) (90) was reported, but mitochondrial $F_1$ does not contain sugars or lipids.

Treatment of $F_1$ with chemical cross-linking agents also gave contradictory results (69, 83, 84). X-ray crystallography suggested twofold symmetry of the $F_1$ molecule, which might be interpreted as indicating a $(\alpha\beta\gamma\delta\epsilon)_2$ structure, although this interpretation was not suggested in the original report (15). The hexagonal structure supports the $\alpha_3\beta_3$ model (14). Chemical analyses of amino acid residues in $F_1$ also gave conflicting results: there is a report that ox heart $F_1$ contains SH in $\alpha\beta$ (not in $\gamma\delta\epsilon$) (28), and another that the SH is in $\alpha\gamma\epsilon$ (not in $\beta\delta$) (85). The most reliable data were obtained on $TF_1$ containing 3SH/$TF_1$ in which it was found that only $\alpha$ contained SH (1SH/subunit), thus supporting the $\alpha_3\beta_3$ model (86). Conclusions on the stoichiometry deduced from the staining intensities of subunits separated by acrylamide gel electrophoresis (30, 32) are not very reliable, but those deduced from $^{14}C$-labeled $F_1$ dissociated into subunits by the same method should be correct. However, the value obtained from chloroplast $F_1$ extracted from plants grown in $^{14}CO_2$ was far from the calculated value: $\alpha$ 34.1% and $\beta$ 31.5% were expected for $\alpha_2\beta_2\gamma_2\delta_2\epsilon_2$, but $\alpha$ 39.6% and $\beta$ 37.6% were obtained, which supported the $\alpha_2\beta_2\gamma\delta\epsilon_2$ model for chloroplast $F_1$. Results obtained by a similar method on $^{14}C$-labeled bacterial $F_1$ [*Salmonella typhimurium* (71),

**Table III.** Controversy on the Stoichiometry of the Subunits in the $F_1$ Molecule

| | $\alpha_2\beta_2$ Model ($\alpha_2\beta_2\gamma_{1\text{-}2}\delta_{1\text{-}2}\epsilon_{1\text{-}2}$) | $\alpha_3\beta_3$ Model ($\alpha_3\beta_3\gamma\delta\epsilon$) |
|---|---|---|
| Calculated molecular weight[a] | 340,000 for $\alpha_2\beta_2\gamma_2\delta_2\epsilon_2$ | 393,000 for $\alpha_3\beta_3\gamma\delta\epsilon$ |
| Calculated composition (%)[a] | $\alpha$ 34.1; $\beta$ 31.5; $\gamma$ 20.3; $\delta$ 8.6; $\epsilon$ 5.5 | $\alpha$ 44.3; $\beta$ 40.8; $\gamma$ 8.8; $\delta$ 3.7; $\epsilon$ 2.4 |
| Method of analysis (reviews) | Nelson (4); Racker (82) | (3), (5), (12), (27), (73), (74) |
| Chemical cross-linking | Baird and Hammes (83)[b] (69)[c] $\alpha_2\beta_2\gamma_2\delta\epsilon$ | Enns and Criddle (84)[b] |
| Crystallography | Amzel and Pedersen (15)[c] $(\alpha\beta\gamma\delta\epsilon)_2$? | Wakabayashi et al. (14)[d] |
| SH content per subunit | Senior (85)[b] $\alpha_2\beta_x\gamma_2\delta_x\epsilon_2$ (8SH, 2SS/$F_1$) | Yoshida et al. (37, 86)[d] (1SH/$\alpha$, 3SH/$F_1$) |
| $^{14}C$-labeled $F_1$ subunit | Nelson (4)[c] $\alpha_2\beta_2\gamma\delta\epsilon_2$ ($\alpha$ 39.6%; $\beta$ 37.6%) | Bragg and Hou (71)[d]; Kagawa et al. (26)[d] |
| Reconstitution of $F_1$ | Vogel and Steinhart (39)[d] $(\alpha\gamma\epsilon)_2\beta_2\delta_{1\text{-}2}$[e] | Yoshida et al. (16)[d]; Abrams et al. (87)[d] |
| Aurovertin binding at $F_1$ | Verschoor et al. (70)[b] $\alpha_2\beta_2\gamma_2\delta_x\epsilon_2$<br>Douglas et al. (88)[b] | — |
| Staining intensity of gel | — | Catterall et al (30)[b]; Takeshige et al. (32)[b] |
| Molecular weight of $F_1$ ($10^3$ daltons) | 340 (39)[d], 347 (28)[b], 320 (79)[c], 324 (90)[b] ($F_1$ contained 8% nonprotein) | 380 (89)[d], 385 (36)[d], 400 (32)[b] 390[b], 380[c], 380[d] (29), 390 (34, 38)[d] |

[a]The molecular weights of subunits are averages of the values reported in Refs 28 and 30 (animal $F_1$).
[b]Mitochondrial $F_1$.
[c]Chloroplast $F_1$.
[d]Bacterial $F_1$.
[e]The yield, purity, and composition of the reconstituted ATPase were not described, and some change was observed in subunit $\gamma$ during the treatment (39).

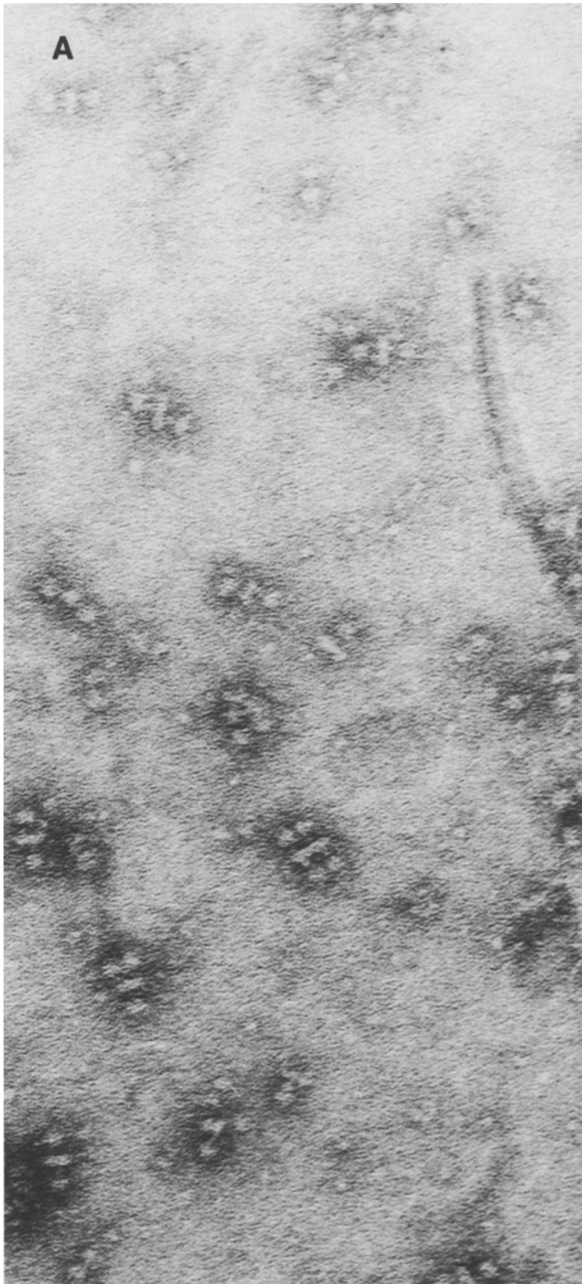


**Fig. 3.** Electron microscopic figure of $H^+$-ATPase of ox heart mitochondria (Y. Kagawa, unpublished). A, negatively stained preparation; B (facing page), tracing of A to show units composed of a headpiece, stalk, and basepiece which form monomers, dimers, trimers, and a tetramer. Basepieces are often smaller than headpieces.

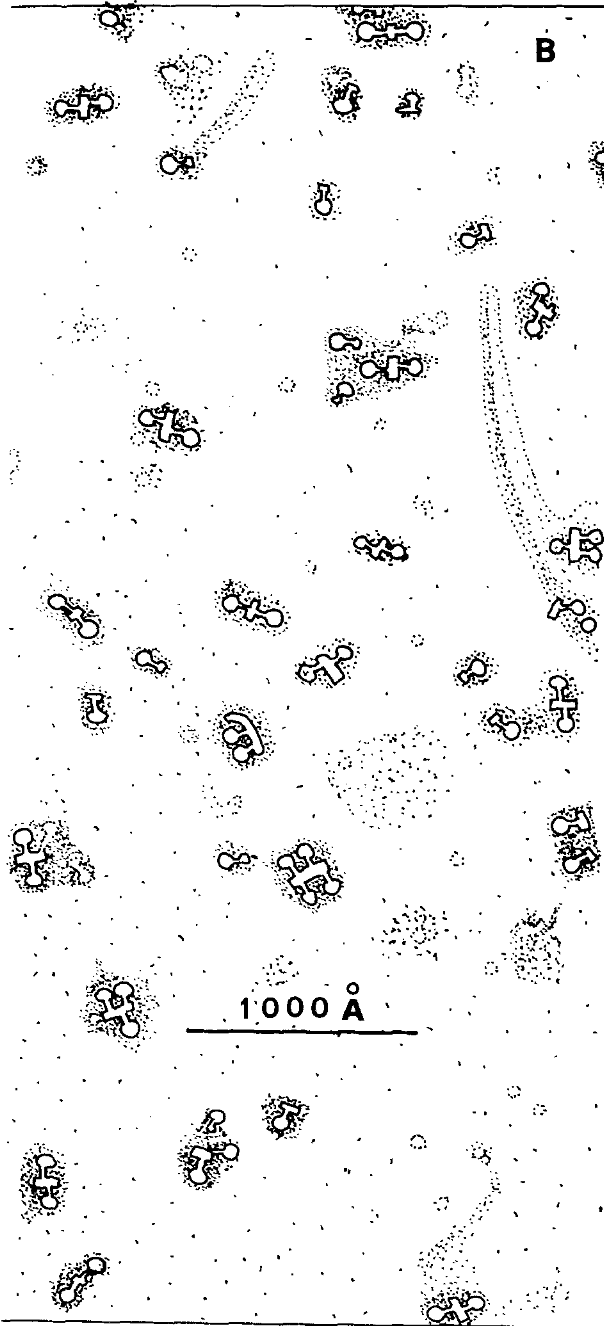


Fig. 3. Continued.

*E. coli* (71), and thermophilic bacterium PS3 (26)] supported the $\alpha_3\beta_3\gamma\delta\epsilon$ model, despite the fact that small, and thus inaccurate, values were obtained for the radioactivities in the minor subunits. Ultraviolet absorption of the eluates from chromatographic column of $F_1$-subunits also supported the latter model (Y. Kagawa).

Reconstitution of $F_1$ of *E. coli* from the subunit assembly $\alpha\gamma\epsilon$ and subunit $\beta$ supports the $(\alpha\gamma\epsilon)_2\beta_2$ model, but the recent reconstitution of the same $F_1$ from $\alpha$, $\beta$, and $\gamma$ showed that the assembly $\alpha\gamma\epsilon$ was not essential (17), and confirmed the number of subunit $\epsilon$ ($1\epsilon/F_1$) (91).

Reconstitution of $TF_1$ from a mixture of $3\alpha + 3\beta + 1\gamma$ resulted in formation of $TF_1$ with ATPase activity (16), but there was little evidence that these subunits were completely associated. The same objection may be directed to other reconstitution experiments in which binding of $\delta$ to the $\alpha_3\beta_3\gamma$ complex was shown to require $Mg^{2+}$ (87).

The binding of aurovertin to subunit $\beta$ supported the $\alpha_2\beta_2$ model, though the binding of a third aurovertin to $F_1$ may be blocked by the first two aurovertin molecules in $F_1$ (70, 88). It is known that one molecule of 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole per $F_1$ can completely block ATPase activity and that the inhibitor is specifically bound to subunit $\beta$ (4, 92). Aurovertin is not bound to $TF_1$.

Several crucial experiments to determine subunit stoichiometry of $F_1$, including sequencing of each subunit (precise molecular weights of subunits), reconstitution of $F_1$ from specifically labeled subunits, detailed X-ray crystallographic analysis, etc., will terminate this controversy. And these data are also needed to elucidate the molecular mechanism of proton translocation (1).

## Structure of $H^+$-ATPase

Crystallographic studies on $H^+$-ATPase are difficult, because the $F_0$ portion is highly hydrophobic and is not easily crystallized. Electron micrograms of $F_0 \cdot F_1$ obtained from ox heart (23) and thermophilic bacterium PS3 (26) show that $H^+$-ATPase is composed of three parts: a headpiece, stalk, and basepiece. The preparation is clearly heterogeneous and in the micrograph of Fig. 3, among about 54 molecules of $H^+$-ATPase, 26 are dimers, 6 are trimers, and 8 ($2 \times 4$) are tetramers. The aggregation takes place at the basepiece ($F_0$), which is hydrophobic and may contain phospholipids. The main portion of the $F_1$ molecule is the headpiece, which can be dissociated with urea or chaotropic anions (23). However, the stalk may contain some minor subunits of $F_1$. There have been many reports and reviews suggesting that the stalk is OSCP (64, 73, 74, 93–95), but recent studies do not support this idea (96–98). One of its components may be $F_6$ (66) and $F_1$-binding protein (65) was isolated from thermophilic $F_0$.

Subunit $\delta$ was shown to be essential for binding the rest of the $F_1$ molecule to $F_1$-deficient particles (81, 99) and $F_0$ (100). As shown in Fig. 3, the stalk is about 50 Å long and 30 Å in diameter (73). Hydrodynamic measurements and small angle X-ray scattering experiments on subunit $\delta$ of $CF_1$ support the hydrodynamic description of a prolate ellipsoid of revolution with gross dimensions of $2a = 25.0$ Å, $2b = 28.0$ Å, and $2c = 90.0$ Å, having a radius of gyration of 21.80 Å, a sedimentation coefficient of 1.70 S, and a translational diffusion constant of $D = 3.92 \times 10^7$ $cm^2 \cdot sec^{-1}$ (101). This long molecule could well be the stalk of $H^+$-ATPase. The other minor subunit of $F_1$, subunit $\epsilon$, is also required for the binding of $TF_1$ to $F_0$ (100). However, in several $F_1$ (4, 81) this may be an ATPase inhibitor subunit. Subunit $\epsilon$ from $CF_1$ was expressed as a prolate ellipsoid of revolution with axes of $2a = 2b =$ 25.4 Å and $2c = 50.8$ Å and with a volume of $17.0 \times 10^3$ $Å^3$ (102). With these dimensions it is too small to constitute the stalk.

It is interesting that $H^+$-ATPase in a preparation from yeast mitochondria was oval (100 × 150 Å) (54), and thus $F_1$ and $F_0$ were directly connected without a stalk. This preparation did not show energy-transforming activity even after its reconstitution into liposomes. Its loss of activity may have been due to structural change, because the typical shape of $H^+$-ATPase is often not seen in the preparation.

The basepiece is composed of $F_0$ and phospholipids (23, 26). However, all $H^+$-ATPase preparations contain detergents that are also bound to the basepiece. This hydrophobic portion is the most heterogeneous of the three parts of $H^+$-ATPase, and it may also contain different amounts of phospholipids and detergents depending on the isolation procedures. Ammonium sulfate fractionation in the presence of 1 to 2% cholate or gel filtration through a column equilibrated with detergents removes most of the phospholipids and thus renders ATPase activity latent. On the contrary, fractionation in the presence of deoxycholate or Triton X-100 does not remove phospholipids and the ATPase in such preparations may not require the addition of phospholipids for activity (55).

The actual molecular weight of $H^+$-ATPase without phospholipids or detergents may be 458,000 (26) or about 480,000 (54). The most hydrophobic protein in $F_0$ was purified as $^{14}C$-DCCD-binding protein and its primary structure (61, 62) and $H^+$-channel activity (63) will be discussed later.

### Reconstitution of ATPase Activity from Purified Subunits of $F_1$

Reviews before 1977 contained only speculations on the roles of the five subunits of $F_1$ (3–5, 24, 25, 27, 73, 74, 82, 103), but recently individual subunits have been purified and analyzed (Table IV).

**Table IV.** Amino Acid Compositions of Subunits of $F_1$ and $F_1$-like ATPases—Data on $F_1$, $CF_1$, and $TF_1$ from Refs. 28, 7, and 29, Respectively

| | Subunit (residues/mol) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Subunit $\alpha$ | | | Subunit $\beta$ | | | Subunit $\gamma$ | | | Subunit $\delta$ | | | Subunit $\epsilon$ | | |
| | $F_1\alpha$ | $CF_1\alpha$ | $TF_1\alpha$ | $F_1\beta$ | $CF_1\beta$ | $TF_1\beta$ | $F_1\gamma$ | $CF_1\gamma$ | $TF_1\gamma$ | $F_1\delta$ | $CF_1\delta$ | $TF_1\delta$ | $F_1\epsilon$ | $CF_1\epsilon$ | $TF_1\epsilon$ |
| Cysteine | 3 | 2 | 1 | 1 | 3 | 0 | 0 | 6 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| Aspartic acid | 42 | 38 | 44 | 35 | 44 | 41 | 27 | 36 | 29 | 12 | 17 | 15 | 3 | 15 | 8 |
| Threonine | 25 | 36 | 24 | 27 | 37 | 22 | 17 | 22 | 18 | 10 | 9 | 5 | 3 | 9 | 3 |
| Serine | 37 | 33 | 21 | 24 | 30 | 15 | 26 | 18 | 11 | 10 | 17 | 9 | 6 | 6 | 4 |
| Glutamic acid | 58 | 82 | 64 | 58 | 58 | 66 | 29 | 40 | 40 | 22 | 23 | 30 | 5 | 15 | 11 |
| Proline | 18 | 17 | 24 | 24 | 26 | 23 | 18 | 15 | 13 | 6 | 4 | 6 | 1 | 4 | 4 |
| Glycine | 57 | 43 | 47 | 46 | 49 | 44 | 18 | 20 | 13 | 10 | 22 | 13 | 4 | 8 | 6 |
| Alanine | 50 | 58 | 42 | 48 | 43 | 38 | 32 | 35 | 24 | 27 | 14 | 21 | 7 | 8 | 12 |
| Valine | 40 | 38 | 42 | 40 | 41 | 41 | 15 | 24 | 20 | 16 | 13 | 12 | 4 | 6 | 9 |
| Methionine | 8 | 11 | 8 | 9 | 14 | 12 | 6 | 7 | 6 | 2 | 1 | 1 | 1 | 1 | 3 |
| Isoleucine | 35 | 40 | 36 | 30 | 27 | 33 | 25 | 17 | 20 | 5 | 9 | 12 | 3 | 11 | 6 |
| Leucine | 45 | 53 | 50 | 41 | 50 | 42 | 24 | 34 | 31 | 15 | 11 | 28 | 2 | 13 | 9 |
| Tyrosine | 9 | 18 | 11 | 11 | 12 | 9 | 10 | 7 | 3 | 1 | 5 | 4 | 2 | 0 | 1 |
| Phenylalanine | 12 | 13 | 15 | 14 | 16 | 16 | 9 | 9 | 8 | 6 | 5 | 7 | 1 | 1 | 2 |
| Lysine | 32 | 20 | 20 | 24 | 20 | 19 | 26 | 23 | 17 | 5 | 9 | 8 | 8 | 5 | 8 |
| Histidine | 6 | 3 | 7 | 8 | 5 | 11 | 5 | 1 | 7 | 2 | 3 | 1 | 1 | 1 | 1 |
| Arginine | 27 | 31 | 27 | 19 | 30 | 27 | 16 | 19 | 17 | 4 | 5 | 16 | 3 | 11 | 7 |

The partial reconstitution of $F_1$ from minor subunits and the remaining aggregates yielded the following information: subunit $\delta$ is essential for binding of bacterial $F_1$ (81, 91, 100, 104) or chloroplast $F_1$ (4, 99) to $F_0$; subunit $\epsilon$ from chloroplast $F_1$ is identical to ATPase inhibitory peptide (4), but subunit $\epsilon$ from ox heart (105) is not identical to the latter (27). Subunit $\epsilon$ is also essential for binding $F_1$ to $F_0$ (91, 100).

Isolated subunit $\beta$ specifically binds the ATPase inhibitor aurovertin (70, 88). Moreover ATP analogues, such as 6[(3-carboxy-4-nitrophenyl) thio]9-$\beta$-D-ribofuranosyl-purine-5′-phosphate (106), and the tyrosine modifier 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (92) were shown to bind specifically to subunit $\beta$. On the other hand, subunit $\beta$ was specifically extracted from $F_1$ in chromatophores with LiCl, and reincorporation of purified subunit $\beta$ into $\beta$-free $F_1$ resulted in restoration of ATPase activity (107).

Thermophilic bacterium PS3 was chosen from among several thermophilic bacteria for use in complete reconstitution of $F_1$ from its purified individual subunits (108). The thermophilic $F_1$ purified from PS3 was not only heat stable (37, 108), but also stable against all dissociation agents tested (Fig. 4). In the proteins of thermophiles, the extra energy of stabilization is usually provided by a few extra salt bridges on the molecular surface (109) or subunit interface (110) without disturbance of the tertiary or quarternary structure essential for enzyme activity. Table IV shows that most subunits of $TF_1$ have higher contents of amino acid residues forming salt bridges (Arg, Glu, and Asp) than those in the subunits of $F_1$. However, the same is not true for $CF_1$. Point mutation of a protein usually results in a temperature-sensitive mutant, but in some cases it results in production of a thermostable protein (111). A very low content of cysteine is a characteristic of $TF_1$ (86).

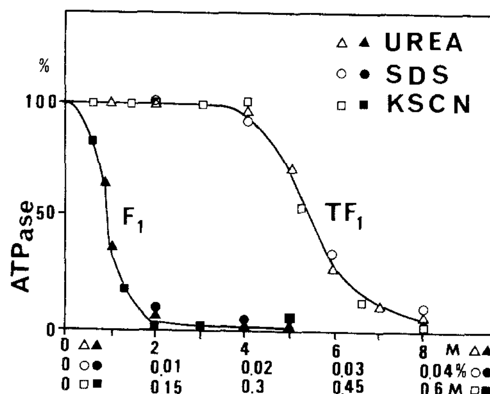


**Fig. 4.** Effects of various dissociating agents on the ATPase activities of beef heart $F_1$ ($F_1$) and thermophilic $F_1$ ($TF_1$) (26).

The $TF_1$ molecule was reconstituted after its complete dissociation into subunits with dodecylsulfate–urea mixture (37) or guanidine hydrochloride (16). After the dissociation each subunit was purified by ion exchange column (DEAE or CM) chromatography (16) and the isolated subunits are shown in Fig. 5.

Mild procedures were developed to obtain active subunits (17). The three major subunits of $F_1$ of *E. coli* were purified by hydrophobic column chromatography (butyl- and phenyl-Sepharose) after dissociating the enzyme by cold inactivation. This gentle procedure for isolating subunits may be applicable to other $F_1$s of mesophiles, which are inactivated at about 50°C

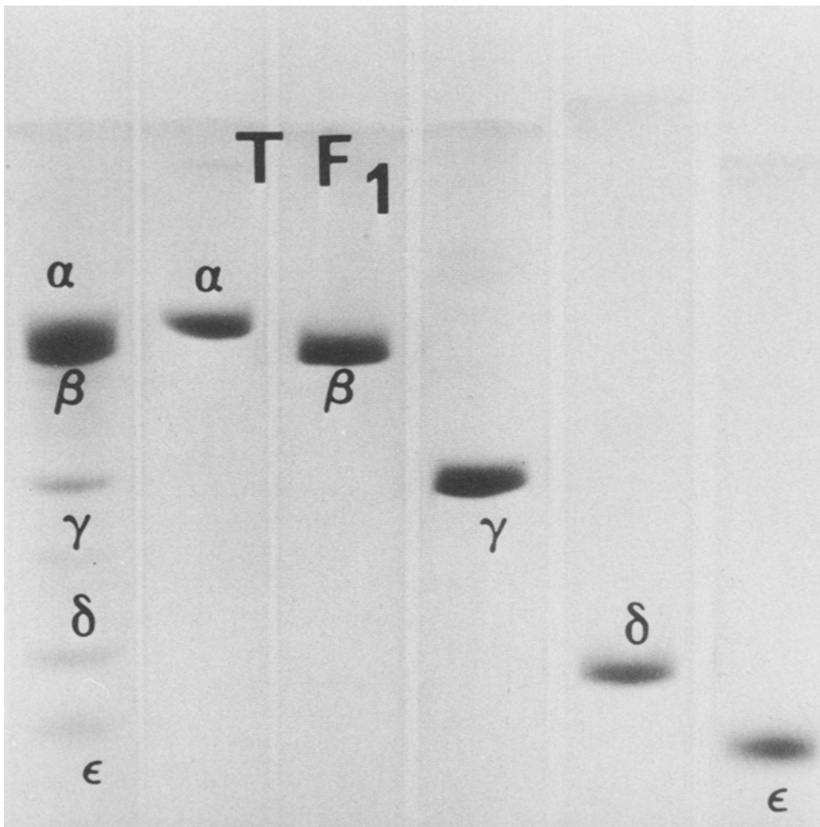


**Fig. 5.** Sodium dodecylsulfate polyacrylamide gel electrophoresis of $TF_1$ (left) and its isolated five subunits.

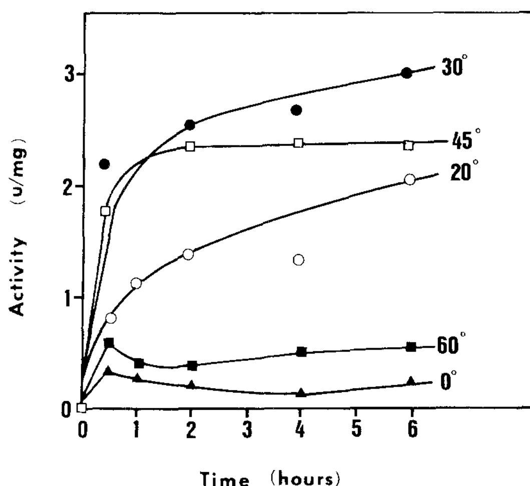


**Fig. 6.** Reconstitution of ATPase activity and the effects of temperature during the reconstitution from purified subunits $\alpha$, $\beta$, and $\gamma$ (16). Solutions containing 80 $\mu$g/ml of subunit $\alpha$, 73 $\mu$g/ml of subunit $\beta$, 21 $\mu$g/ml of subunit $\gamma$, 5 mM $MgSO_4$, and 50 mM Tris-sulfate at pH 8.0 were incubated at the temperatures indicated. At appropriate times, 30-$\mu$l samples were removed for assay of ATPase activity in 15 min at 60°C.

(37, 112). Moreover, agents that increase the stability of $F_1$, such as $D_2O$ (113), may be used in future work.

It is now established that both minor subunits are not necessary for the ATPase activity of $F_1$ (16, 17), because their removal did not affect the reconstituted ATPase activity. Subunit $\gamma$ is effective in all kinds of reconstitution (16), although it is not essential in reconstitution of $TF_1$. Assembly $\alpha\beta\delta$ did not show the azide sensitivity characteristic of most $F_1$ preparations (16). This assembly was thermolabile and differed in electrophoretic mobility from the original $TF_1$ (16).

Subunits $\alpha$, $\beta$, and $\gamma$ of *E. coli* $F_1$ are all required for ATPase (17). Moreover, the addition of ATP was essential during reconstitution of this kind of $F_1$ (17). However, nucleotides of $TF_1$, if present, were completely removed during column chromatography on DEAE-cellulose or Dowex-1 to remove dodecylsulfate. The time courses of reconstitution of subunits $\alpha$, $\beta$, and $\gamma$ are shown in Fig. 6.

## Reconstitution of $H^+$-Channel ($F_0$)

$F_0$ has been assumed to be an $H^+$-channel (2). In fact, passive $H^+$-translocation in $F_1$-depleted submitochondrial particles or vesicles containing

crude $F_0$ was shown to be inhibited by addition of an energy-transfer inhibitor (114–116). However, these $F_0$ preparations contained considerable amounts of impurities, including electron carriers, and were not stable enough to permit quantitative analysis of $H^+$-conduction through $F_0$ under various conditions.

When purified thermostable $F_0$ ($TF_0$) was incorporated into liposomes loaded with $K^+$, the addition of valinomycin caused rapid uptake of $H^+$ through $TF_0$ (Fig. 7) (63). Both the velocity and extent of $H^+$-translocation were greatly enhanced by increasing the amount of $TF_0$ added.

The extent of $H^+$-translocation in $TF_0$-liposomes or simple liposomes was not affected by addition of FCCP as an $H^+$-carrier, irrespective of the amount added. Since a preparation of $TF_0$-liposomes contained simple liposomes without $TF_0$, FCCP could induce further $H^+$-uptake by preparation that had already attained equilibrium after addition of valinomycin (63).

The initial velocity of uptake was calculated as $6H^+$ per second per $F_0$ molecule per 100 mV (63). The velocity of $H^+$-conductance through $F_0$ obeys ohmic law, if there is no gate. The velocity corresponds to a unit conductance of $9.5 \times 10^{-18}$ mho per $F_0$ at pH 8.0 and the maximum unit conductance is 1.6 $\times 10^{-16}$ mho, calculated from results obtained by titration at different pHs. The effect of external pH on passive $H^+$-uptake in $F_0$-liposomes showed that

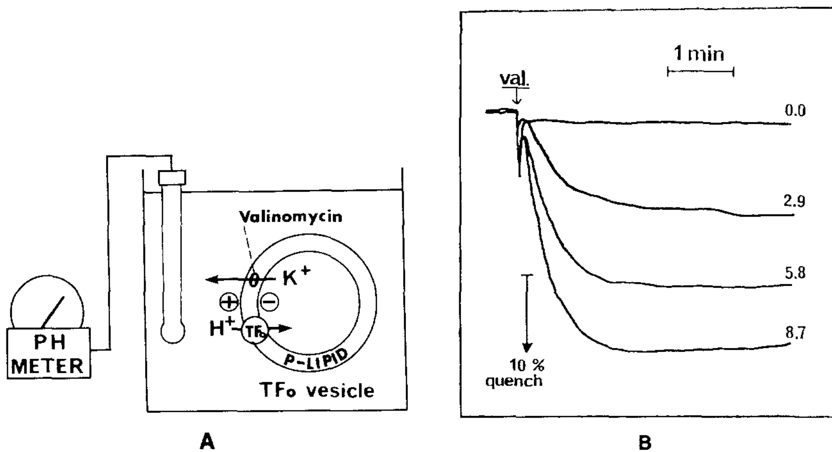


**Fig. 7.** The measurement of $H^+$-channel activity of $TF_0$ reconstituted into liposomes loaded with KCl. The addition of valinomycin causes efflux of $K^+$ and thus produces an inside negative membrane potential ($\Delta\Psi$) and drives $H^+$ through $TF_0$. The concentration of $H^+$ is measured with the glass electrode of a pH meter (A) or $\Delta pH$ across the liposome membrane is calculated from the fluorescence quenching of 9-aminoacridine (63, 133). The time course of $H^+$-uptake expressed as quenching of the fluorescence is shown in B. $TF_0$ was added in microgram amounts.

$H^+$ but not $OH^-$ was the ionic species actually conducted (63). The pH–velocity curve of $H^+$-conductance suggested the presence of an $H^+$-binding site and one-to-one stoichiometry of $H^+$-binding. This observation is interesting since a stoichiometry of $2H^+$ to $3H^+$ per ATP has been reported for $H^+$-ATPase in crude membrane preparations (117, 118).

Reconstitution experiments confirmed the hypothetical role of $F_0$ in ATP synthesis (2, 10, 119). As shown in Fig. 8, uptake or release of $H^+$ from $TF_0$-liposomes and $^{32}$Pi-ATP exchange in the presence of $TF_1$ were all proportional to the amount of $TF_0$ added to the liposomes. And both activities were inhibited by the addition of DCCD (Fig. 9). Net ATP synthesis with $F_0 + F_1$ will be described in the following section.

The highly hydrophobic peptide that specifically binds DCCD was extracted from the $F_0$ portion of the $H^+$-ATPase of several species (65, 120–122). The primary structure of this DCCD-binding protein was determined (61, 62) (Fig. 10), and DCCD was shown to be bound to glutamic acid residues (61, 62). The DCCD-binding proteins (8000 daltons) from the two microorganisms seen in Fig. 10 show extensive homology in their amino acid sequences despite the fact that one is translated outside, and the other inside, the mitochondria (62).

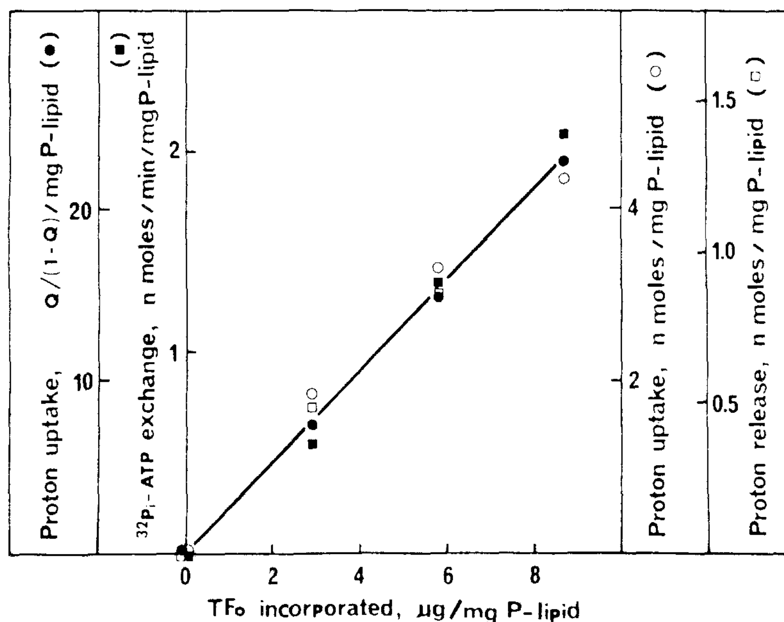


**Fig. 8.** The parallel relation between $H^+$-translocation and the $^{32}$Pi-ATP exchange reaction (63).

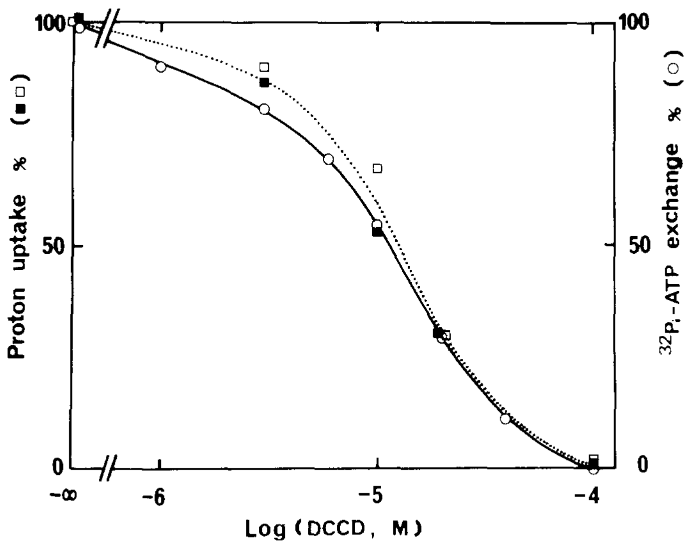


**Fig. 9.** The effects of DCCD on $H^+$-translocation and $^{32}$Pi-ATP exchange reaction (63).

Purified DCCD-binding protein of chloroplast was shown to conduct $H^+$ through liposomes containing bacteriorhodopsin (123), and a similar observation was also reported on the protein of *E. coli* (122). The remaining subunits of $TF_0$ of 19,000 and 13,500 daltons may correspond to those of 19,000 and 11,000 daltons in $F_0$ from *Neurospora cassa* (124). The reproducibility of results on DCCD-sensitive $H^+$-translocation by DCCD-binding protein was not satisfactory in our laboratory, but that of results on $TF_0$ was excellent (63). Moreover, pure DCCD-binding protein did not bind or inhibit $TF_1$, but the addition of a 13,500-dalton subunit did (65). Genetically, $F_0$ is composed of at least two peptides (125).

The reconstitution of $TF_0$ into liposomes suggests that $TF_0$ is not a mobile carrier, such as FCCP, but a channel or pore, such as gramicidin (12). If an ion is conducted via a mobile carrier, its conduction is strongly affected by temperature, which controls the fluidity of lipids in the membrane (126). On the other hand, if it is conducted through a channel, the effect of fluidity is rather small (126). The phospholipids obtained from thermophilic bacterium PS3 contained no unsaturated fatty acids and had a transition temperature of 22°C. These phospholipids are composed of phosphatidyl-glycerol, phosphatidylethanolamine, and cardiolipin (26), and their molecular species are mainly of the 1-15-methylhexadecanoyl-2-13-methyltetradecanoyl-*sn*-glycerol-3-phosphoryl type and 1,2-di-13-methyltetradecanoyl-*sn*-glycerol-3-phosphoryl type (127). The break in the Arrhenius plot of $H^+$-translocation through $TF_0$

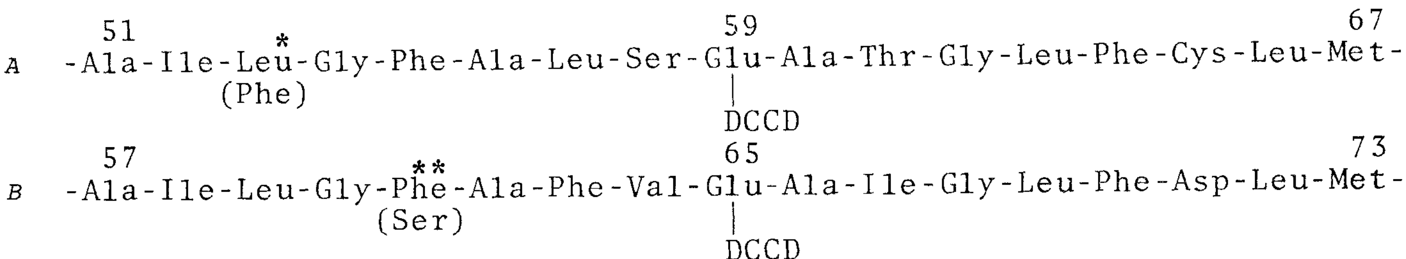


**Fig. 10.** Amino acid sequence of the middle portion of DCCD binding proteins in $F_0$. A, *Saccharomyces cervisiae*, *mutant D 273-10B/A21 (oligomycin-resistant mutant of mitochondrial DNA); B, *Neurospora crassa*, ***Mutant AP-2* (oligomycin-resistant mutant of nuclear DNA). Data from Sebald et al. (61, 62).

was small, but that through FCCP was large (63). Moreover, $TF_0$ is a large molecule of $7.3 \times 10^4$ daltons, and may not be able to move or rotate as a whole in the membrane, especially when $F_1$ is attached to one side of the membrane.

A proton is a unique ion since it has no electrons surrounding its nucleus, and thus the ionic radius of $H^+$ is only $10^{-5}$ Å (the radii of $Na^+$ and $K^+$ are about 1 Å). This small size is associated with a large electric field at the $H^+$ surface. The individual ion equivalent conductivity at infinite dilution is 315.2 for $H^+$ (43.2 for $Na^+$), because of the $H^+$-jump through water molecules. The mechanism of $H^+$-translocation through $F_0$ is controversial. $H^+$ may jump from one to another of the protonated amino acid residues, perhaps glutamyl residues which may serve as an $H^+$-filter. It is interesting that only one DCCD molecule can block $H^+$-translocation through $F_0$ which is composed of several copies of DCCD-binding protein (122).

## Reconstitution of the $H^+$-Gate

Addition of $F_1$ to $F_1$-depleted particles is known to block $H^+$-leakage from the particles (82, 114). The so-called structural role of inactivated $F_1$ that improves oxidative phosphorylation of $F_1$-depleted particles may be attributed to this gating function of $F_1$ (82, 114). Since catalytic activity is localized on subunit $\beta$, assisted by subunits $\alpha$ and $\gamma$, and these three subunits, alone or in combination, did not block $H^+$-uptake into $TF_0$-liposomes, attempts were made to reconstitute the $H^+$-gate separately from the catalytic activity of the ATPase molecule (100).

Figure 11 shows that complete assembly ($\alpha + \beta + \gamma + \delta + \epsilon$) as well as $TF_1$ reduced the $H^+$-uptake of $TF_0$-vesicles loaded with 0.5 M KCl, which were suspended in assay medium containing 0.5 M sucrose, 2.5 mM $MgSO_4$, 10 mM N-[-2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine-NaOH (pH 8.0), and 5 $\mu$M 9-aminoacridine. The reaction was started by addition of 20 ng of valinomycin. Subunit $\gamma$ with $\delta$ and $\epsilon$ significantly lowered $H^+$-uptake. Other combinations lacking one of these subunits were ineffective. Thus, the $H^+$-gate is composed of these subunits. At the same time, the experiment indicated that the $TF_1$-binding site of $TF_0$ molecules in the liposomes must be on the outside because otherwise complete block of $H^+$-uptake by the combination of $TF_1$ components would not be possible.

The gating process of the $H^+$-pump has been related directly to the process of ATP synthesis (128). In chloroplasts, a sharp 1000-fold increase in $H^+$-conductance was observed when $\Delta\mu H^+$ reached a certain threshold value ($\Delta pH = 3.0$) (129). This threshold for $H^+$-conductance was lowered by the addition of ADP. DCCD reduced the conductance sevenfold. The character-

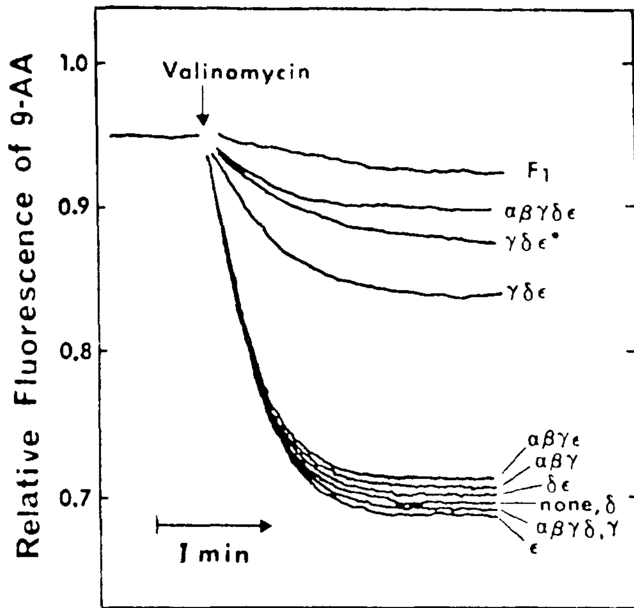


**Fig. 11.** Reconstitution of the H$^+$-gate on TF$_0$-liposomes. Influx of H$^+$ into TF$_0$-liposomes was assayed by the methods described in Fig. 7. The following amounts of subunits were added to 50 $\mu$l of TF$_0$-liposome suspensions which contained 17.5 $\mu$g of TF$_0$, as indicated; $\alpha$, 31 $\mu$g; $\beta$, 45 $\mu$g; $\gamma$, 15 $\mu$g; $\delta$, 11 $\mu$g; $\epsilon$, 11 $\mu$g. In the experiment designated as $\gamma\delta\epsilon$*, double concentrations of the three subunits were used (100).

istics of the flux force were similar to those of the potential barrier at the PN junction of the "Zener-diode" (129). The low H$^+$-conductance (0.1 $\mu$mho/cm$^2$) allows the formation of a high $\Delta\tilde{\mu}H^+$ at low electron flux.

## Electrochemical Potential Difference of H$^+$ in H$^+$-ATPase-Liposomes

According to the chemiosmotic theory (130, 131) the difference in the electrochemical potential of H$^+$ across the membrane ($\Delta\tilde{\mu}H^+$) is generated by electron transport, and the flow of H$^+$ driven by $\Delta\tilde{\mu}H^+$ through F$_0$ · F$_1$ results in synthesis of ATP. Although there have been many reports on the $\Delta\tilde{\mu}H^+$ across the membranes of mitochondria, chloroplasts, and bacteria [see reviews (5, 132)], no quantitative data on $\Delta\tilde{\mu}H^+$ in the reconstituted F$_0$ · F$_1$-liposomes have been reported until recently. The use of stable F$_0$ · F$_1$ (26, 53) and PS3 phospholipids (127) enabled us to measure $\Delta\tilde{\mu}H^+$ (19, 133). $\Delta\tilde{\mu}H^+$ is composed of a membrane potential ($\Delta\Psi$) and a pH gradient ($\Delta$pH) across the membrane (1, 2, 5, 132):

$$\Delta\bar{\mu}H^+ = \Delta\Psi - Z\Delta pH$$

where $Z$ is approximately 63 at 45°C.

In order to measure $\Delta\Psi$, negatively charged 8-anilinonaphthalene-1-sulfonate (ANS) was used, because limited intramolecular rotation of ANS attracted to the membranes results in enhancement of fluorescence intensity (134, 135). The relative enhancement of fluorescence was proportional to the diffusion potential that was artificially imposed by the addition of KCl and valinomycin to the reconstituted liposomes. The diffusion potential of $K^+$ mediated by valinomycin was calculated by Nernst's equation as follows:

$$\Delta\Psi = RT \ln [K^+]_o/[K^+]_i$$

where $[K^+]_i$ and $[K^+]_o$ are the concentrations of $K^+$ inside and outside the liposomes, respectively. This calibration has to be made for each preparation (133).

In order to measure ΔpH, the weak base 9-aminoacridine (9AA) was used, because with decrease in pH inside the liposomes, 9AA was accumulated and showed self-quenching of its fluorescence (136). When the external pH is below 9, ΔpH can be calculated (136) by the following equation:

$$\Delta pH = \log Q/(1-Q) + \log 1/V$$

where $Q$ is the fraction of the total fluorescence that is quenched in response to ATP added to the $F_0 \cdot F_1$-liposomes, and $V$ is the volume of the osmotic compartment as a fraction of the total volume of the assay.

Figure 12 shows schemes for $H^+$-translocation by $H^+$-ATPase in the reconstituted liposomes. Hydrolysis of ATP via $F_0 \cdot F_1$ oriented in liposomes resulted in formation of $\Delta\bar{\mu}H^+$ (more positive or acidic inside). The reaction was followed with the fluorescent probes ANS and 9AA. Uncharged Tris

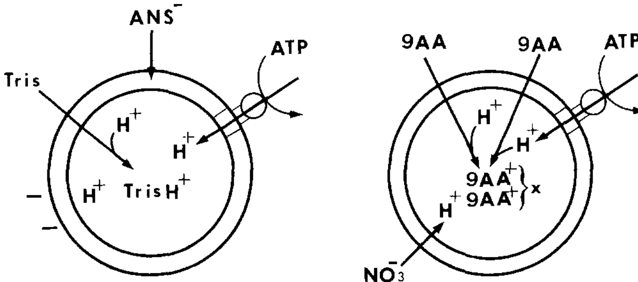


**Fig. 12.** $H^+$-translocation by $H^+$-ATPase incorporated in liposomes. Left, determination of $\Delta\Psi$ by ANS fluorescence in the presence of Tris (a permeant buffer); right, determination of ΔpH by 9AA fluorescence in the presence of $NO_3^-$ (a permeant anion).

**Table V.** Estimations of $\Delta\mu H^+$, $\Delta\Psi$, and ΔpH of $H^+$-ATPase Liposomes[a]

| Medium | Enhancement of fluorescence of ANS (%) | $\Delta\Psi$ (mV) | Quenching of fluorescence of 9AA $[Q/(1-Q)]$ | ΔpH | $\Delta\mu H^+$ (mV) |
|---|---|---|---|---|---|
| Tris | 102 | 145 | 0 | 0 | 145 |
| Tricine | 48 | 70 | 0.67 | 2.9 | 253 |
| Tricine + $NaNO_3$ | — | — | 2.45 | 3.5 | 221 |

[a]Liposomes were reconstituted from PS3-phospholipid mixture containing 0.25 mg of $F_0 \cdot F_1$ protein as described (133). The $V$ value was 0.83 μl/ml, and the amount of ATP added was 0.5 μmol.

molecules, which can pass through the PS3 lipid bilayer, increased the magnitude of $\Delta\Psi$ caused by ATP hydrolysis by depressing pH changes in the liposomes. In contrast, the permeable anion $NO_3^-$ apparently increased ΔpH by decreasing $\Delta\Psi$. In either case, the ATPase reaction proceeded until a certain $\Delta\tilde{\mu}H^+$ was reached (Table V).

Figure 13 shows tracings of typical experiments (133). The addition of ATP induced quenching of 9AA fluorescence (trace A), which was enhanced by the presence of $NO_3^-$. After development of maximal quenching, the fluorescence slowly returned to the original level as added ATP was hydrolyzed. The addition of Tris (trace B), FCCP (trace C), or nigericin (trace D) after maximal quenching dissipated the ΔpH. The presence of an energy-transfer inhibitor, tributyltin-chloride ($Bt_3SnCl$ in trace E) or DCCD (trace F), inhibited the development of ΔpH. In these experiments, the maximal quenching due to ATP hydrolysis was about 50%. These tracings suggest that the properties of $H^+$-ATPase-liposomes are similar to those of the original energy-transforming membranes.

Energy transformation may not require a specific membrane structure. For example, when $F_1$ is adsorbed on the octane–water interface, ATP-induced, $F_1$-mediated changes in the Volta potential difference in the interface were observed (5). This experiment is interesting because it shows that the $H^+$-channel ($F_0$) may not be necessary for energy transformation at the catalytic site. $^{32}$Pi-ATP exchange reaction of Complex V is reported to occur without membrane structure (55) and there have been many similar reports, which are discussed in a recent book (82). This exchange reaction was observed in reconstituted $F_0 \cdot F_1$ liposomes (11), and was shown to be inhibited by uncouplers (mobile carriers of $H^+$) and $K^+$ plus valinomycin plus nigericin. The exchange reaction of Complex V was lost on addition of these agents, but at least part of the inhibition was explained by a special

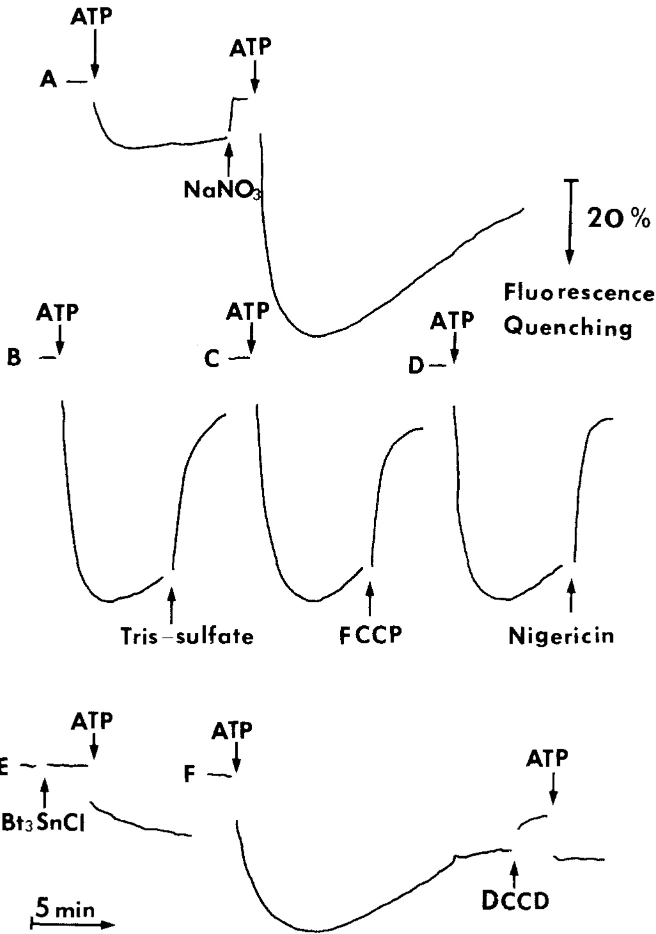


**Fig. 13.** $H^+$-translocation by $H^+$-ATPase-liposomes measured by 9AA. $H^+$-ATPase-liposomes (0.25 mg protein) were in the presence of 0.1 mmol of $NaNO_3$ (except at the beginnings of traces A and F). For details, see text and Ref. 133.

"uncoupler binding protein" (55). However, it is very difficult to imagine a protein which can bind a wide variety of uncouplers with different chemical structures.

The $H^+$-translocating activity of $H^+$-ATPase in liposomes (11) has been studied in detail in the proteoliposome-planar membrane system by measuring the potential directly with an electrode (137). One advantage of this system is that it permits studies on the role of phospholipids in $H^+$-ATPase liposomes. Phospholipids are required to restore (1) ATPase activity of the delipidated $H^+$-ATPase (8, 138), and (2) $^{32}$Pi-ATP exchange activity and

$H^+$-translocation into liposomes (138, 139). Phosphatidylcholine (lecithin) was shown to be effective in restoring ATPase but not $^{32}$Pi-ATP exchange (139). This discrepancy was explained by the $H^+$-conductivity of the reconstituted lipid bilayer (11), and, in fact, direct measurements showed that the $H^+$-conductivity of the membrane of phosphatidylcholine was $3 \times 10^{-8}$ $ohm^{-1}$ $cm^{-2}$, while those of phosphatidylethanolamine and mitochondrial phospholipids were 1.0 and $0.5 \times 10^{-8}$ $ohm^{-1}$ $cm^{-2}$, respectively (137). Mitochondrial phospholipids that were mixtures of phosphatidylcholine, phosphatidylethanolamine, and cardiolipin were the most effective for restoring $H^+$-translocation and $^{32}$Pi-ATP exchange reaction (11, 138, 139).

Thus, it has been established that $H^+$-ATPase can translocate $H^+$ through the membrane against a $\Delta\tilde{\mu}H^+$ of about 253 mV, and a value of as much as 312 mV (19) was obtained under favorable conditions. This maximum value is reached by one of the following mechanisms: (1) the steady-state level of $\Delta\tilde{\mu}H^+$ is established as result of balance between $H^+$-influx through $H^+$-ATPase and $H^+$-efflux through leaks in the membrane, or (2) a specific $H^+$-gate is opened when a certain threshold value of $\Delta\tilde{\mu}H^+$ is reached.

Since the $\Delta\tilde{\mu}H^+$ of 210 mV should result in the synthesis of ATP by $H^+$-ATPase in a stoichiometry of $2H^+$ per ATP (2), and recent experiments do not support this value (117, 118), detailed measurements of $\Delta\tilde{\mu}H^+$ and the $H^+$/ATP ratio in a simple reconstituted $H^+$-ATPase are necessary. All of these discussions are based on results with intact mitochondria (1, 2, 117, 118) and chloroplasts (140), and the experiments are complicated by antiport, symport, and leakage of anions and cations including ATP, ADP, substrates, Pi, $K^+$, $Ca^{2+}$, and $Na^+$.

### Net Synthesis of ATP in $H^+$-ATPase-Liposomes by an Artificially Imposed Electrochemical Potential of $H^+$

Mitchell's chemiosmotic hypothesis (130, 131) predicts the synthesis of ATP by imposing $\Delta\tilde{\mu}H^+$ across the $H^+$-ATPase through which $H^+$ is driven (1, 2). In fact, net ATP synthesis driven by $\Delta$pH was demonstrated in broken chloroplasts that were first incubated in weak acid and then put in alkaline solution containing ADP and $^{32}$Pi (141). This finding was confirmed with intact mitochondria (142) and submitochondrial particles in the presence of $K^+$ and valinomycin to form the $\Delta\Psi$ component of $\Delta\tilde{\mu}H^+$ (143).

However, these membranes contained many components, including electron carriers, translocators, and other energy transformers, and so it is difficult to conclude from the results that only $H^+$-ATPase was responsible for the synthesis of ATP by this acid–base treatment. Net synthesis of ATP was also observed in reconstituted vesicles containing crude $H^+$-ATPase in

the presence of electron components called Complexes (144) or bacterial rhodopsin (145). But ATP synthesis in these preparations has been explained by the presence in them of the lipoate–oleate system (146). $H^+$-ATPase preparations were unstable and net ATP synthesis driven by "acid–base transition" in reconstituted $H^+$-ATPase-liposomes has never been reported.

As shown in Table II, $H^+$-ATPase preparation from thermophilic bacterium PS3 is the simplest in its subunit composition and the most stable (53, 65). Moreover, it was confirmed that there is no oleic acid in phospholipids of PS3 (127) and no lipoic acids in either PS3 phospholipids or PS3 $H^+$-ATPase that could be titrated with 5,5′-dithiobis (2-benzoic acid) after complete reduction of SH-groups (3SH per $F_0 \cdot F_1$ are attributed to cysteine).

The $H^+$-ATPase liposomes reconstituted from PS3 phospholipids and $F_0 \cdot F_1$ were first incubated in acidic malonate buffer at pH 5.5 with valinomycin. Then a base stage solution (glycylglycine buffer, final pH 8.33) containing KCl was rapidly injected. This instantaneous transition should create $\Delta\tilde{\mu}H^+$, composed of both ΔpH (2.38 unit, acidic inside) and $\Delta\Psi$ (125 mV, positive inside) across the liposome membrane (18, 19). Esterification of $^{32}$Pi occurred at a velocity of 650 nmol per mg $F_0 \cdot F_1$ per minute. Considering the content of $F_0 \cdot F_1$, this velocity should be faster than that coupled to substrate oxidation. The primary role of $H^+$-translocation in oxidative phosphorylation is thus substantiated (11, 18, 143).

The maximum level of Pi esterified was about 100 nmol per mg $F_0 \cdot F_1$ in the reconstituted liposomes, but less than 2.5 nmol per mg protein was synthesized by mitochondria (142), submitochondrial particles (143), or bacterial membranes (147).

Figure 14 shows the effect of the pH in the acid stage and base stage. The decreases in the yield of ATP at pH below 5.5 (Fig. 14A) and above 8.5 (Fig. 14B) were probably due to inactivation of the proteins. The optimal KCl concentration was 0.15 M at a pH difference of 2.8 pH units. The reaction was inhibited by energy-transfer inhibitors, uncouplers, and permeant anions. In this experiment the minimum $\Delta\tilde{\mu}H^+$ required to synthesize ATP was 200 mV, irrespective of its component ΔpH and $\Delta\Psi$ (18). Thermodynamic calculations indicate that a value of 204 mV is necessary for equilibration at a stoichiometry of $2H^+$ per ATP, when the reaction is carried out at an ADP:ATP ratio of 50 in 2 mM Pi, assuming that the standard free energy change during ATP hydrolysis is −8.0 kcal/mol at pH 8.0 (148).

## Reconstitution of $H^+$-Pump and Mechanism of ATP Synthesis

Preceding sections described reconstitutions of essential parts of $H^+$-ATPase (148), namely, the catalytic site of $F_1$, $H^+$-channel ($F_0$), $H^+$-gate,

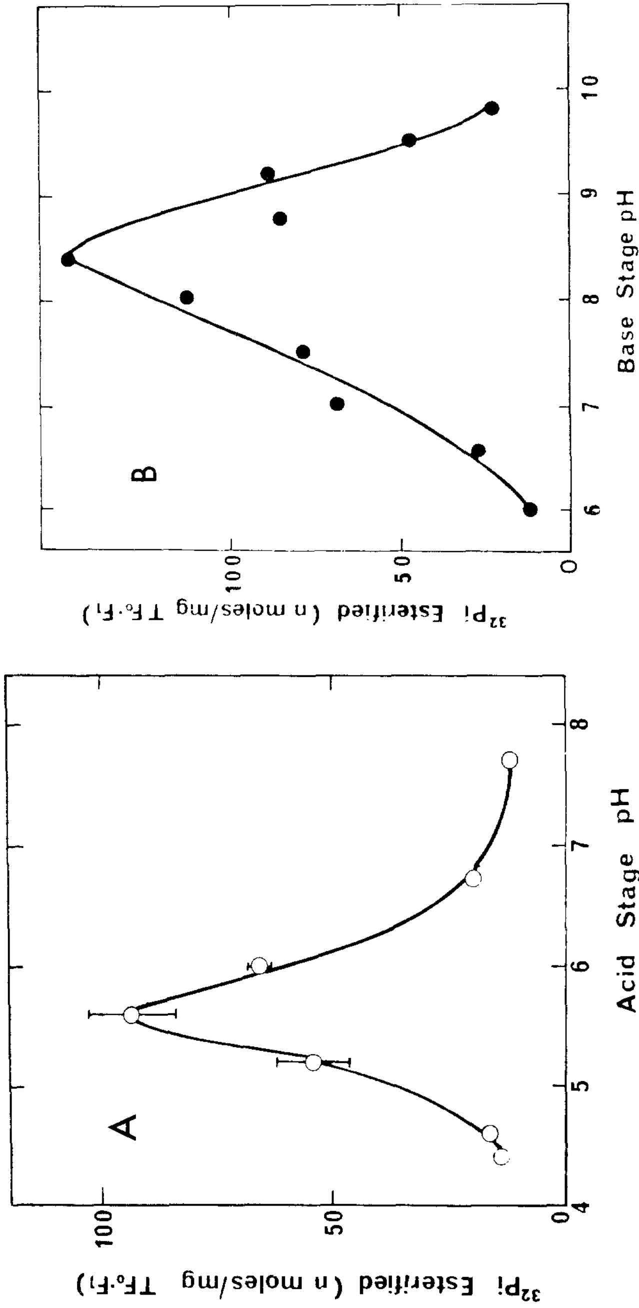


**Fig. 14.** Net ATP synthesis by H+-ATPase-liposomes by an artificially imposed electrochemical potential gradient. A, dependence of ATP yield on the acid stage (first incubation pH; B, dependence of ATP yield on the base stage (second incubation) pH (18).

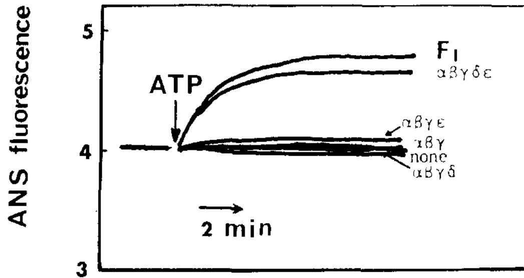


**Fig. 15.** Reconstruction of $H^+$-ATPase and restoration of $H^+$-pump activity from $TF_0$-liposomes and purified five subunits of $TF_1$. Time course of ANS fluorescence after the addition of 0.5 μmol of ATP to 5.3 μg $TF_0$ in liposomes preincubated with subunit assemblies (each 12.5 μg).

and liposomes capable of $H^+$-retaining activity (148). Finally, reconstitution of $H^+$-ATPase-liposomes from the subunits is described. Figure 15 shows that all of the subunits α, β, γ, δ, and ϵ are essential for the ATP-driven $H^+$-translocation. Table VI summarizes the role of the subunits of $F_1$. Most of the activities have been reconstituted only in the case of $TF_1$ (11). A tentative model of $H^+$-ATPase is shown in Fig. 16, but the model will probably be changed after further analyses.

The important question still to be answered is the precise molecular

**Table VI.** The role of Subunits of $F_1$

| Subunit | α | β | γ | δ | ϵ |
|---|---|---|---|---|---|
| Net ATP synthesis[a] | (+) | (+) | (+) | (+) | (+) |
| $H^+$-transport by ATPase[a] | (+) | (+) | (+) | (+) | (+) |
| $^{32}$Pi-ATP exchange[a] | (+) | (+) | (+) | (+) | (+) |
| ATPase catalytic activity | (±) | (+) | (−)[a] | (−) | (−) |
| $H^+$-gate activity[a] | (−) | (−) | (+) | (+) | (+) |
| Binding to $F_0$ | (−) | (−) | (−) | (+) | (+)[c] |
| Conferral of $N_3$ sensitivity[a] | (−) | (−) | (+) | (−) | (−) |
| Stabilization of ATPase[a] | (−) | (−) | (+) | (−) | (−) |
| $H^+$-channel activity | (−) | (−) | (−) | (−) | (−) |
| ATP, ADP binding activity | (+) | (+) | (−) | (−) | (−) |
| Aurovertin binding activity[b] | (±) | (+) | (−) | (−) | (−) |
| ATPase inhibitory activity[b] | (−) | (−) | (−) | (−) | (+)[d] |

[a]Confirmed only in $TF_1$ by reconstitution.
[b]Activity not present in $TF_1$.
[c]For *E. coli*, see Ref. 93.
[d]Confirmed only in $CF_1$ and *E. coli* $F_1$.

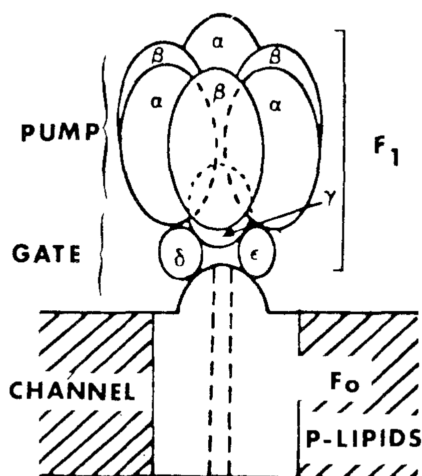


**Fig. 16.** A tentative model of $H^+$-ATPase (100).

events that take place in $H^+$-ATPase. The simplest mechanism, proposed by Mitchell (131), is as follows: $2H^+$ attack the O of $PO_4^-$ in a complex with $ADPO^-$ and $Mg^{2+}$ at the active site of $F_1$ (subunit $\beta$), from the $H^+$-channel ($F_0$) side, so that $H_2O$ is released, and $P^+O_3$ remains while $ADPO^-$ makes a nucleophilic attack on the $P^+$ center, thereby producing ATP. One judges from the $pK_a$ of each $O^-$ of Pi that this kind of protonation of Pi could not take place in aqueous solution, but it is difficult to make a theoretical prediction about an unknown catalytic site in the $H^+$-pump. A conformational change of $F_1$ has been suggested to smooth the energy profile of the chemical transition state (131), and, in fact, a conformational change of chloroplast $F_1$ was detected with $^3H$ during photophosphorylation (149).

Conformational theory of oxidative phosphorylation has been interpreted in several ways, but energy transfer from the electron transport system by direct contact between proteins is improbable (1). Conformational changes of proteins have been observed during a drastic pH change of the solution or ligand binding. But, the question remains: are the reported conformational changes of $F_1$ directly involved in energy transfer to ATP?

Light-induced incorporation of N-ethylmaleimide into subunit $\gamma$ of $CF_1$ (150), and rapid ATP-induced change in flourescence of $F_1$-aurovertin complex have been attributed to an energized state of $F_1$ (151, 152). But in $TF_1$ there is no SH in subunit $\gamma$ (Table IV) and no aurovertin binding and thus these reactions *per se* are not related to ATP synthesis.

The large conformational change (90 $^3H$ exchanged/$CF_1$) observed during photophosphorylation (149) is unusual for enzyme reactions. But pH change caused rapid $^2H$–$^1H$-exchange in $TF_1$ with a rate constant $k$: $k$ = 50

$(10^{-pH} + 10^{pH-6})\ 10^{0.05(\theta-20)}\ \text{min}^{-1}$ where $\theta$ is the temperature in °C (153). It is still not clear whether this rapid H-exchange is due to ΔpH or energized conformational change.

The roles of bound nucleotide of $F_1$ and conformational change of $F_1$ in ATP synthesis have been discussed in detail (1), but there is a report that the initial $^{32}$P-ATP formed derived from ADP in the medium and not from ADP bound to $CF_1$ (154). On the other hand, transitorily tightly bound ATP was shown to be on the catalytic pathway for ATP synthesis (1, 155, 156). Tightly bound nucleotide was rapidly released from thylakoid membrane on "acid–base transition" (155) and "external electric field treatment" (157). The energy of conformational change was supposed to be necessary for release of bound ATP synthesized on $F_1$ (1). Conformational changes of $TF_1$ (152) and subunits $\alpha$ and $\beta$ were induced by nucleotide (ATP and ADP) binding (158). Circular dichroic spectra showed stacking of tyrosine residue and adenine portion (158), and they were not explained by the formation of *syn*-type nucleotide on the subunits during *anti-syn* transition (159). In fact, labeling of $F_1$ with *p*-fluorosulfonyl [$^{14}$C]-benzoyl-3′ adenosine resulted in a labeling of tyrosine residue in both $\alpha$ and $\beta$ subunits. A tryptic digest of the labeled $\beta$ subunit contained a single radioactive peptide, the amino acid sequence of which was: Ile-Met-Asp-Pro-Asn-Ile-Val-Gly-Ser-Glu-His-Tyr*-Asp-Val-Ala-Arg, where Tyr* was the O-[$^{14}$C]-sulfonylated derivative of the tyrosine residue (160). Moreover, the rate of $^2$H–$^1$H exchange of $TF_1$ (153) and $CF_1$ (161) decreased in the following order: free $F_1$, $F_1 \cdot$ ADP, $F_1 \cdot$ ATP, and $F_1 \cdot$ AMPPNP.[2]

Net synthesis of ATP by applying an external electric field on thylakoid membrane may still be explained by the movement of $H^+$ by $\Delta\Psi$ (157, 162). This electrical method has high time resolution, and the method revealed that $\Delta\Psi$ increased the number of activated $CF_1$ that released nucleotides, but not the turnover number (5 msec) of $CF_1$ (157). Pure $H^+$ATPase reconstituted in macroliposomes also synthesized ATP by applying an external electric field (163).

However, we still do not know the mechanism of energy transformation in $H^+$-ATPase.

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[2]If $F_1$ dissociates in the absence of nucleotides, this dissociation will affect any measurement of H-exchange. $TF_1$ is highly stable (Fig. 4).

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