J. Membrane Biol. 55, 1-8 (1980)

The Journal of
Membrane Biology

Topical Review

Energy-Transducing Proteins in Thermophilic Biomembranes

Yasuo Kagawa

Department of Biochemistry, Jichi Medical School, Minamikawachi, Tochigi-ken, Japan 329-04

Summary. Biomembranes are the major site of energy transduction. The chemisomotic theory of energy transduction is based on the following four major systems (i) H^+-ATPase which is composed of a catalytic portion (F_1) and a H^+-channel (F_o), (ii) electron transport components, (iii) H^+-linked porters, and (iv) a H^+-impermeable lipid bilayer which is plugged through by systems *i* to *iii* that are specially oriented to translocate H^+.

Studies on the molecular mechanism of energy transduction have been hampered by the impurity, instability and complexity of preparations of membrane proteins from mesophilic organism. However, using stable, simple membrane proteins from a thermophilic bacterium, we obtained the following results:

1) Thermophilic H^+-ATPase was dissociated into 5 subunits of F_1 and 3 subunits of F_o and their functions and structures were studied by reconstitution. F_1 was crystallized.

2) Thermophilic cytochrome oxidase, cytochrome *c* and NADH-dehydrogenase were purified. In contrast to the complex mitochondrial cytochrome oxidase (7 subunits) and NADH-dehydrogenase (3 subunits), the purified thermophilic proteins were shown to be composed of single components.

3) H^+-linked porters such as a H^+-driven amino acid carrier and a Na^+-H^+ antiporter were characterized.

4) Thermophilic lipids were shown to be completely saturated. Using these stable lipids, liposomes capable of H^+-driven vectorial reactions including net ATP synthesis and alanine transport were reconstituted.

The electrochemical potential difference of protons ($\Delta\bar{\mu}H^+$)[1] across the biomembrane is the driving force of oxidative phosphorylation, photophosphorylation and transport of many substrates [3, 36]. In studies on the molecular mechanism of this energy transduction, pure and stable membrane proteins responsible for this reaction are necessary for the following purposes:

1) To observe the reaction of isolated proteins. In a complex system, measurements may be affected by side reactions of contaminants.

2) To differentiate energy transfer by conformational change of connected protein, local electrochemical change in the membrane, and $\Delta\bar{\mu}H^+$ between two bulk water phases [37].

3) To isolate subunits of the protein in an active state and determine their roles and physicochemical properties [61].

4) To reorganize subunits into an oligomer, so that stepwise restoration of higher functions can be observed [23, 60].

5) To reconstitute oligomers into proteoliposomes, so that their vectorial energy transducing reactions can be examined [21, 23, 45].

Most preparations of mesophilic proteins obtained so far from energy transducing biomembranes have been unstable, impure, and complex [3, 15], except for preparations of cytochrome *c*. Thus, reconsti-

[1] *Abbreviations:* DCCD, N,N′-dicyclohexylcarbodiimide; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone; F_1, catalytic portion of H^+-ATPase; F_o, H^+-channel portion of H^+-ATPase; TF_1, thermophilic F_1; TF_o, thermophilic F_o; $\Delta\psi$, membrane potential; ΔpH, pH difference across the membrane; $\delta\bar{\mu}H^+$, electrochemical potential difference of H^+ across the membrane; OSCP, oligomycin sensitivity conferring protein.

0022-2631/80/0055-0053 $03.20

tution of the component subunits is difficult and it is uncertain which of the complex components in the preparations are essential and which are contaminants. This review describes energy transduction by stable, pure, and simple proteins obtained from thermophilic bacteria for the purposes listed above.

Thermophilic Proteins

If an organism is to survive in a hot spring, all its proteins must be stable [9]. These proteins are also resistant to dissociation agents that, even at very low concentration, denature mesophilic counterparts. As a source of stable energy-transducing proteins, the aerobic thermophilic bacterium PS3 was chosen [22]. Figure 1 shows the difference in stabilities of F_1 ATPase [30] and cytochrome oxidase [49] from mesophiles and the thermophile. Essentially similar results were obtained with many other dissociation agents, including chaotropic anions such as SCN^- and ClO_4^-, LiCl, organic solvents, detergents such as sodium dodecylsulfate, and heavy metals. The stability of TF_1 was confirmed with ^1H-^2H-exchange [40].

The greater stability of the enzymes from thermophiles is mainly due to an increased number of salt bridges (Arg-Asp, Arg-Glu, etc.). This idea is supported by X-ray crystallography of thermophilic ferredoxins [43] and other thermophilic proteins [62]. In the thermophilic proteins, the extra energy of stabilization is provided by a few extra salt bridges on the molecular surface or subunit interface without disturbance of the tertiary or quarternary structure, which is essential for the function common to thermophiles and mesophiles [9, 62]. In contrast to hydrogen- and hydrophobic-bondings, which are short range, the electrostatic force of the salt bridges is long range and facilitates structural reconstitution of a protein after its complete denaturation. This property has been utilized in reconstitution of H^+-ATPase ($F_o \cdot F_1$) [23, 30]. $F_o \cdot F_1$ was dissociated into F_o (H^+-channel protein) [22, 26, 41], and F_1 (catalytic protein) [42, 44]. TF_1 was completely dissociated into its 5 nonidentical subunits (α, β, γ, δ and ε) and then reconstituted into active subunit complexes as will be described in a later section [23, 60].

The contents of SH and S-S groups are extremely low in thermophilic proteins [9, 61, 62]. This has proved useful in determining the subunit stoichiometry of TF_1. In mesophilic F_1, there are more than 10 SH groups per molecule, and so it is difficult to determine the subunit stoichiometry from them. But in TF_1, onle the α subunit contains an SH group (1 SH/1 α subunit) [61], and since there are 3 SH groups per TF_1 molecule, there must be 3 α subunits per TF_1 molecule [61]. Moreover, the fact that TF_o has no SH group proves the idea that SH groups or lipoate do not participate in the functioning of TF_o. Although lipoate [13] and SH [35] have been reported to be involved in ATP synthesis of other biomembranes, the considerable homology in the functioning portion of one of the subunits of mesophilic F_o's and TF_o [47] suggests that these proteins have the same mechanism of H^+-translocation.

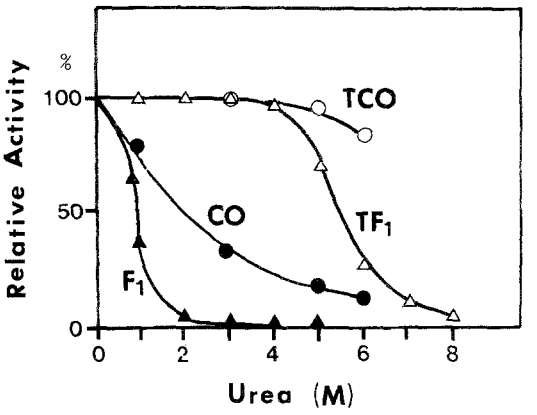

Fig. 1. Effects of urea concentration on the activities of ATPase and cytochrome oxidase [31, 49]. F_1, F_1 purified from beef heart mitochondria; TF_1, F_1 purified from thermophilic bacterium PS3; *CO*, cytochrome oxidase purified from beef heart mitochondria; *TCO*, cytochrome oxidase purified from thermophilic bacterium PS3.

Thermostable Lipids

In the biomembranes of thermophiles, lipids, as well as proteins, are physically and chemically stable. As shown in Fig. 2*A*, glycerophospholipids of mitochondria and mesophilic bacteria, and glycerogalactolipids of chloroplasts form a bilayer which is highly fluid and leaky to solutes. These properties of mesophilic biomembranes have been explained by the presence of unsaturated acyl groups in the glycerolipids, especially in the second position. The most mobile portion of the bilayer is the end of the hydrocarbon chains in the middle of the bilayer, as shown by nuclear magnetic resonance of ^{13}C, and electron paramagnetic resonance of spin-labeled lipids.

However, in thermophiles, the bilayers formed by lipids are compact and prevent ion leakage [9]. As shown in Fig. 2*B–D*, there is no unsaturation in these lipids, so their hydrocarbon chains in a trans-staggered conformation are tightly packed. This absence of unsaturation prevents oxidation and extreme thermal agitation of lipid molecules at high tempera-

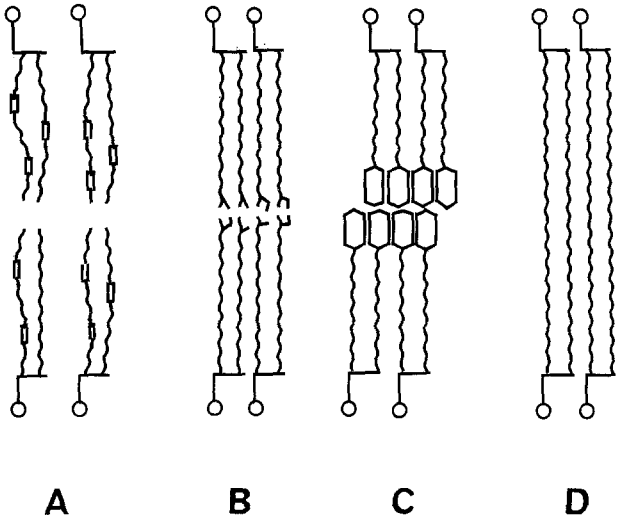

Fig. 2. Structure of lipid bilayer of thermophilic glycerolipids. (*A*): Mesophilic lipids containing unsaturated fatty acyl groups mainly at the second position of glycerol. (*B*): Phospholipids of thermophilic bacterium PS3 with iso and anteiso-groups at the end of the acyl groups [24]. (*C*): Glycerolipids of *Bacillus acidocardarius* with ω-cyclohexyl groups at the end of acyl groups [39]. (*D*): Diglycerol tetraether of *Thermoplasma acidophilum*. A single C_{40} hydrocarbon is combined through ether linkages with *sn*-2,3-glycerol [33]. The open circles indicate polar groups attached to glycerol. Note the wider intermolecular spaces and mid-membrane spaces in *A* than in *B*, *C* or *D*.

ture. The movement of the end of hydrocarbon chains is also restricted by iso- and anteiso-groups of thermophilic bacterium PS3 (Fig. 2*B*) [24], ω-cyclohexyl groups of *Bacillus acidocardarius* (Fig. 2*C*) [39], and the diglycerol tetraether structure of *Thermoplasma acidophilum* (Fig. 2*D*) [33] which grows well at pH 1 at 56 °C.

The phospholipids of PS3 were found to consist of 57.6% phosphatidylethanolamine, 19.2% cardiolipin, 12.9% phosphatidylglycerol, and 10.3% unidentified material [31]. The molecular species are mainly of the 1-15-methylhexadecanoyl 2-13-methyltetradecanoyl-*sn*-glycerol-3-phosphoryl type (31.5%) and 1,2-di-13-methyltetradecanoyl-*sn*-glycerol-3-phosphoryl type (24.9%) [24].

These stable phospholipids are useful in reconstitution of liposomes capable of ATP synthesis [25, 51], H^+-transport through F_o [41], and amino acid transport by a carrier [18]. Thus the widely accepted idea that unsaturation of the lipids is essential for fluidity and functioning of biomembranes is not applicable to thermophiles. The possibility of energy transfer through oleic acid and lipoic acid [13] in the membrane is also excluded by the fact that neither was detected in reconstituted H^+-ATPase liposomes [23, 30].

Reconstitution of H^+-ATPase from its Subunits

H^+-ATPase was first extracted from beef heart mitochondria with cholate [27] and later shown to be a universal component of all cells [30]. As described in recent reviews, this ATPase has been found in mitochondria [42], chloroplasts [35, 38], and prokaryotic plasma membranes [5]. H^+-ATPase was shown to be composed of a catalytic portion (F_1) and a membrane portion (F_o), which renders F_1 sensitive to energy transfer inhibitors [26].

Mitchell postulated that H^+-ATPase is responsible for ATP synthesis using energy of H^+-flow through the membrane [36]. In fact, when crude H^+-ATPase was incorporated into liposomes, they accumulated protons at the expense of ATP hydrolysis [28] and were capable of oxidative phosphorylation in the presence of crude electron transport complexes [45].

However, the mitochondrial H^+-ATPase preparations are unstable and impure, even when obtained by recently developed methods [54]: Typical preparations gave 13 bands on electrophoresis [54]. Moreover, mitochondrial and chloroplast F_1's have never been reconstituted from their subunits. The roles of the subunits have been studied by the use of anti-subunit-antibodies, but the results [38] obtained have been different from those obtained by reconstitution of thermophilic F_1 [60] and with antibodies against subunits of thermophilic F_1 [61]. Our conclusions on the structure and functions of each subunit of thermophilic F_1 are summarized in Table 1 [30, 59].

There are only three subunits in thermophilic F_o [29, 31]: an OSCP-like protein (19,000 daltons), F_1-binding proteins (13,500 daltons) and DCCD-binding proteins (7,300 daltons) [47, 50]. Although the subunit composition of F_o of mitochondria is very complex [54] and is still controversial, recent reports on prokaryotic H^+-ATPases suggest that there are also only three subunits in F_o. H^+-ATPases have been obtained from *Escherichea coli* [7, 8], *Streptococcus faecalis* [1], and *Rhodospirillum rubrum* [2], but functioning F_o has not been dissociated from any of them. Thermophilic F_o was reconstituted into liposomes capable of specific H^+-translocation that was inhibited by energy transfer inhibitors such as DCCD [41]. The DCCD-binding protein in both TF_o and F_o was completely sequenced [47] and [^{14}C]-DCCD was found on Glu^{56} of TF_o as shown below:

$$-Gly^{51}-Val-Ala-Leu-Val-Glu^{56}-Ala-Leu-Pro-Ile^{60}-$$

There are a total of 72 amino acid residues in TF_o and 64 of them are hydrophobic. This explains the solubility of the DCCD-binding protein in organic

Table 1. Structures and functions of subunits of TF_1

Subunit	α	β	γ	δ	ε
Mol wt	54,600	51,000	30,200	21,000	16,000
α-Helix content (%)	31	34	49	65	33
β-Sheet content (%)	19	23	4	15	24
Cysteine content (mol/subunit)	1.0	0.0	0.0	0.0	0.0
Net ATP synthesis and H^+-transport required in reconstitution	(+)	(+)	(+)	(+)	(+)
P_i-ATP exchange					
Required in reconstitution	(+)	(+)	(+)	(+)	(+)
Inhibition by each antibody	(+)	(+)	(−)	(−)	(−)
ATP hydrolysis					
Required in reconstitution	(±)[a]	(+)	(±)[a]	(−)	(−)
Inhibition by each antibody	(+)	(+)	(−)	(−)	(−)
Nucleotide binding to isolated pure subunits					
ATP and ADP	(+)	(+)	(−)	(−)	(−)
ITP and IDP	(−)	(+)	(−)	(−)	(−)
CTP	(+)	(−)	(−)	(−)	(−)
H^+-gate activity	(−)	(−)	(+)	(+)	(+)
Direct binding to TF_o	(−)	(−)	(−)	(+)	(+)
N_3-sensitivity	(−)	(−)	(+)	(−)	(−)

[a] Not always required.

solvents and its tight binding to the hydrocarbon region of membranes. When only one of its ionizable residues (4 arginyl, 3 glutamyl, and a tyrosyl) was chemically modified, the H^+-channel activity of F_o was lost [48]. H^+-translocation through F_o may occur by protonation and deprotonation of the polar groups in the DCCD-binding protein [30].

Further information of F_1 was obtained by physical methods. Thermophilic F_1 has been crystallized [31], and computerized image reconstruction of thermophilic F_1 revealed a hexagonal molecular shape [57]. Although there are many hypotheses on the molecular structure of F_1, exact determination of its molecular weight has been hindered by its ready dissociation [42]. Since TF_1 scarcely dissociates even at low temperature, its mol wt could be determined as 380,000 by low speed sedimentation equilibrium method [61]. This value supports a stoichiometry of the subunits of $\alpha_3\beta_3\gamma\delta\varepsilon$ [61].

The ATP binding site of the β subunit in F_1 has the following sequence [6]:

Ile–Met–Asp–Pro–Asn–Ile–Val–Gly–Ser–Glu–
His–Tyr*–Asp–Val–Ala–Arg–,

where Tyr* is the binding site of the ATP analogue. The large negative elipticity of the ATP-β subunit complex at 275 nm may be the result of stacking of protonated tyrosine residues and adenine bases. It is interesting that the tyrosine residue is surrounded by an imidazole, a hydroxyl and two caroxyl groups that may transfer H^+ during the interaction of ADP, P_i and Mg^{2+}. The synthetic nonapeptide corresponding to residues 32 to 40 of adenylate kinase, i.e.,

Tyr–Gly–Tyr–Thr–His–Leu–Ser–Thr–Gly,
also binds Mg-AT(D)P

by a similar tyrosine-adenine interaction [14].

Single Band Cytochrome Oxidase, Cytochrome C and NADH-Dehydrogenase

Mitochondrial cytochrome oxidase is composed of 7 subunits (36,000, 19,000 daltons and 5 others), but none of them has been purified in a reconstitutably active state [34]. The mol wt of this enzyme has been reported to be 220,000 to 530,000, but that determined with electron irradiation was only 67,000 [20]. There is a report that the largest subunit was an ion channel [10]. The electron transport system of the thermophilic bacterium is highly sensitive to CN^-, and its absorption spectrum reveals the presence of cytochromes *a* (605 nm), *b* (562 nm), and *c* (550 nm) in the α-band region just as in mitochondria. Therefore purification of its components has been attempted [49].

First, the substrate of cytochrome oxidase, i.e., cytochrome *c*, was purified [19]. In contrast to mito-

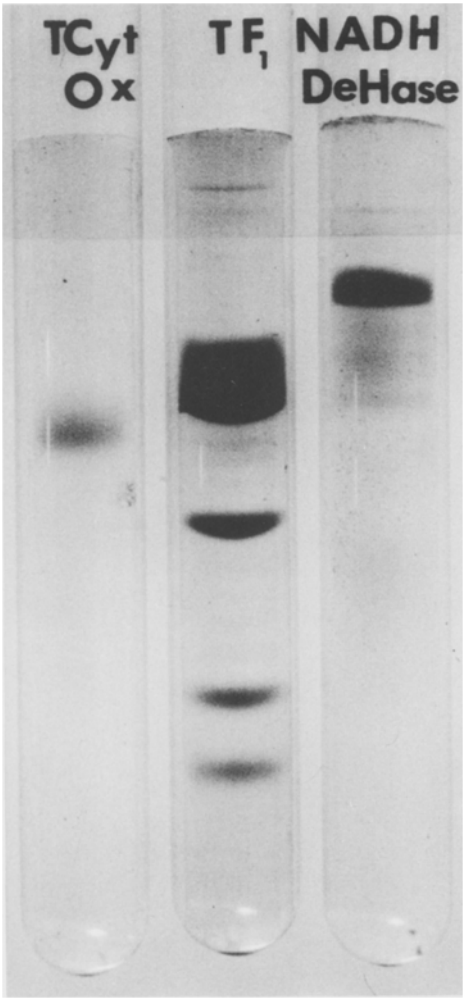

Fig. 3. Subunits of cytochrome oxidase, F_1, and NADH dehydrogenase of thermophilic bacterium PS3. Proteins were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate as described in ref. [61].

chondrial cytochrome *c*, which is basic, PS3 cytochrome *c* has an isoelectric point of pH 5.6. The preparation was homogeneous, and its mol wt was 10,400 as determined by equilibrium sedimentation. The molar extinction coefficients at 550 and 416 nm of the reduced form and at 550 nm of the reduced-minus oxidized difference spectrum were 27.5 and 174.2, and 19.3×10^3 $M^{-1}cm^{-1}$, respectively. Its pyridine ferrohemochrome showed absorption peaks at 415, 521 and 550 nm, indicating that this cytochrome is *c*-type. PS3 cytochrome *c* was oxidized by cytochrome oxidase of PS3, but not by that of beef heart. PS3 cytochrome *c* was denatured at 96 °C in the presence of 2 M guanidine HCl, while horse cytochrome *c* was denatured at 60 °C in the same solution, as judged by loss of elipticity at 222.5 nm [19].

Cytochrome oxidase of PS3 was extracted with Triton-X100 (4%) from the residue obtained by washing with 4 M LiCl-cholate-deoxycholate [49].

The extract was then purified by DEAE-cellulose and hydroxyapatite column chromatographies in the presence of 0.5% Triton X-100, and the greenish-brown eluate was fractionated with ammonium sulfate in the presence of 1.5% sodium cholate, to remove most of Triton X-100.

As shown in Fig. 3, on sodium dodecylsulfate polyacrylamide gel electrophoresis, PS3 cytochrome oxidase gave a single band at a position of 38,000 daltons. The difference spectrum (dithionite reduced minus oxidized) showing absorption peaks at 445 and 604 nm, which were identified as those of heme *a*, and at 418, 521, and 550 nm, which were identified as those of heme *c*. PS3 cytochrome oxidase differs in composition from mitochondrial cytochrome oxidase, which has no covalently bound heme *c*, although it contains tightly bound cytochrome *c* in the membrane. The chemical composition of PS3 cytochrome oxidase is heme *a* 13.5, heme *c* 13.9, instrinsic copper 13.6 nmol, and phospholipids 0.06 mg, per mg protein [49]. Probably, two 38,000-dalton peptides (a heterodimer), contain 1 heme *a*, 1 heme *c*, and 1 Cu [49]. The infrared absorption band appears to reach a maximum at about 790 nm, supporting the fact that PS3 oxidase has only one kind of Cu (B. Chance, *personal communication*). The electron paramagnetic resonance spectrum of PS3 oxidase was similar to that of mitochondrial oxidase (T. Yamano and N. Sone, *unpublished*).

PS3 oxidase was able to generate a membrane potential when reconstituted into liposomes. The potential was monitored with a cyanine dye, the absorption ($\Delta_{630-670}$) of which changes according to the membrane potential [49].

There is strong NADH-oxidase activity in the membrane of PS3 which can drive protons through the membrane [17]. Complex I of mitochondria contains many peptides, but NADH-ferricyanide dehydrogenase of mitochondria is composed of 3 subunits (51,000, 24,000, and 10,000 daltons) [15]. NADH-dehydrogenase (NADH-ferricyanide reductase) of PS3 was extracted from the membranes with a solution of 2% cholate and 1% deoxycholate in 50 mM Tris-sulfate, pH 8.0. After partial dialysis of the solution to precipitate carriers of amino acids and many hydrophobic proteins, it was fractionated with ammonium sulfate, and the active fraction obtained was subjected to column chromatographies on Sepharose 6B, DEAE-cellulose, hydroxylapatite, and then Sepharose 4B. As shown in Fig. 3, a single band of 73,000 daltons was found on sodium dodecylsulfate gel electrophoresis (Y. Kagawa, *to be published*). The

FMN content measured from the light absorption at 450 nm, was 1.0 mole per mole of enzyme.

H^+-Driven Amino Acid Carriers and Na^+-H^+-Antiporter

Several lines of evidence indicate the presence of H^+-driven amino acid carriers [36] and antiporters of ions [56] in bacteria. Although the glutamate carrier was partially purified from mitochondria [11], H^+-driven amino acid carriers have never been isolated. PS3 lives in hot water (pH 7.1, 78 °C, Na^+ 730 ppm, Cl^- 1058 ppm) containing substances that react with ninhydrin in the order of μM, including alanine and glycine, which may be derived from the nearby sea or sedimentary rocks [22]. In fact, alanine and glycine are transported at the expense of energy derived from NADH oxidation [17].

Since alanine carrier in the liposomes is separated from the energy supply system, the carrier reconstituted into liposome requires energy artificially supplied by an ion gradient of K^+ across the liposomes, which is started by the addition of valinomycin [16]. The carrier was extracted from the membranes with 2% cholate/1% deoxycholate in 0.2 M Na_2SO_4 at pH 8.0. The supernatant fraction ($140{,}000 \times g$, 1 hr) was dialyzed, and the resulting precipitate was extracted with 2% Triton. The extract was further purified by column chromatographies on DEAE cellulose and CM-cellulose in the presence of 0.2% Triton X100 and 4 M urea. The final preparation gave two major protein bands with mol wt of 36,000 and 9,400 [18], by gel electrophoresis in the presence of dodecylsulfate. This carrier also translocates glycine, but not the other amino acid tested, and it is driven by H^+, Na^+ and Li^+, but not by K^+, NH_4^+ or choline [18]. The K_m for alanine was 10 μM with H^+ and 14 μM with Na^+. The initial velocity of alanine transport was 20 nmol per mg of carrier per min with either a Na^+ or H^+ gradient. Na^+-driven transport of purified carrier was inhibited by Na^+ ionophores such as monensin but not by H^+-carriers such as FCCP. Membrane permeant anions, including tetraphenylboron and NO_3^-, enhanced alanine uptake driven by Na^+, since this alanine-Na^+-cotransport accumulated positive charges inside the reconstituted liposomes.

Since amino acids are cotransported with Na^+ in Na^+-rich medium, there must be either a Na^+ pump or a Na^+-H^+ antiporter, depending on the H^+-translocating NADH-oxidation system [12]. In fact, the addition of Na^+ to membrane vesicles of PS3 caused rapid decrease in the pH of the medium. NADH oxidation by inside-out vesicles caused rapid uptake of ^{22}Na (40 pmol/mg/min). This Na^+-H^+ antiporter was extracted with a mixture of cholate and deoxycholate. The activity of the antiporter was measured with a pH meter, after reconstituting each fraction into proteoliposomes [12]. The K_m of this antiport for Na^+ at pH 8.5 was 1.4 mM. Further studies are still necessary to characterize this antiporter.

Reconstitution of Vectorial Processes

As described in the previous sections, we purified the major components of four systems of the chemiosmotic theory as stable proteins and lipids. To achieve five purposes described in the introduction, we then reconstituted these components into proteoliposomes that showed vectorial reactions [23, 30].

1) In the absence of contaminating peptides, transport activities of H^+-ATPase, cytochrome oxidase and alanine carrier were confirmed. The electrochemical potential difference across the membrane ($\Delta\bar{\mu}H^+$) [32, 37] obtained in $TF_o \cdot F_1$ liposomes was as high as 253 mV [53]. If TF_1 portion was removed from them, the resulting TF_o-liposomes showed an electrophoretic translocation of H^+, which was specifically blocked with energy transfer inhibitors such as DCCD [41]. The single band cytochrome oxidase-liposomes showed some indication of H^+-pumping activity, in contrast to the original half-loop role of the cytochrome oxidase [36]. This extra-proton translocation may change the classical $2H^+/2e^-$/site stoichiometry of oxidative phosphorylation, and, in fact, much higher values have been reported [4]. However, these experiments were performed with intact mitochondria or complicated proteins.

2) Energy transfer by conformational change of connected proteins [3] or local electrochemical change [3] in the membrane were shown to be not necessary in the ATP synthesis and solute transports, because $\Delta\bar{\mu}H^+$ between two bulk water phases on both sides of the membrane [37] could drive these reactions. Since TF_1, TF_o and PS3 lipids were stable during drastic treatments, direct ATP synthesis by flow of H^+ through H^+-ATPase could be demonstrated in the proteoliposomes by imposing an artificial acid- ($\Delta pH = 2.38$) and KCl- ($\Delta\psi = 125$ mV) gradient [51], or by applying an external electric field (1000 V/cm) [46]. For ATP synthesis a $\Delta\bar{\mu}$-H^+ of 200 mV, irrespective of its component $\Delta\psi$ and ΔpH, was necessary [51]. The maximum level of ATP synthesis was about 100 nmol per mg H^+-ATPase in the reconstituted liposomes, but less than 2.5 nmol per mg protein was synthesized in submitochondrial particles [55] and bacterial membranes [56]. ATP synthesis occurred at a rate of 650 nmol per mg H^+-ATPase per min, which is faster than the rate of substrate oxidation in the original membranes, thus primary role of H^+-ATPase in ATP synthesis was established.

3) Subunits of TF_1 and TF_o were isolated in an

active state and their roles and physicochemical properties were determined as shown in Table 1 and the previous sections. *CD* and *IR* spectra of subunits of TF_1 revealed that the isolated α and β subunits bound AT(D)P, and their relaxation spectrum in the presence of ^2H_2O showed their conformational changes. The antibodies against subunits of TF_1 clarified the controversial functions of subunits of F_1 which are easily denatured [38] (Table 1). Crystallographic analysis of TF_1 [57] is in progress. Single-band cytochrome oxidase and NADH-dehydrogenase changed the complicated ideas on the structure of electron carriers [49]. Details of their structures are now analyzed by several new methods including K-edge analysis of Cu with synchrotron. The elucidation of the molecular structure of these subunits are essential for the study of molecular events during the vectorial processes.

4) Stepwise restoration of higher functions was observed when subunits of TF_1 were reorganized into complexes [23]. The conformational interaction between α and β subunits was examined by reconstituting deuterated subunits (α^* and β^*) into hybrid complexes ($\alpha^*\beta$ and $\alpha\beta^*$) which had no ATPase activity. The results showed that α subunit rendered the conformation of β subunit tight only when ATP was present. Another new approach of is reconstitution of subunits of TF_1 on TF_o-liposomes. The F_1-binding protein of TF_o [52] bound δ and ε subunits. When the γ subunit was added to the $\delta\varepsilon$-TF_o complex, leakage of H^+ through TF_o was blocked. This activity of the $\gamma\delta\varepsilon$-complex is a H^+-gate function [54] (Table 1).

5) Finally, when these oligomers were reconstituted into proteoliposomes, energy-transducing reactions could be observed. Addition of the catalytic site of TF_1, the $\alpha\beta\gamma$-complex, to the $\delta\varepsilon$-TF_o-liposomes completely restored the original H^+-ATPase activity both in the direction of ATP synthesis and in H^+-accumulation [30]. In contrast to the kinetic analysis of chloroplasts with light pulse [58], the time-resolution of above described H^+-transport studies has been insufficient. However, time resolution studies on ATP synthesis with external electric field has been developed [46]. As calculated by the Laplace equation of the electric field (1,000 V/cm) around the liposomes, to create a membrane potential of 200 mV, the diameter of the liposomes should be larger than 5.3 μm. Experiments with macroliposomes of this size are now in progress.

Because of their stability and simplicity, these thermophilic membrane components will be useful in studies on bioenergetics and biology of membranes.

This work was supported by grants from the Ministry of Education of Japan (grants # 311909 and 412011).

References

1. Babakov, A.V., Vasilov, R.G. 1979. Mg^{2+}-dependent adenosine triphosphatase from *Streptococcus faecalis* membrane. I. Isolation and subunit composition. *Biorg. Khimiya* **5**:119
2. Bengis-Garber, C., Gromet-Elhanan, Z. 1979. Purification of the energy-transducing adenosine triphosphatase complex from *Rhodospirillum rubrum*. *Biochemistry* **18**:3577
3. Boyer, P.D., Chance, B., Ernster, L., Mitchell, P., Racker, E., Slater, E.C. 1977. Oxidative phosphorylation and photophosphorylation. *Annu. Rev. Biochem.* **46**:955
4. Brand, M.D., Lehninger, A.L. 1977. H^+/ATP ratio during ATP hydrolysis by mitochondria: Modification of the chemiosmotic theory. *Proc. Nat. Acad. Sci. USA* **74**:1955
5. Downie, J.A., Gibson, F., Cox, G.R. 1979. Membrane adenosine triphosphatases of prokaryotic cells. *Annu. Rev. Biochem.* **48**:103
6. Esch, S.F., Allison, W.S. 1978. Identification of a tyrosine residue at a nucleotide binding site in the β subunit of a mitochondrial ATPase with *p*-fluorosulfonyl-[^{14}C]-benzoyl-5′-adenosine. *J. Biol. Chem.* **253**:6100
7. Foster, D.L., Fillingame, R.H. 1979. Energy-transducing H^+-ATPase of *Escherichia coli;* Purification, reconstitution, and subunit composition. *J. Biol. Chem.* **254**:8230
8. Friedle, P., Friedl, C., Schairer, H.U. 1979. The ATP synthetase of *Escherichia coli* K12: Purification of the enzyme and reconstitution of energy-transducing activities. *Eur. J. Biochem.* **100**:175
9. Friedman, S.M. (editor). 1978. Biochemistry of Thermophily. Academic Press, New York
10. Fry, M., Green, D.E. 1979. Ion-channel component of cytochrome oxidase. *Proc. Nat. Acad. Sci. USA* **76**:2664
11. Gautheron, D.C., Julliard, J.H. 1979. Isolation of glutamate carrier system from pig heart mitochondria and incorporation into liposomes. *In:* Methods in Enzymology. S. Fleischer and L. Packer, editors. Vol. 56, p. 430. Academic Press, New York
12. Goto, K., Hirata, H., Kagawa, Y. 1979. Na^+-H^+ antiport and substrate transport in thermophilic bacterium PS3. *Seikagaku (abstr.)* **51**:896
13. Griffith, D.E., Hyamus, R.L., Bertoli, E. 1977. Studies of energy linked reactions: Dihydrolipoate- and oleate-dependent ATP synthesis in yeast promitochondria. *FEBS Lett.* **74**:38
14. Hamada, M., Palmieri, R.H., Russell, G.A., Kuby, S.A. 1979. Studies on adenosine triphosphate transphosphorylases XIV. Equilibrium binding properties of the crystalline rabbit and calf muscle ATP–AMP transphosphorylase (adenylate kinase) and derived peptide fragments. *Arch. Biochem. Biophys.* **195**:155
15. Hatefi, Y., Galante, Y.M., Figeri, L., Stiggall, D.L. 1978. Structure and composition of the mitochondrial energy transduction system. *In:* Structure and Function of Biomembrane. K. Yagi, editor. p. 167. Japan Scientific Society Press, Tokyo
16. Hirata, H. 1979. Solubilization and purification of alanine carrier from thermophilic bacteria and reconstitution into vesicles capable of transport. *Methods Enzymol.* **56**:430
17. Hirata, H. 1979. Characterization of alanine transport by reconstituted proteoliposomes. *In:* Functions and Molecular Aspects of Biomembranes. E. Quagliariello, editor. p. 505. Elsevier, Amsterdam
18. Hirata, H., Sone, N., Yoshida, M., Kagawa, Y. 1977. Isolation of the alanine carrier from the membranes of a thermophilic bacterium and its reconstitution into vesicles capable of transport. *J. Supramol. Struct.* **6**:77
19. Hon-nami, K., Ohshima, T., Kihara, H., Kagawa, Y. 1979. Cytochrome *c*-550 from a thermophilic bacterium PS3. *Biochem. Biophys. Res. Commun.* **87**:1066
20. Kagawa, Y. 1967. Target size of components in oxiative phosphorylation. Studies with a linear accelerator. *Biochim. Biophys. Acta* **131**:586

21. Kagawa, Y. 1972. Reconstitution of oxidative phosphorylation. *Biochim. Biophys. Acta* **265**:297
22. Kagawa, Y. 1976. Transport activity of proteoliposomes reconstituted from crystalline ATPase or from solubilized alanine carrier. *J. Cell. Physiol.* **89**:569
23. Kagawa, Y. 1978. Reconstitution of the energy transformer, gate and channel: subunit reassembly, crystalline ATPase and ATP synthesis. *Biochim. Biophys. Acta* **505**:45
24. Kagawa, Y., Ariga, T. 1977. Determination of molecular species of phospholipids of thermophilic bacterium PS3 using mass-chromatography. *J. Biochem. (Tokyo)* **81**:1161
25. Kagawa, Y., Ohno, K., Yoshida, M., Takeuchi, Y., Sone, N. 1977. Proton translocation by ATPase and bacteriorhodopsin. *Fed. Proc.* **36**:1815
26. Kagawa, Y., Racker, E. 1966. Partial resolution of the enzymes catalyzing oxidative phosphorylation: VIII. Properties of a factor conferring oligomycin sensitivity on mitochondrial adenocatalyzing oxidative phosphorylation: IX. Reconstitution of oligomycin-sensitive adenosine triphosphatase. *J. Biol. Chem.* **241**:2467
28. Kagawa, Y., Racker, E. 1971. Partial resolution of the enzymes catalyzing oxidative phosphorylation: XXV. Reconstitution of vesicles catalyzing ^{32}Pi-adenosine triphosphate exchange. *J. Biol. Chem.* **246**:5477
29. Kagawa, Y., Sone, N. 1979. DCCD-sensitive ATPase ($TF_o \cdot F_1$) from a thermophilic bacterium: Purification, dissociation into functional subunits, and reconstitution into vesicles capable of energy translocation. *Methods Enzymol.* **55**:364
30. Kagawa, Y., Sone, N., Hirata, H., Yoshida, M. 1979. Structure and function of H^+-ATPase. *J. Bioenerg. Biomembr.* **11**:39
31. Kagawa, Y., Sone, N., Yoshida, M., Hirata, H., Okamoto, H. 1976. Proton translocating ATPase of a thermophilic bacterium: Morphology, subunits and chemical composition. *J. Biochem (Tokyo)* **80**:141
32. Kozlov, I.A., Skulachev, V.P. 1977. H^+-adenosine triphosphatase and membrane energy coupling. *Biochim. Biophys. Acta* **465**:29
33. Langworthy, T.A. 1977. Long chain diglycerol tetraethers from *Thermoplasma acidophilum*. *Biochim. Biophys. Acta* **487**:37
34. Ludwig, B., Downer, N.W., Capaldi, R.A. 1979. Labeling of cytochrome *c* oxidase with [^{35}S] diazobenzene sulfonate. Orientation of this electron transfer complex in the inner mitochondrial membrane. *Biochemistry* **18**:1401
35. McCarty, R.E. 1979. Roles of a coupling factor for photophosphorylation in chloroplasts. *Annu. Rev. Plant Physiol.* **30**:79
36. Mitchell, P. 1966. Chemiosmotic coupling in oxidative phosphorylation. *Biol. Rev.* **41**:455
37. Mitchell, P. 1976. Vectorial chemistry and molecular mechanics of chemiosmotic coupling. Power transmission by proticity. *Biochem. Soc. Trans.* **4**:399
38. Nelson, N. 1976. Structure and function of chloroplast ATPase. *Biochim. Biophys. Acta* **456**:314
39. Ohshima, M., Ariga, T. 1975. ω-cyclohexyl fatty acids in acidophilic thermophilic bacteria. Studies on their presence, structure, and biosynthesis using precursors labeled with stable isotopes and radioisotopes. *J. Biol. Chem.* **250**:6963
40. Ohta, S., Nakanishi, M., Tsuboi, M., Yoshida, M., Kagawa, Y. 1978. Kinetics of hydrogen-deuterium exchange in ATPase from a thermophilic bacterium PS3. *Biochem. Biophys. Res. Commun.* **80**:929
41. Okamoto, H., Sone, N., Hirata, H., Yoshida, M., Kagawa, Y. 1977. Purified proton conductor in proton translocating adenosine triphosphatase of a thermophilic bacterium. *J. Biol. Chem.* **252**:6125
42. Penefsky, H.S. 1979. Mitochondrial ATPase. *Adv. Enzymol.* **49**:223
43. Perutz, M.E., Raidt, H. 1975. Stereochemical basis of heat stability in bacterial ferredoxins and haemoglobin A_2. *Nature (London)* **255**:256
44. Pullman, M.E., Penefsky, H.S., Datta, A., Racker, E. 1960. Partial resolution of the enzymes catalyzing oxidative phosphorylation. I. Purification and properties of soluble dinitrophenol stimulated adenosine triphosphatase. *J. Biol. Chem.* **235**:3322
45. Racker, E. 1976. A New Look at Mechanisms in Bioenergetics. Academic Press, New York
46. Rögner, M., Ohno, K., Hamamoto, T., Sone, N., Kagawa, Y. 1979. Net ATP synthesis in H^+-ATPase macroliposomes by an external electric field. *Biochem. Biophys. Res. Commun.* **91**:362
47. Sebald, W., Hoppe, J., Wachter, E. 1979. Amino acid sequence of the ATPase proteolipid from mitochondria, chloroplasts and bacteria (wild type and mutant). *In:* Function and Molecular Aspects of Biomembrane Transport. E. Quagliariello, editor. p. 63. Elsevier, Amsterdam
48. Sone, N., Ikeba, K., Kagawa, Y. 1979. Inhibition of proton conduction by chemical modification of the membrane moiety of proton translocating ATPase. *FEBS Lett.* **97**:61
49. Sone, N., Ohyama, T., Kagawa, Y. 1979. Thermostable single-band cytochrome oxidase. *FEBS Lett.* **106**:39
50. Sone, N., Yoshida, M., Hirata, H., Kagawa, Y. 1975. Purification and properties of a dicyclohexyl-carbodiimide sensitive adenosine triphosphatase from a thermophilic bacterium. *J. Biol. Chem.* **250**:7917
51. Sone, N., Yoshida, M., Hirata, H., Kagawa, Y. 1977. Adenosine triphosphatase synthesis by an electrochemical proton gradient in vesicles reconstituted from purified adenosine triphosphatase and phospholipids of a thermophilic bacterium. *J. Biol. Chem.* **252**:2956
52. Sone, N., Yoshida, M., Hirata, H., Kagawa, Y. 1978. Resolution of the membrane moiety of the H^+-ATPase complex into two kinds of subunits. *Proc. Nat. Acad. Sci. USA* **75**:4219
53. Sone, N., Yoshida, M., Hirata, H., Okamoto, H., Kagawa, Y. 1976. Electrochemical potential of protons in vesicles reconstituted from purified, proton translocating adenosine triphosphatase. *J. Membrane Biol.* **30**:121
54. Stiggall, D.L., Galante, Y.M., Hatefi, Y. 1978. Preparation and properties of an ATP-P_i exchange complex (Complex V) from bovine heart mitochondria. *J. Biol. Chem.* **253**:956
55. Thayer, W.P., Hinkle, P.C. 1975. Kinetics of adenosine triphosphate synthesis in bovine heart submitochondrial particles. *J. Biol. Chem.* **250**:5336
56. Tsuchiya, T., Rosen, B.P. 1976. Adenosine 5′-triphosphate synthesis energized by an artificially imposed membrane potential in membrane vesicles of *Escherichia coli*. *J. Bacteriol.* **127**:154
57. Wakabayashi, T., Kubota, M., Yoshida, M., Kagawa, Y. 1977. Structure of ATPase (coupling factor TF_1) from a thermophilic bacterium. J. Mol. Biol. **117**:515
58. Witt, H.T. 1979. Energy conversion in the functional membrane of photosynthesis. Analysis by light pulse and electric pulse methods. The central role of the electric field. *Biochim. Biophys. Acta* **505**:355
59. Yoshida, M., Okamoto, H., Sone, N., Hirata, H., Kagawa, Y. 1977. Reconstitution of thermostable ATPase capable of energy coupling from its purified subunits. *Proc. Nat. Acad. Sci. USA* **74**:936
60. Yoshida, M., Sone, N., Hirata, H., Kagawa, Y. 1977. Reconstitution of adenosine triphosphatase of thermophilic bacterium from purified individual subunits. *J. Biol. Chem.* **252**:3480
61. Yoshida, M., Sone, N., Hirata, H., Kagawa, Y., Ui, N. 1979. Subunit structure of adenosine triphosphatase: Comparison of the structure in thermophilic bacterium PS3 with those in mitochondria, chloroplasts and *Escherichia coli*. *J. Biol. Chem.* **254**:9525
62. Zuber, H. 1978. Comparative studies of thermophilic and mesophilic enzymes: Objectives, problems, results. *In:* Biochemistry of Thermophily. S.M. Friedman, editor. p. 267. Academic Press, New York