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Electrochemical Potential of Protons in Vesicles Reconstituted from Purified, Proton-Translocating Adenosine Triphosphatase

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Summary. Measurements were made of the difference in the electrochemical potential of protons ($\Delta\bar{\mu}H^+$) across the membrane of vesicles reconstituted from the ATPase complex ($TF_0 \cdot F_1$) purified from a thermophilic bacterium and P-lipids. Two fluorescent dyes, anilinonaphthalene sulfonate (ANS) and 9-aminoacridine (9AA) were used as probes for measuring the membrane potential ($\Delta\Psi$) and pH difference across the membrane (Δ pH), respectively.

In the presence of Tris buffer the maximal $\Delta\Psi$ and no Δ pH were produced, while in the presence of the permeant anion NO_3^- the maximal Δ pH and a low $\Delta\Psi$ were produced by the addition of ATP. When the ATP concentration was 0.24 mM, the $\Delta\Psi$ was 140–150 mV (positive inside) in Tris buffer, and the Δ pH was 2.9–3.5 units (acidic inside) in the presence of NO_3^-. Addition of a saturating amount of ATP produced somewhat larger $\Delta\Psi$ and Δ pH values, and the $\Delta\bar{\mu}H^+$ attained was about 310 mV.

By trapping pH indicators in the vesicles during their reconstitution it was found that the pH inside the vesicles was pH 4–5 during ATP hydrolysis.

The effects of energy transfer inhibitors, uncouplers, ionophores, and permeant anions on these vesicles were studied.

Vesicles capable of energy transfer reactions, such as ^{32}Pi-ATP exchange [9], ANS fluorescence enhancement [9], and proton accumulation [7, 8] have been reconstituted from crude mitochondrial ATPase complex and phospholipids. According to the chemiosmotic theory of oxidative phosphorylation, the difference in the electrochemical potential of protons ($\Delta\bar{\mu}H^+$) across the membrane is generated by substrate oxidation, and the flow of protons driven by this potential through an "anisotropic ATPase" results in synthesis of ATP [11]. To date, no quantitative data on $\Delta\bar{\mu}H^+$ of the reconstituted vesicles have been

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reported and no such ATPase has been purified. Thus, it is important to determine $\Delta\bar{\mu}H^+$, which is the sum of the membrane potential ($\Delta\psi$) and pH difference ($Z\Delta$ pH) across the membrane of reconstituted vesicles containing only pure ATPase complex and phospholipids.

Recently a stable ATPase complex ($TF_0 \cdot F_1$) was purified from a thermophilic aerobic bacterium PS3 [16], and vesicles capable of energy transfer reactions were reconstituted from P-lipids and $TF_0 \cdot F_1$ [16]. This paper is on the measurement of $\Delta\psi$ and Δ pH in these vesicles. Negatively charged 8-anilinonaphthalene-1-sulfonate (ANS) was used to measure $\Delta\psi$, because limited intramolecular rotation of ANS attached to the membranes results in enhancement of fluorescence intensity [1, 3, 6]. The weak base 9-aminoacridine (9AA) was used to measure Δ pH, because on progressive accumulation of 9AA inside the vesicles with decrease in pH, the 9AA shows self-quenching of its fluorescence [4, 13–15].

Materials and Methods

Materials

The ATPase complex $TF_0 \cdot F_1$ from the thermophilic bacterium PS3, P-lipid fraction from PS3 and soybean P-lipids were obtained as described in the preceding report [16]. Nigericin and monensin were kindly donated by Dr. J.W. Chamberlin of Lilly Research Laboratories, Indianapolis, and SF-6847 (3,5-di-tert-butyl-4-hydroxy benzylidenemalononitrile) by Dr. Y. Anraku of the University of Tokyo. Valinomycin was purchased from P-L Biochemicals, Inc., FCCP (carbonylcyanide *p*-trifluoromethoxy-phenylhydrazone) from Boehringer Mannheim, ANS from Eastman, 9AA and DCCD(N, N′-dicyclohexylcarbodiimide) from Nakari Chemicals Ltd., Kyoto, and Bt_3SnCl (tri-*n*-butyltin chloride) from Wako Pure Chemical Co., Osaka.

Reconstitution of Active Vesicles

Vesicles were reconstituted by the standard method described in the previous paper [16], except that the content of the dialysis bag (0.5 ml) was composed of dithiothreitol (2.5 μmoles) and EDTA (0.1 μmole), $TF_0 \cdot F_1$ (0.2–0.4 mg), P-lipids (20 mg), sodium cholate (8 mg), sodium deoxycholate (4 mg) and Tricine-NaOH buffer, pH 8.0 (5 μmoles). In some experiments, vesicles were reconstituted by sonication. For this, a suspension of 40 mg of soybean P-lipids in 4 ml of 0.1 M sucrose containing 10 mM Tris-sulfate buffer, pH 8.0, 1 mM dithiothreitol and 0.2 mM EDTA was subjected to sonic oscillation in a Tomy probe sonic oscillator, model UR-150P, at 20 kHz and 150 W in an ice bath for 5 min.

Measurement of ANS and 9AA Fluorescence

ANS Fluorescence was measured in a Hitachi fluorometer, model 204, using an excitation wave length of 365 nm and measuring emission at 480 nm at 45 °C, described previously [16]. The reconstituted vesicles (0.05 ml, containing 15–30 μg protein) were placed in a cuvette containing 2.0 ml of 50 mM Tris-sulfate, pH 7.6, 2 mM $MgSO_4$, and 20 μg of ANS at 45 °C.

After 10–15 min the reaction was started by adding a mixture of 0.5 μmole of ATP and 0.25 μmole of $MgSO_4$. The enhanced fluorescence due to addition of 0.5 μmole ATP (ΔF) was calculated as a fraction of the total fluorescence (F). Fluorescence of aminoacridine was measured in the same way, except that 365 and 451 nm were chosen as excitation and emission wave-lengths, respectively. The reconstituted vesicles (0.05–0.1 ml, containing 0.02–0.06 mg protein) were placed in a cuvette containing 2.0 ml of 20 mM Tricine-NaOH, pH 7.7 at 45 °C, 50 mM $NaNO_3$, 2 mM $MgSO_4$, and 4 μM 9AA.

Other Analytical Methods

The absorbance due to the presence of pH indicator was measured in Hitachi Dual beam spectrophotometer, model 356, at 45 °C. Wavelength-pairs of 575–610 nm, 590–625 nm and 588–620 nm were employed for chlorphenol red, bromphenol blue and bromcresol purple, respectively. ATP hydrolysis, protein and lipid phosphorus were assayed as described previously [16]. The dextran trapping volume of vesicles was measured by counting the radioactivity of [^{14}C]dextran retained in vesicles which had been prepared in the presence of [^{14}C]dextran (0.1 μCi, 0.25 mg) and then washed twice with 8 ml of fresh dialyzing medium by centrifugation at 198,000 × g for 30 min [9]. Radioactivity was counted in Bray's solution using an Aloka liquid scintillation counter, model LSC-653. The radioactivity due to adsorption was estimated by adding [^{14}C]dextran after dialysis and the value (below 0.2% of the total count) was subtracted from the results.

Results and Discussion

Conditions for Reconstitution of Active Vesicles

Fig. 1 shows tracings of the enhancement of ANS fluorescence induced by addition of ATP to vesicles reconstituted from purified $TF_0 \cdot F_1$ and P-lipids under various conditions. The membrane potential (positive inside) created by ATP hydrolysis attracts negatively charged ANS, and this results in increased fluorescence [6]. The vesicles reconstituted from either P-lipids of the thermophilic bacterium PS3 (trace *A*) or of soybean (trace *B*) showed greatly enhanced fluorescence on addition of ATP. When deoxycholate (trace *C*) or cholate (trace *D*) was omitted during the reconstitution procedures, the resulting vesicles showed little enhancement of fluorescence. Omission of Mg^{2+} from the dialysis buffer had a similar effect (trace *E*). The vesicles prepared using an alternative sonication method were far less active (trace *F*) than those prepared by dialysis under the standard conditions.

ANS as a Probe for Measuring Membrane Potential

Fig. 2 shows the relationship between the concentration of Tris in the assay medium and the enhancement of ANS fluorescence. The stim-

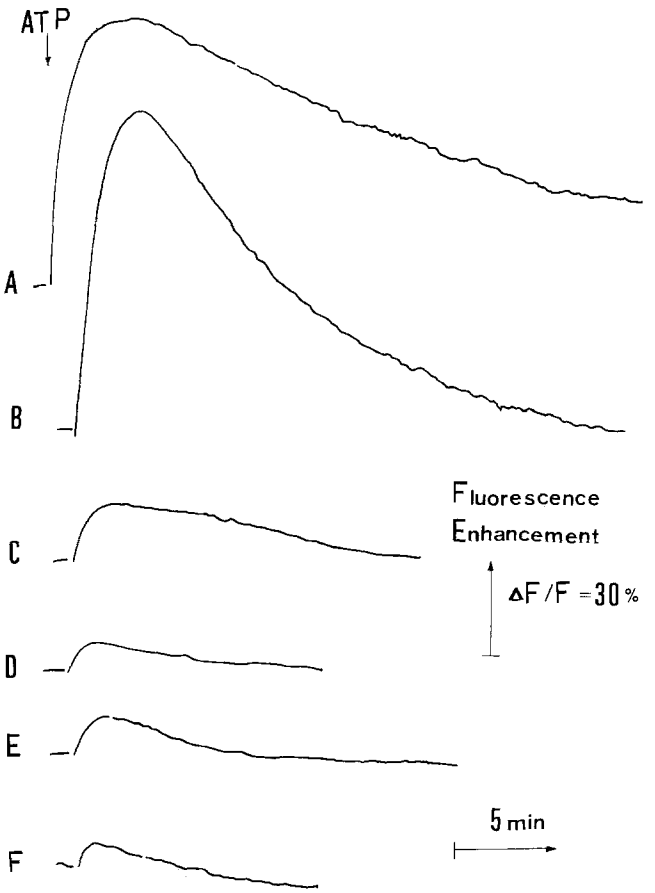

Fig. 1. Enhancement of ANS fluorescence on addition of ATP to the reconstituted vesicles. The vesicles were reconstituted from $TF_0 \cdot F_1$ (0.62 mg protein) and the P-lipids mixture under indicated conditions. The assay method and the standard reconstitution method were as described under *Materials and Methods*. *A*, PS3 P-lipids, standard; *B*, soybean P-lipids, standard; *C*, soybean P-lipids, standard minus deoxycholate; *D*, soybean P-lipids, standard minus cholate; *E*, soybean P-lipids, standard minus $MgSO_4$; *F*, vesicles prepared by sonication

Fig. 2. Effect of concentration of Tris on ANS fluorescence enhancement. The relative enhancement of ANS fluorescence on addition of ATP ($\Delta F/F$) was measured as in Fig. 1 (*A*) except that the indicated amount of Tris-sulfate at pH 7.7 was added to the Tricine-NaOH buffer and $TF_0 \cdot F_1$ used in the 0.5 ml of the reconstitution mixture was 0.25 mg

Fig. 3. Effects of uncouplers, inhibitors, permeant anions and ionophores on ANS fluorescence enhancement. Experimental conditions were as for Fig. 1 (*B*) ($TF_0 \cdot F_1$, 0.31 mg) in 50 mM Tris-sulfate, pH 7.6, 2 mM $MgSO_4$ and 10 μg/ml ANS. Additions were as follows: ATP (0.50 μmole containing 0.25 μmole of $MgSO_4$), SF-6847 (1.4 nmoles), $(Bt)_3$ SnCl (8 nmoles), $NaNO_3$ (0.10 nmole), valinomycin (0.5 μg), KCl (40 μmoles), nigericin (0.4 μg)

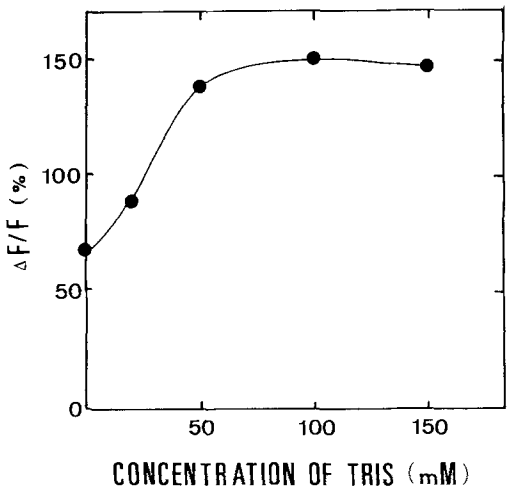
150
100
50
0
ΔF/F (%)
50
100
150
CONCENTRATION OF TRIS (mM)

Fig. 2

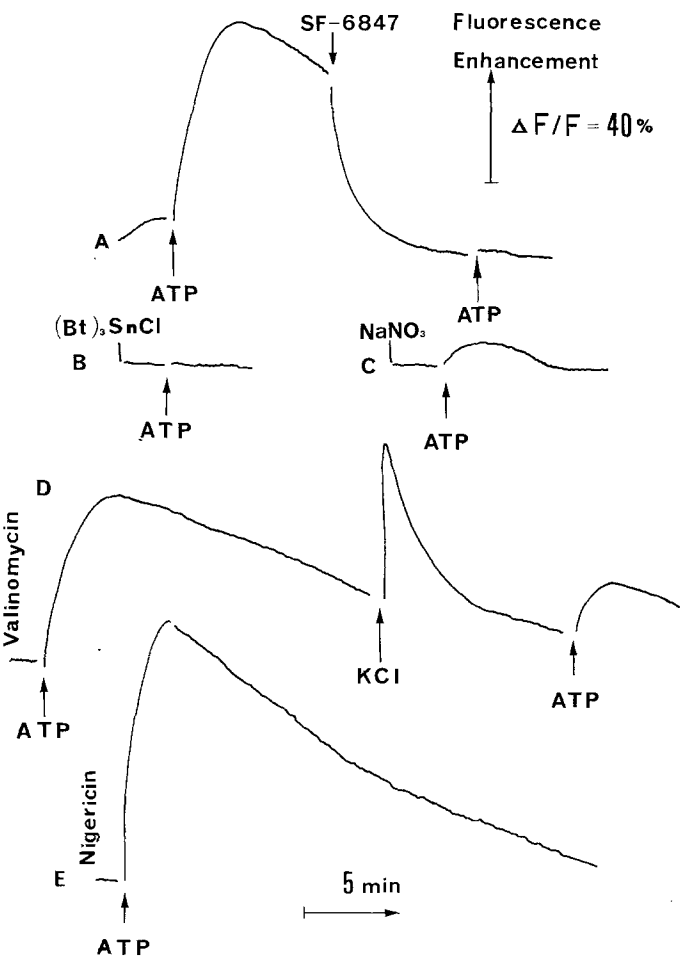
SF-6847
Fluorescence
Enhancement
ΔF/F = 40%
A
ATP
ATP
(Bt)₃SnCl
B
ATP
NaNO₃
C
ATP
D
Valinomycin
ATP
KCl
ATP
Nigericin
E
ATP
5 min

Fig. 3

ulating effect of Tris is probably due to the following reaction which dissipates free H^+ without altering the net charge and which may occur inside the vesicles: $Tris + H^+ = Tris\,H^+$. The membrane may be permeable to Tris in uncharged form and impermeable to its charged form so that the response is larger. Assay mixture of pH 7.6 was used because preliminary experiments showed that this was the optimal pH for enhancement of ANS fluorescence.

Under these conditions effects of biochemical reagents on the enhancement of ANS fluorescence due to ATP were examined (Fig. 3). The ATP-dependent enhancement of ANS fluorescence was rapidly lost on addition of an uncoupler, such as FCCP [16]. Another uncoupler SF-6847 had a similar effect (trace *A*). In the presence of energy transfer inhibitors, such as DCCD [16] and Bt_3SnCl (trace *B*), the ATP-dependent enhancement of ANS fluorescence was totally abolished. The enhancement was also greatly suppressed by the ionophorous antibiotic valinomycin in the presence of K^+ (trace *D*) and by membrane-permeant anions, such as NO_3^- (trace *C*) and ClO_4^- (not shown). On the contrary, nigericin and monensin, which both catalyze electrically neutral exchange of H^+/K^+ or Na^+, respectively, failed to inhibit the reaction (trace *E*). These results qualitatively satisfy the postulated modes of action of these uncouplers and ionophores [5].

Enhancement of ANS Fluorescence Caused by K^+ Inflow

Fig. 3*D* also shows the enhancement of ANS fluorescence introduced by addition of valinomycin and K^+. Since valinomycin catalyzes an electrogenic diffusion of K^+ across the membrane, the magnitude of ANS fluorescence enhancement may be dependent on the external concentration of K^+.

Results obtained with vesicles reconstituted from $TF_0 \cdot F_1$ and soybean P-lipids are shown in Fig. 4, where the relative fluorescence change ($\Delta F/F$) is plotted against the KCl concentration of the external medium. The linear relationship indicates that $\Delta F/F$ is proportional to the diffusion potential across the membrane. The diffusion potential of K^+ mediated by valinomycin may be calculated by the following Nernst's equation:

$$\Delta\psi = RT \ln (K^+)\,o/(K^+)\,i \qquad (1)$$

where $(K^+)\,i$ and $(K^+)\,o$ are the concentrations of K^+ inside and outside the vesicles, respectively. The slope of the line differed for different prep-

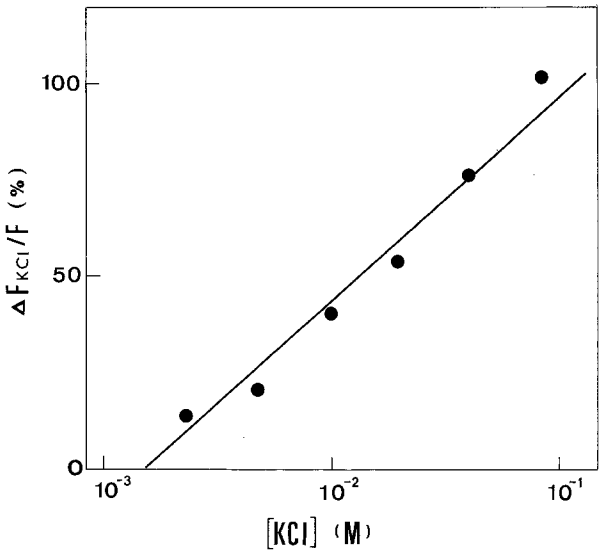

Fig. 4. Plot of the relative enhancement of ANS fluorescence ($\Delta F/F$) against the KCl concentration. Vesicles (0.05 ml), reconstituted as described in the legend for Fig. 1*B* were placed in a cuvette containing 2.0 ml of 50 mM Tris · H_2SO_4 (pH 7.6) with 2 mM $MgSO_4$, 20 μg ANS and 0.2 μg valinomycin. After 10–20 min preincubation 5–40 μl of KCl solution (1–4 M) were added. ΔF_{KCl} was measured as the increase in ANS fluorescence on KCl addition extrapolated to zero time

arations and at higher concentrations of KCl, $\Delta F/F$ tended to deviate from the linear relationship, so the calibration must be made for each preparation.

Probe for Measuring Δ pH

Fluorescent amines, such as 9AA, are known to respond to pH difference (Δ pH) across the the membrane [4, 15]. When a low concentration of 9AA is used and the external pH is below 9, Δ pH can be calculated by the following equation [13, 15]:

$$\Delta \text{ pH} = \log Q/(1-Q) + \log 1/V \tag{2}$$

where Q is the fraction of the total fluorescence which is quenched in response to ATP addition, and V is the volume of the osmotic compartment as a fraction of the total volume.

Fig. 5 shows tracings of typical experiments. The addition of ATP induced quenching of 9AA fluorescence (trace *A*), which was enhanced by the presence of $NaNO_3$. The effect of the permeant anion, NO_3^- on

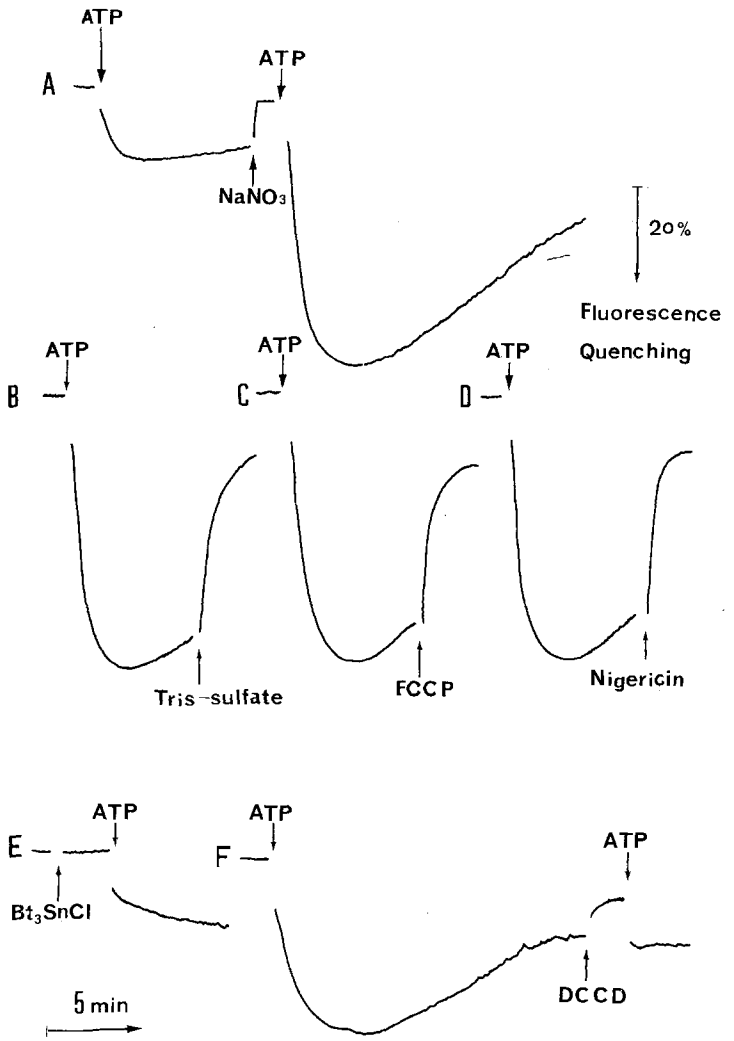

Fig. 5. Quenching of 9-aminoacridine fluorescence. Vesicles were reconstituted from PS3 P-lipid mixture containing $TF_0 \cdot F_1$ (0.25 mg protein). Other conditions were as described under *Materials and Methods*, except that $NaNO_3$ was added later as indicated (*A*) or replaced by valinomycin (0.5 μg) and KCl (20 μmoles) (*F*). Additions were as follows: ATP (0.50 μmole containing 0.25 μmole of $MgSO_4$), $NaNO_3$ (0.10 mmole), Tris-sulfate (10 μmoles), FCCP (2 nmoles), nigericin (0.2 μg), Bt_3SnCl (20 nmoles), DCCD (100 nmoles)

Δ pH will be discussed later. After development of maximal quenching (3–4 min after ATP addition), the fluorescence slowly returned to the original level. As predicted above, addition of Tris-sulfate after maximal quenching greatly accelerated the dissipation of Δ pH (trace *B*). The same effect was observed with the uncoupler FCCP (trace *C*) or SF-6814 (not

shown) and with nigericin (trace *D*) and monensin (not shown). The presence of an energy transfer inhibitor, Bt_3SnCl (trace *E*) or DCCD (trace *F*), inhibited the development of Δ pH. In these experiments quenching due to ATP hydrolysis was about 50%. Based on measurement of trapping of [^{14}C]dextran [9], the internal volume of vesicles was calculated as about 0.05% of the total volume of the assay mixture. From this value the Δ pH formed by ATP hydrolysis was calculated from the above equation to be 3.3 units.

Measurement of Intravesicular pH

The pH profile of Δ pH formation by reconstituted vesicles on addition of ATP is shown in Fig. 6. The optimal pH for Δ pH formation was around 8 under the standard conditions, and thus the intravesicular pH must be between 4.5 and 5. The intravesicular pH can also be measured using pH indicators with a suitable pKa. More lipophilic indicators, such as bromthymol blue [2, 12], are unsuitable because they move across the lipid membrane [12]. Vesicles containing pH indicators, such as bromcresol purple, chlorophenol red, and bromphenol blue, were obtained by reconstituting vesicles in the presence of these dyes. Fig. 7 shows tracings of the optical absorption changes of the pH indicators trapped in the reconstituted vesicles.

About 80% of chlorphenol red was bleached on addition of 0.20 mM ATP (Fig. 7*A*), indicating that the dye was transformed into the undisso-

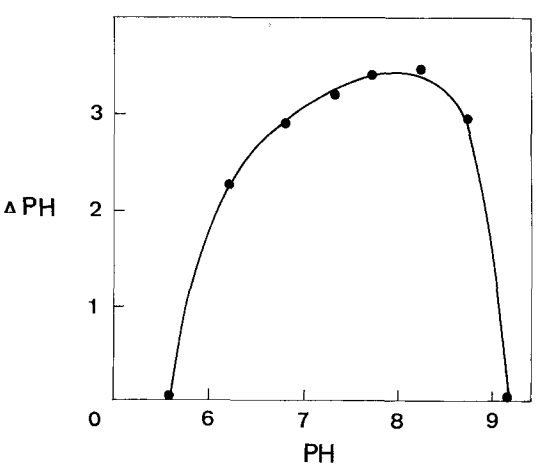

Fig. 6. Dependence of Δ pH on external pH. Conditions were as for Fig. 5*A* except that the pH of the reaction medium was as indicated

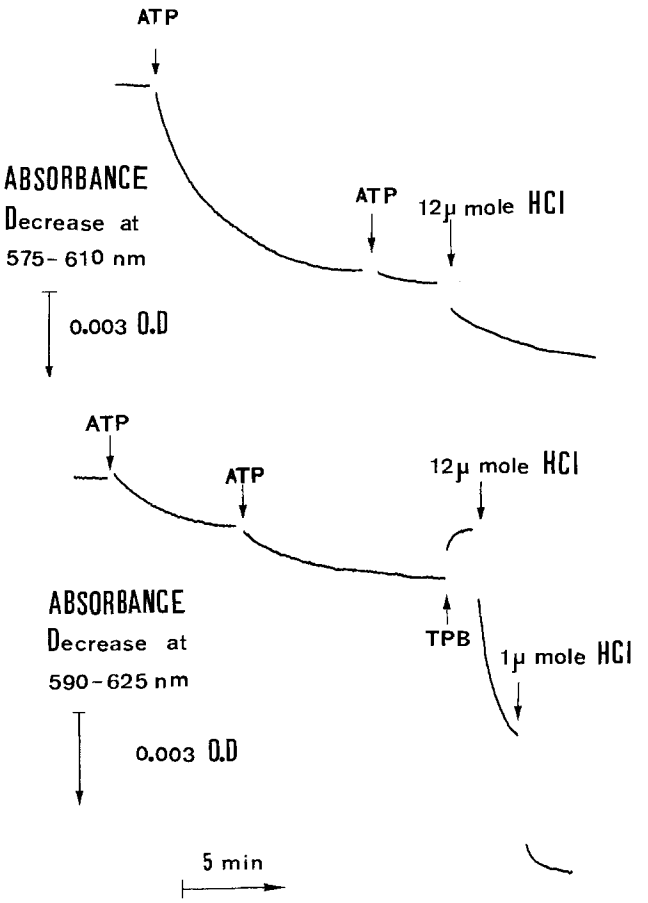

Fig. 7. Response of chlorophenol red (*A*) and bromphenol blue (*B*) trapped in vesicles. Vesicles (1.0 ml) were prepared from soybean P-lipid mixture containing $TF_0 \cdot F_1$ (0.25 mg protein) as described under *Materials and Methods*, except that both the mixture in the dialysis bag and the dialysis medium contained about 0.1 mg/ml of chlorophenol red (upper) or bromphenol blue (lower). The dialysis medium was replaced by medium containing no pH indicator at 5 hr and dialysis was continued for another 20 hr at 5 °C. The vesicles were then diluted with 7 ml of dialysis medium and centrifuged for 30 min at 140,000 × g. The pellet was resuspended in its original volume of dialysis medium. Vesicles containing pH indicator (0.2 ml) were placed in a cuvette (1 cm light path) containing 2.3 ml of 20 mM Tricine-NaOH at pH 7.7, 2 mM $MgSO_4$ and 40 mM $NaNO_3$. Other assay conditions were described under *Materials and Methods*. Additions were as follow: ATP (0.5 μmole containing 0.25 μmole $MgSO_4$) and TPB (sodium tetraphenylborate 29 nmoles)

ciated form. This dye changes color between pH 5.0 and 6.6, so the intravesicular pH must be around 5.0. Similarly, about 20% of the bromphenol blue was converted to the free acid on addition of 0.2 mM ATP, indicating that the internal pH must be around the upper limit of the range of color change (pH 2.8–4.6).

Estimation of Membrane Potential and Δ pH

The results described above show that ATP hydrolysis by these reconstituted vesicles resulted in the creation of either a membrane potential ($\Delta\psi$) or a pH gradient (Δ pH) across the membrane. Table 1 summarizes the $\Delta\psi$ and Δ pH values of the reconstituted vesicular membranes produced under various conditions. The data necessary for calculation of $\Delta\psi$ and Δ pH are also tabulated. As described above the maximal $\Delta\psi$ was obtained in Tris buffer and the maximal Δ pH in Tricine buffer containing $NaNO_3$. In Tricine buffer without $NaNO_3$, both $\Delta\psi$ and Δ pH could be estimated. Based on the observed values, the maximal electrochemical potential difference of H^+ ($\Delta\bar{\mu}\,H^+$) is about 250 mV from the equation:

$$\Delta\bar{\mu}\,H^+ = \Delta\psi - Z\Delta\,\mathrm{pH} \tag{3}$$

where Z is approximately 63 at 45 °C [10].

Effect of ATP Concentration

As reported previously, the K_m value of $TF_0 \cdot F_1$-ATPase for ATP is 0.4 mM [16]. Table 2 summarizes the effects of ATP concentration on

Table 1. Estimation of $\Delta\psi$ and Δ pH following addition of 0.5 μmole of ATP.[a]

Exp	P-lipid	Medium	$\Delta F/F$ (%)	$\Delta\psi$ (mV)	$Q/1-Q$	Δ pH	$\Delta\bar{\mu}H^+$
1	PS3	Tris	102	145	0	0	145
		Tricine	48	70	0.67	2.9	253
		Tricine $+NaNO_3$			2.45	3.5	221[b]
2	Soybean	Tris	51	83	0	0	83
		Tricine	24	33	0.15	2.3	178
		Tricine $+NaNO_3$			0.36	2.7	170[b]
3	Soybean	Tris	141	140	0	0	140
		Tricine	74	74	0.29	2.6	238
		Tricine $+NaNO_3$			0.58	2.9	183[b]

[a] Vesicles were reconstituted from PS-3 P-lipid mixture containing 0.25 mg of $TF_0 \cdot F_1$ protein (Exp. 1), from soybean P-lipid mixture containing 0.31 mg of $TF_0 \cdot F_1$ protein (Exp. 2), or from soybean P-lipid mixture containing 0.25 mg of $TF_0 \cdot F_1$ protein (Exp. 3). $\Delta F/F$ of ANS fluorescence and $Q/1-Q$ of 9-aminoacridine were assayed as described in Fig. 2 and Fig. 5, respectively. The V values under the assay conditions were 0.83 (Exp. 1), 0.80 (Exp. 2) and 0.74 μl/ml (Exp. 3).

[b] A very low $\Delta\psi$ was observed but was ignored (*see* Fig. 3 *C*).

Table 2. Effect of ATP concentration on $\Delta\psi$ and Δ pH[a]

ATP (mM)	Exp. 1		Exp. 2	
	$\Delta F/F$ (%)	$\Delta\psi$ (mV)	$Q/1-Q$	Δ pH
0	0.0	0	0	0
0.12	0.57	71	0.71	2.9
0.24	0.88	109	2.73	3.5
0.49	1.17	145	3.17	3.6
0.97	1.26	156	3.54	3.6

[a] In Exp. 1, the vesicles were reconstituted from soybean P-lipid mixture containing 0.31 mg of $TF_0 \cdot F_1$ protein and in Exp. 2, from PS3 P-lipid mixture containing 0.25 mg of $TF_0 \cdot F_1$ protein. The V value in the assay was quenching of 0.80 µl/ml. ANS fluorescence enhancement (Exp. 1) was measured in Tris buffer and quenching of 9AA fluorescence (Exp. 2) in Tricine buffer with $NaNO_3$.

the ANS fluorescence enhancement in Tris-sulfate buffer and the quenching of 9AA fluorescence in Tricine-NaOH buffer containing $NaNO_3$. The $\Delta\psi$ and Δ pH were also calculated. $\Delta\psi$ and Δ pH formed by the vesicles reconstituted from $TF_0 \cdot F_1$ and PS3 P-lipids were also measured in Tricine buffer without $NaNO_3$ under similar conditions to those for experiment 1 of Table 1; on addition of 2 µmoles of ATP (0.97 mM in final concentration) to the vesicles, 113 mV of $\Delta\psi$ and 3.1 units of Δ pH were observed, giving a value of 308 mV for $\Delta\bar{\mu}\,H^+$.

General Discussion

Fig. 8 shows schematic models for the mechanisms of energy transduction observed in the present investigation. Hydrolysis of ATP *via* $TF_0 \cdot F_1$ oriented in P-lipid vesicles resulted in formation of an electrochemical gradient of H^+ (more positive or acidic inside). The reaction was followed by the fluorescent probes ANS and 9AA. The former was used for detection of $\Delta\psi$ (left hand side of Fig. 8) and the latter for that of Δ pH (right hand side of Fig. 8). Uncharged Tris molecules which can pass through the lipid membrane increased the magnitude of $\Delta\psi$ due to ATP hydrolysis by dissipating free H^+ in the vesicles (*see* Fig. 5 *B*). In contrast, the permeable anion NO_3^- apparently increased Δ pH by decreasing $\Delta\psi$ (*see* Fig. 3 *C*). In either case, the translocation of H^+ by the ATPase complex was the initial event in energy transduction. Thus, the ATPase complex ($TF_0 \cdot F_1$) from the thermophilic bacterium is a proton-translocating ATPase (H^+-ATPase) similar to that from mitochondria ($CF_0 \cdot F_1$) [7, 10].

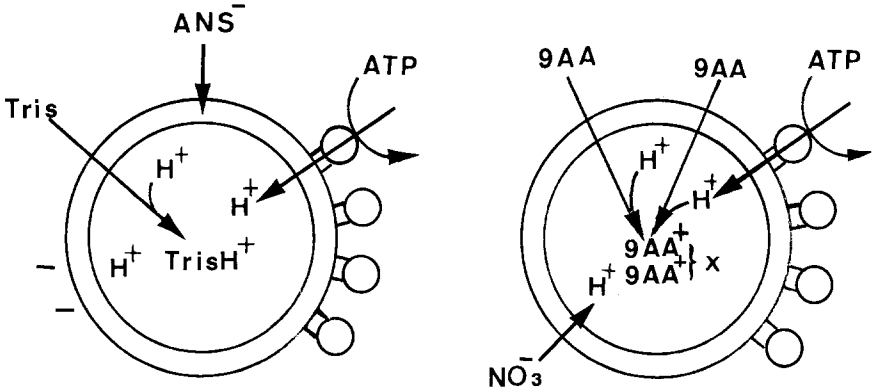

Fig. 8. H^+-Translocation by $TF_0 \cdot F_1$ and detection of $\Delta\psi$ by ANS fluorescence in the presence of Tris (left), or Δ pH by 9-aminoacridine (9AA) fluorescence in the prescence in the presence of NO_3^- (right)

Under the experimental conditions a thermodynamic calculation predicts that the maximal $\Delta\bar{\mu}\,H^+$ formed is about 300 mV supposing that 2 H^+ are transported on hydrolysis of 1 ATP, that the concentrations of ATP, ADP and Pi are all 0.12 mM, and that the free energy change during ATP hydrolysis is -8.0 kcal/mole. This theoretical value corresponds fairly well with the observed value (*see* Table 1). Thus, energy transduction in these reconstituted vesicles occurred efficiently under the present conditions.

The stoichiometry of H^+ translocated per ATP hydrolyzed was not clarified in the present investigation. However, preliminary experiments (N. Sone, *unpublished data*) showed that 2 H^+ were transported into the vesicles with hydrolysis of 1 ATP, although this stoichiometry rapidly decreased with time due to nonspecific leakage of H^+. A stoichiometry of 2 H^+/1 ATP has been reported for rat liver mitochondria [11] and bovine heart submitochondrial particles [17].

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