Biochimica et Biophysica Acta, 501 (1978) 136—149

BBA 47425

MECHANISM OF 3,5-DI-*tert*-BUTYL-4-HYDROXYBENZYLIDENE-MALONONITRILE-MEDIATED PROTON UPTAKE IN LIPOSOMES

KINETICS OF PROTON UPTAKE COMPENSATED BY VALINOMYCIN-INDUCED K^+-EFFLUX

AKIHITO YAMAGUCHI and YASUHIRO ANRAKU *

Department of Botany, Faculty of Science, University of Tokyo, Bunkyo-Ku, Tokyo 113 (Japan)

(Received June 14th, 1977)

Summary

1. Kinetic studies were made on proton translocation across the phospholipid bilayer membrane in liposomes; this proton translocation is mediated by the uncoupler 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile (SF6847) and compensated by valinomycin-induced potassium efflux. The initial rate of proton uptake was directly proportional to the concentration of SF6847 in the presence of excess valinomycin, indicating that the monomer of SF6847, at least, is a proton carrier and participates in limiting the rate of the proton uptake cycle.

2. The partition constant of SF6847 between the liposomal membranes, prepared from *Escherichia coli* phospholipids, and the aqueous phase was determined kinetically to be 21 mM^{-1} in medium of pH 7.0 and 4.5 mM^{-1} in medium of pH 8.0. The turnover rates of SF6847 across the bilayer membrane at pH 7.0 and 8.0 were determined as 140 and 550 times/molecule per s, respectively.

3. The initial rate of proton uptake depended hyperbolically on the concentration of valinomycin in the presence of a limiting amount of SF6847. It also depended on the concentration of potassium ion (K^+) inside the liposomes. These results suggest strongly that a ternary complex valinomycin · K^+ · SF^- (SF^-, anionic form of SF6847) is a direct intermediate, participating in limiting the rate of the proton uptake cycle.

* To whom correspondence should be addressed.
Abbreviations used: SF 6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile; SF^-, anionic form of SF6847; SFH, neutral form of SF6847; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

4. The equilibrium constants of the following equilibria,

valinomycin + $K^+ \overset{K_1}{\rightleftharpoons}$ valinomycin · K^+ and

valinomycin · K^+ + $SF^- \overset{K_2}{\rightleftharpoons}$ valinomycin · K^+ · SF^-

were determined: K_1 was $3.8 \cdot 10^{-1}$ M and K_2/A (A, constant; see text) was $4.0 \cdot 10^{-9}$ M under the conditions employed. From these results a model is proposed for the mechanism of the proton uptake cycle mediated by SF6847 in liposomes.

5. The mechanism of proton uptake mediated by carbonylcyanide *m*-chlorophenylhydrazone (CCCP) was also studied. It was concluded that the mechanism was essentially the same as that mediated by SF6847.

Introduction

In the chemiosmotic theory the mechanism of action of an uncoupler dissipating proton motive force in energy-transducing membranes is predicted to be of primary importance [1,2]. Translocation of molecules of uncoupler through a phospholipid bilayer and the proton conduction coupled with this process can be studied quantitatively using artificial phospholipid membranes, because these are impermeable to proton and many anions, such as phosphate ion.

Certain weak acids, known to be uncouplers of oxidative phosphorylation, act as proton conductors in lipid bilayer membranes [3—9]. Uncouplers can be classified into two groups by their mode of action in proton conduction in black lipid membranes. CCCP belongs to the group in which both A^- (anionic form) and HA (neutral form) of the molecule are translocated [10,11]. 2,4-Dinitrophenol and derivatives of benzimidazoles belong to the other group in which the charge carrier translocated may be the dimer of HA · A^- [12,13].

In liposomes, uncouplers act as proton conductors only when there is a compensating flux of charges, such as valinomycin-induced K^+ flux [14] or ferrocene-induced electron flux [15]. Recently, van Deenen and co-workers [16] suggested that the valinomycin-induced K^+ leak from liposomes containing KSCN was mediated by the ternary complex of valinomycin · K^+ · SCN^-, while this type of mechanism was not directly applicable to black lipid membranes. Correlation between uncoupling activity and proton conduction has been studied by several investigators [17—19]. These papers stimulated us to study the mechanism of proton conduction by uncouplers at molecular level.

In this study we used the uncoupler SF6847, because it is one of the strongest uncouplers known [20] and because the spectrum of the neutral form of the molecule, SFH, is distinctly different from that of anionic form, SF^-, [21]. Quantitative determinations of the partition constants of SF6847 in liposomes at different pH values and of the initial rates of proton uptake as functions of concentrations of valinomycin, potassium ions and liposomes were carried out kinetically. Theoretical considerations of these kinetic data suggested the presence of a ternary complex valinomycin · K^+ · SF^- in liposomes which acts as a direct intermediate carrying SF^- back across the liposomal

membrane. A preliminary account of this work has appeared (Yamaguchi, A., Ikegami, S. and Anraku, Y. (1976) Japan Bioenergetics Group Abst. 2, 100–102).

Materials and Methods

Chemicals. SF6847 was a product of Sumitomo Chemical Industry, Co., Osaka and was kindly supplied by Dr. Y. Nishizawa. Valinomycin and CCCP were obtained from Calbiochem., Los Angeles. Cholesterol was obtained from Sigma, Co. All other reagents were of analytical reagent grade.

Preparation of phospholipid from Escherichia coli. Phospholipids were prepared from freshly harvested cells of *E. coli* strain W3092 by the method of Bleigh and Dyer [22] and purified by subsequent chromatography on silica gel (Unisil, Clarkson Chemical).

The purified phospholipids consisted of 80% phosphatidylethanolamine, 10% phosphatidylglycerol and 10% cardiolipin. Phospholipid was determined by measuring its phosphorus by the method of Gerlach and Deuticke [23].

Preparation of liposomes containing potassium ion. K^+-containing liposomes were prepared by dispersing the *E. coli* phospholipids in 150 mM potassium chloride or 200 mM potassium phosphate at a given pH with a vortex mixer (Taiyo Bussan Co., Tokyo) and then sonicating the mixture for 30 s with a bath type sonifier (Branson model 220). The final concentration of liposomal phospholipids was approx. 10 mM. The K^+ outside the liposomes was removed by dialysis against 150 mM NaCl at the same pH (adjusted with NaOH), at room temperature. These liposomes are multi-lamellar vesicles of about 0.1–0.5 μm diameter. (Electron micrograph not shown).

Measurement of pH change. The reaction mixture for measuring the pH change consisted of 70 μl of the K^+-containing liposomes and 630 μl of 150 mM of NaCl adjusted at the same pH. The pH change immediately after the addition of a μl volume of an ethanolic solution of SF6847 and valinomycin was measured with a pH electrode (Yuasa Instrument, SE 1600 GC) equipped with a recorder at 25°C. The amount of protons taken up was calculated from the titration curve with standard HCl.

Release of potassium ion from K^+ liposomes was measured with a K^+ electrode (Nitsushin Instrument) at 25°C.

Results

(1) Valinomycin-K^+-dependent uptake of proton by SF6847 in liposomes

Fig. 1A shows that SF6847 induced uptake of proton into K^+ liposomes in the presence of valinomycin. A rapid increase in the pH of the medium was observed on addition of SF6847 to K^+-liposome solution containing 1 μg of valinomycin (0.9 μM). The pH increased to a plateau and returned rapidly to the original level on addition of 0.1% Triton X-100 to the medium (Fig. 1A). This rise in the pH was due to the accumulation of protons inside the liposomes and it only occurred when valinomycin was present.

Fig. 1B shows that the pH of the medium decreased when SF6847 was added to Na^+ liposomes suspended in K^+-containing medium. These results indi-

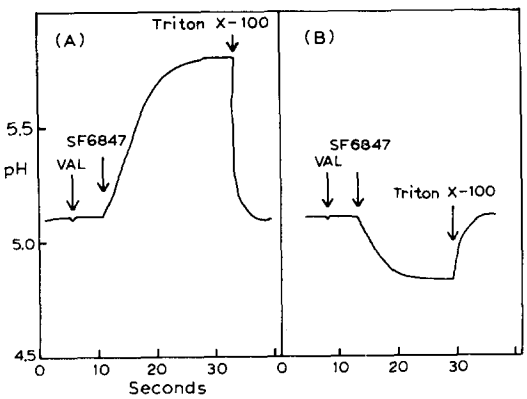

Fig. 1. Time course of SF6847-induced pH change of reaction medium containing K^+- and Na^+-liposomes. (A) K^+ liposomes were prepared from phospholipids of *E. coli* in 150 mM KCl, pH 5.1 and dialyzed against isotonic NaCl solution, pH 5.1, for 4 h at room temperature. The change of pH was monitored as described in Materials and Methods at 25°C in a pH meter equipped with a recorder. The liposome concentration used was 1.0 μmol phospholipid/ml. Valinomycin (0.9 μM), and SF6847 (1 μM) were added to the reaction mixture at the times indicated by arrows. Triton X-100 (0.1%) was added when the reaction reached a plateau. (B) Experimental conditions were the same as for A except that the Na^+ liposomes were prepared in 150 mM NaCl, pH 5.1, and dialyzed against 150 mM KCl, pH 5.1. VAL, valinomycin.

cate that proton flux takes place against a concentration gradient of potassium ions and that in the presence of valinomycin SF 6847 induces K^+/H^+ exchange across the phospholipid bilayers in liposomes.

Next we examined the K^+/H^+ exchange process quantitatively. K^+ liposomes were prepared and dialyzed against 0.3 M sucrose, pH 7.0. The initial rate of proton uptake was nearly equal to that of K^+-release from the liposomes (Fig. 2). The ratio of the amount of H^+ accumulated to the amount of K^+ released was found to be $H^+/K^+ = 1.1$.

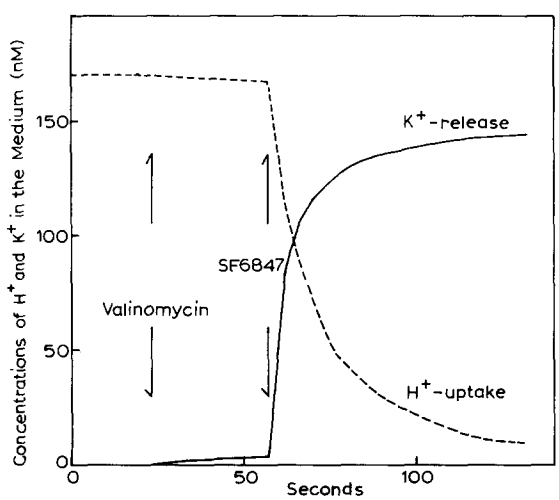

Fig. 2. Stoichiometry of K^+/H^+ exchange reaction in K^+-containing liposomes. K^+ liposomes (1.0 mM phospholipid) were prepared in 200 mM potassium phosphate (pH 7.0), dialyzed against 0.3 M sucrose (pH 7.0) for 4 h, and suspended in the same sucrose solution. Valinomycin (1.8 μM), and SF6847 (1.4 μM) were added to the reaction mixture at the times indicated by arrows. The change of pH and the release of K^+ were monitored (see Materials and Methods) and expressed as concentration of H^+ and K^+ in the medium.

(2) Dependence of the initial rate of proton uptake on the SF6847 concentration and pH of the medium

Fig. 3 shows the initial rates of proton uptake in the K^+ liposomes as functions of the concentrations of the uncouplers SF6847 and CCCP in the presence of excess valinomycin (0.3 μM). The initial rates are directly proportional to the concentrations of the uncouplers at concentrations of up to about 5 nM. This indicates that one molecule of SF6847 or CCCP carries proton. The result with CCCP is not incompatible with that obtained using black lipid membranes [11].

The initial rate of proton uptake mediated by SF6847 increased with increase in the pH of the medium from 6.0 to 8.0, while that mediated by CCCP decreased (Fig. 4). The reason for this interesting difference is discussed later. On the basis of the pH profile of SF6847 (Fig. 4), it is noted that the binding of proton to SF^- in liposomes is not rate limiting. We also found that the initial rate of proton uptake in the liposomes prepared in KCl medium was less than that in the liposomes prepared in potassium phosphate (see Fig. 5). This suggests that the pH inside the liposomes affects the initial rate.

(3) Effect of cholesterol

Scarpa and de Gier [24] observed that valinomycin-induced translocation of potassium ion in liposomes was inhibited by cholesterol, possibly because the fluidity of the membranes decreased. As shown in Fig. 5 the initial rate of proton uptake was significantly less when the liposomes contained cholesterol. Fig.

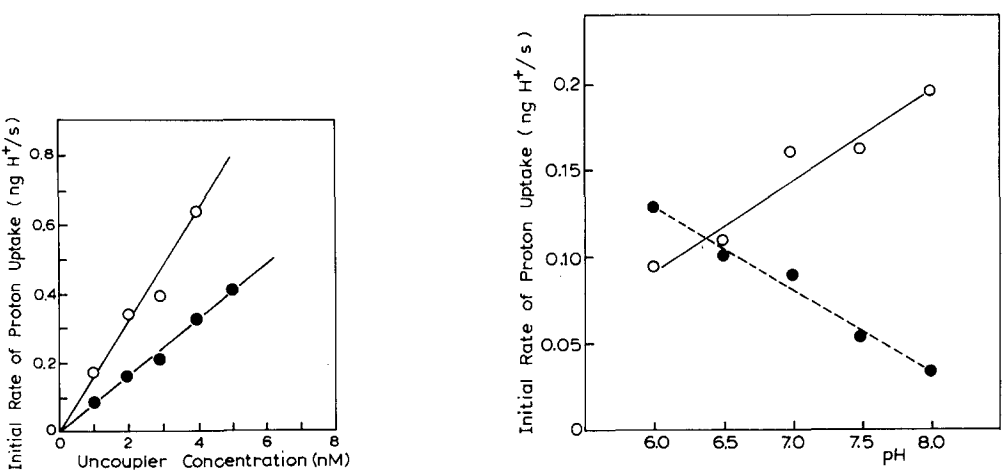

Fig. 3. Dependence of the initial rates of proton uptake on the concentrations of uncouplers SF6847 and CCCP. The initial rates of proton uptake into K^+ liposomes, prepared in 200 mM potassium phosphate (pH 7.0) (1 mM phospholipid), were determined by monitoring the change of pH for 1—3 s after adding the uncoupler with a pH electrode in 0.7 ml of 0.15 M NaCl. For details, see Materials and Methods. The final concentration of valinomycin added was 0.3 μM. SF6847 (○) and CCCP (●) were added at the concentrations indicated.

Fig. 4. Effects of pH on the initial rates of proton uptake mediated by uncouplers. K^+ liposomes were prepared in 200 mM potassium phosphate as described in Materials and Methods and the pH of the medium used was as indicated. The uncouplers used were SF6847 (○) and CCCP (●) at concentrations of 1 nM. For other conditions see legend to Fig. 3).

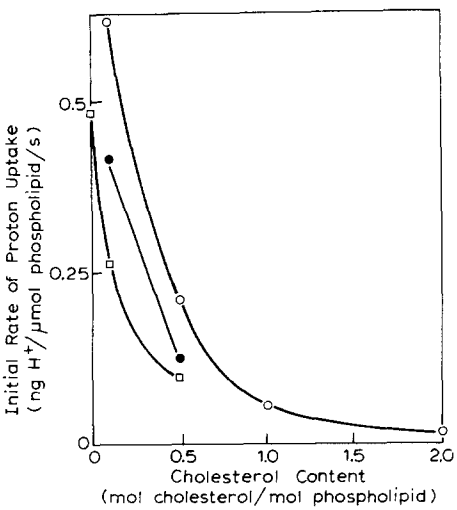

Fig. 5. Effect of cholesterol on the initial rate of proton uptake into K^+ liposomes. K^+ liposomes were prepared as for Fig. 1, except that they contained the amount of cholesterol indicated. The pH change was monitored in 1.0 ml of medium containing 1 mM K^+-liposome phospholipids, 150 mM NaCl and 2 μM valinomycin. The final concentrations of SF6847 were 0.1 μM (□), 1 μM (●) and 10 μM (○), respectively.

5 also shows that the initial rate of proton uptake depended on the concentration of SF6847, even when cholesterol was present. These findings suggest that the fluidity of the membranes affects, not only the rates of valinomycin-induced release of potassium ion, but also SF6847-mediated proton uptake. Therefore, the rate limiting step of proton uptake seems to be translocation of SF6847 through the liposomal membrane.

(4) Partition equilibrium and turnover rate of SF6847

If the binding of SF6847 to liposomes follows partition equilibrium [21] and the initial rate of proton uptake is directly proportional to the amount of bound SF6847, the initial rate of proton uptake should be hyperbolically dependent on the liposome concentration. The partition constant K_p at a particular pH in a constant volume of reaction mixture is defined as follows:

$$K_p = \frac{SF_b}{SF_f \cdot [lip]} \tag{1}$$

where SF_b and SF_f are the amounts of bound and free SF6847, respectively, and [lip] represents the liposome concentration. When the total amount of added SF6847 is expressed as SF_t, SF_f can be written as follows:

$$SF_f = SF_t - SF_b \tag{2}$$

Substituting Eqn. 2 into Eqn. 1 and rearranging the latter, we obtain:

$$\frac{1}{SF_b} = \frac{1}{K_p \cdot SF_t} \cdot \frac{1}{[lip]} + \frac{1}{SF_t} \tag{3}$$

Assuming that the initial rate of proton uptake depends upon the amount of

SF_b, then:

$$v_{\Delta H^+} = U \cdot SF_b \tag{4}$$

where $v_{\Delta H^+}$ represents the initial rate of proton uptake and U is a constant. Substituting Eqn. 4 into Eqn. 3, we obtain:

$$\frac{1}{v_{\Delta H^+}} = \frac{1}{U \cdot K_p \cdot SF_t} \cdot \frac{1}{[lip]} + \frac{1}{U \cdot SF_t} \tag{5}$$

If the assumption presented by Eqn. 4 is correct, a plot of the reciprocal of $v_{\Delta H^+}$ against the reciprocal of the liposome concentration according to Eqn. 5 should give a straight line. Fig. 6 shows that this is indeed the case.

From Eqn. 5, we can obtain the V and K_p values by extrapolating $1/[lip] = 0$ and $1/v_{\Delta H^+} = 0$, respectively. Table I summarizes the V and K_p values determined in the presence of various amounts of SF6847. The turnover rate of SF6847 across the membranes is obtained by dividing the V value by the amount of added SF6847. The rate is affected greatly by the pH of the medium. It is also comparable to the value for the turnover rate of SF6847 in mitochondrial membranes estimated from the value for 50% inhibition of oxidative phosphorylation [25]. The V increased with increase in pH value, suggesting that not all the bound SF6847 participated in the rate limiting step. Thus it seems very likely that the dissociated form of SF6847 (SF^-) participates in the rate limiting step of proton uptake. Confirmation of this possibility is given in the following paper [26]. The ratio of dissociated to undissociated

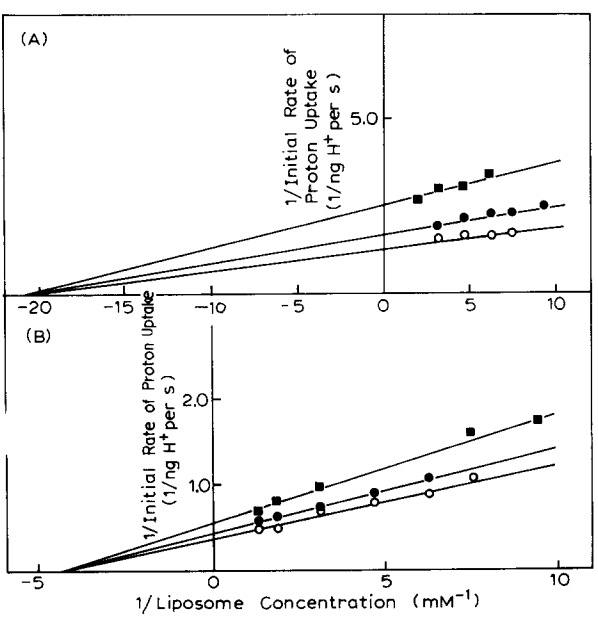

Fig. 6. Double reciprocal plots of the initial rate of proton uptake against the liposome concentration at pH 7.0 (A) and pH 8.0 (B). K^+ liposomes were prepared in 120 mM potassium phosphate, and dialyzed against 150 mM NaCl at the same pH. SF6847 was added at concentrations of 8 nM (○), 6 nM (●) and 4 nM (■), in the presence of 1 μM valinomycin.

TABLE I

PARTITION CONSTANT AND TURNOVER RATE OF SF6847

For definition, see Eqn. 5 and Fig. 6.

pH	K_p (mM^{-1})	SF6847 (nM)	Turnover rate * (numbers of H^+ translocated per molecule of SF6847 per s)
7.0	21	4	$1.4 \cdot 10^2$
		6	$1.4 \cdot 10^2$
		8	$1.4 \cdot 10^2$
8.0	4.5	4	$6.4 \cdot 10^2$
		6	$5.4 \cdot 10^2$
		8	$4.8 \cdot 10^2$

* The amounts of SF6847 bound to the liposomes were about 90% or more of the total SF6847 added. (For details, see the following paper [26]).

SF6847 varies greatly in the pH range examined, since the pK_a of SF6847 is about 6.7 [21].

(5) Kinetic evidence for the formation of the ternary complex, valinomycin · K^+ · SF^-

In the presence of excess SF6847, the initial rate of proton uptake is directly proportional to the amount of added valinomycin (Fig. 7). This suggests that valinomycin participates directly in the process of proton translocation. Accordingly we examined the dependence of the initial rate of proton uptake

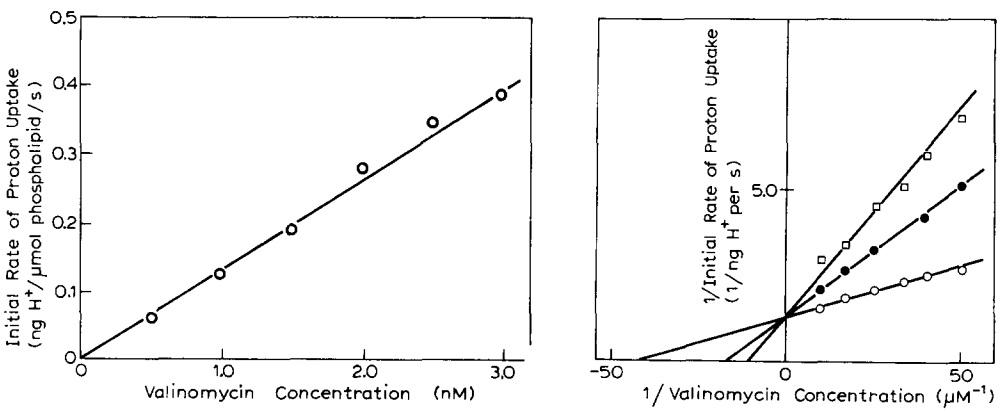

Fig. 7. Dependence of the initial rate of proton uptake on the concentration of valinomycin. K^+ liposomes (1.2 mM phospholipid) were prepared as in Fig. 3. The reaction was started by adding SF6847 (5 μM) to mixture containing K^+ liposomes in the presence of the indicated amount of valinomycin.

Fig. 8. Double reciprocal plots of the initial rate of proton uptake against the valinomycin concentration in the presence of various concentrations of a combination of potassium and sodium ions inside the liposomes. Liposomes (1.2 mM phospholipid) were prepared in potassium phosphate (pH 7.0) at concentrations of 200 mM (○), 67 mM (●) and 33 mM (□) with 0 mM, 133 mM, and 167 mM sodium phosphate, pH 7.0, respectively. The liposomes were dialyzed separately against 150 mM NaCl, pH 7.0. The concentration of SF6847 used was 6 nM.

on the valinomycin concentration in the presence of a limiting amount of SF6847. In Fig. 8, the reciprocal of the initial rate of proton uptake is plotted against the reciprocal of the valinomycin concentration. The initial rate of proton uptake depended hyperbolically on the valinomycin concentration. Therefore, it was difficult to explain this phenomenon simply by electrical coupling, because potassium ions can cross the liposomes much more freely than protons under these conditions. Furthermore, as shown in Fig. 9, the initial rate of proton uptake also depended hyperbolically on the K^+ concentration inside the liposomes in the presence of excess SF6847 and a limiting amount of valinomycin. It is not due to alteration in the amount of bound valinomycin since the affinity of valinomycin for liposomes is not affected by the K^+ concentration inside the liposomes [16]. Therefore, the results strongly suggest that a ternary complex of valinomycin $\cdot$ K^+ $\cdot$ SF^- participates in limiting the rate of the proton uptake cycle.

Thus we assume that the following equilibria exist on the inside surface of the outermost bilayer of multi-layered liposomes:

$$\text{valinomycin} + K^+ \overset{K_1}{\rightleftharpoons} \text{valinomycin} \cdot K^+$$

$$\text{valinomycin} \cdot K^+ + SF^- \overset{K_2}{\rightleftharpoons} \text{valinomycin} \cdot K^+ \cdot SF^-$$

where K_1 and K_2 represent equilibrium constants in the respective reactions.

The valinomycin $\cdot$ K^+ $\cdot$ SF^- is a complex which undergoes fast transmembrane diffusion while translocation of single molecule of SF^- is relatively slow. On the basis of this assumption, immediately after addition of valinomycin in the presence of a limiting amount of SF6847, the following equation holds (see Appendix):

$$\frac{1}{v_{\Delta H^+}} = \frac{K_m}{U \cdot SF_t} \cdot \frac{1}{[\text{valinomycin}]_t} + \frac{1}{U \cdot SF_t} \tag{6}$$

where K_m represents $(2/A) \cdot (K_1 \cdot K_2/[K^+]_i + K_2)$. $[\text{Valinomycin}]_t$ and SF_t are the total amounts of added valinomycin and SF6847, respectively, $[K^+]_i$ is the concentration of K^+ inside the liposomes, and A and U are the constants defined in the Appendix.

Eqn. 6 shows that a double reciprocal plot of the initial rate of proton uptake against the concentration of added valinomycin in a constant volume of reaction mixture gives a straight line. To test this, we prepared liposomes containing various concentrations of K^+ and Na^+, but with constant total osmolarity. These preparations were then dialyzed separately against isotonic NaCl solutions for 4 h. These liposome preparations had various concentrations of K^+ inside but their concentration gradients of K^+ across the liposomal membrane were approximately the same. Using these preparations we found that the K_m value depended on the K^+ concentration inside, whereas the V value was constant (Fig. 8). These results show that the above assumption is correct.

The K_m values were obtained by extrapolating the curves to $1/v_{\Delta H^+} = 0$ (Fig. 8). A plot of the K_m values against the reciprocals of $[K^+]_i$ gave a straight line, as shown in Fig. 10. The intercept on the ordinate and the slope of this curve represent the value of $2K_2/A$ and $K_1 \cdot 2K_2/A$, respectively. Using these values,

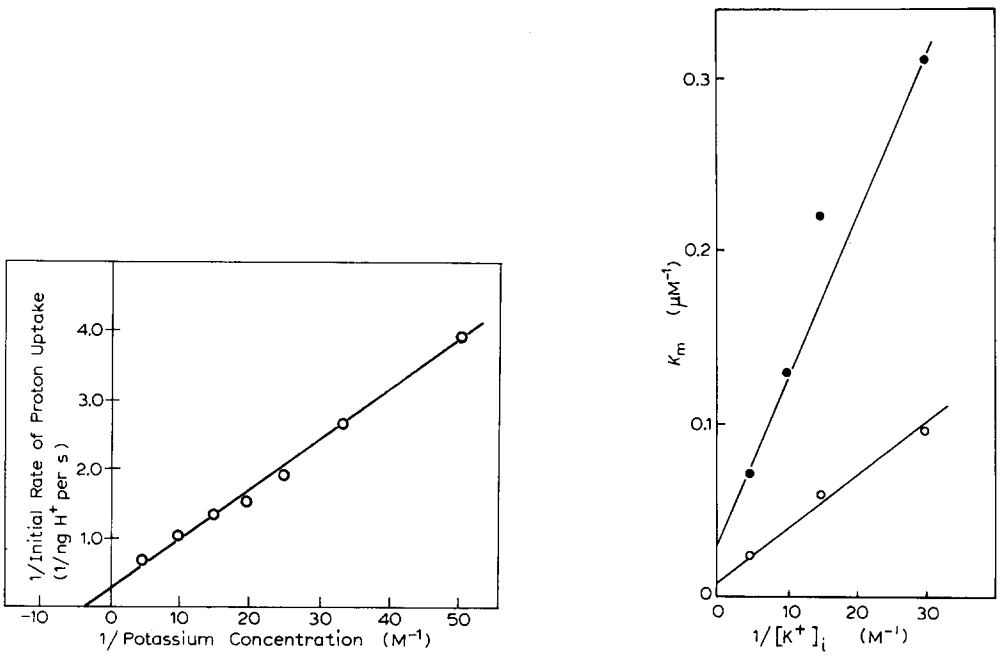

Fig. 9. Double reciprocal plot of the initial rate of proton uptake against the concentration of potassium ions inside the liposomes. Liposomes were prepared in solutions containing the indicated concentrations of potassium phosphate with sufficient sodium phosphate to keep the ionic strength constant. The final concentration of phosphate buffer used was 120 mM and the pH was 7.0. The pH change was monitored as in Fig. 8 using 1 nM valinomycin and 10 μM SF6847.

Fig. 10. Plot of the K_m value against the reciprocal of the concentration of potassium ion inside the liposomes. The K_m values were determined from the intercepts on the abscissa of plots of $1/v_{\Delta H^+}$ vs. 1/[valinomycin] in Fig. 8. ○, SF6847; ●, CCCP. For further details, see Eqn. 6 in text.

K_1 was calculated to be $3.8 \cdot 10^{-1}$ M, and K_2/A to be $4.0 \cdot 10^{-9}$ M. *

The mechanism of proton uptake mediated by CCCP was also examined on the basis of Eqn. 6. As the plot of the K_m values against the $1/[K^+]_i$ values gave a straight line (Fig. 10) we concluded that the mechanism with CCCP is essentially the same as that with SF6847 and that CCCP can form a ternary complex with valinomycin · K^+. With CCCP the K_1 value was $2.7 \cdot 10^{-1}$ M and K_2/A was $1.5 \cdot 10^{-8}$ M.

Discussion

In this work we determined the initial rate of proton uptake mediated by SF6847 in liposomes kinetically. This uptake is compensated by valinomycin-induced potassium efflux. As shown by Eqn. 6, the mechanism of the reaction can be best explained by supposing that a ternary complex valinomycin · K^+ · SF^- acts as a direct intermediate in the proton uptake cycle. The scheme in Fig.

* The value A is a constant representing $[SF^-_b]_i/[SF]_t$ (see Appendix). The order and range for A are estimated in the following paper [26].

11 summarizes the mechanism of the proton uptake cycle mediated by SF6847.

This model is unique in the following points: (1) Translocation of SF^- in the phospholipid bilayer membrane is mediated by a neutral ternary complex valinomycin · K^+ · SF^- (see Eqn. 6). We did not measure transmembrane conductivity of SF^-. However, our results suggest strongly that the transmembrane diffusion of uncomplexed SF^-, if it would exist, is not a main pathway in the model. (2) The stoichiometry of the constituents in the ternary complex is suggested to be valinomycin : K^+ : SF^- = 1 : 1 : 1 (Figs. 3,7). (3) Accordingly, this cycle should mediate stoichiometric counter exchange of potassium ion and proton. This was confirmed to be the case (Fig. 2). (4) The rate-limiting step in the proton uptake cycle is the back diffusion of this ternary complex in the phospholipid bilayer membranes. Therefore, in the presence of excess valinomycin, the initial rate of proton uptake depends on the concentration of SF^- in the membranes.

CCCP, which is a typical weak acidic uncoupler, also translocates protons across the membrane (Fig. 10) by the mechanism shown in Fig. 11, but the K_2/A value with CCCP and valinomycin · K^+ is about four times that with SF6847 and valinomycin · K^+. This difference corresponds to the difference in the potencies of SF6847 and CCCP as proton-conducting agents (Fig. 3).

On the other hand, the initial rate of proton uptake mediated by SF6847 increased with increase in pH, while that by CCCP decreased (Fig. 4). Namely, the pH dependences of the abilities of these two uncouplers to take up protons differed, although both uncouplers are weak acidic compounds. We suggest that these differences are due to differences of the interactions of SF^- and $CCCP^-$ with liposomal membranes, because the rate limiting step in the proton uptake cycle is the rate of reverse diffusion of the ternary complex (see Fig. 11) and because the amounts of the complexes are proportional to the amounts of bound SF^- and $CCCP^-$. The amount of the uncouplers bound to liposomes is affected by their pK_a values and partition constants. However, the pH dependences observed in Fig. 4 can not be explained simply by differences of the pK_a values reported (pK_a of SF6847, 6.7; pK_a of CCCP, 6.0 [21]). Thus, it is

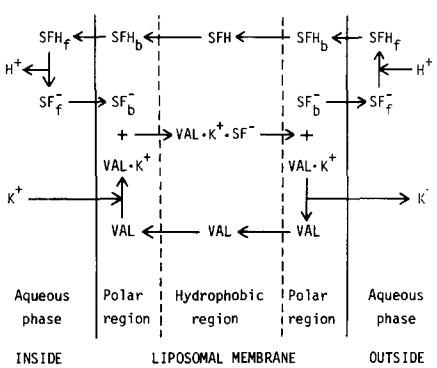

Fig. 11. Mechanism of the proton uptake cycle mediated by SF6847 in liposomes. SF_b^- and SF_f^- are bound and free SF^-, and SFH_b and SFH_f are bound and free SFH, respectively. VAL, valinomycin. For other symbols and explanation, see text.

most important to determine the partition constants of uncoupler in its anionic and protonated forms separately. This problem is treated extensively in the following paper [26].

In studies on the effects of anions of valinomycin dependent K^+ leak, van Deenen and co-workers found that thiocyanate ion stimulated K^+ leak greatly and they showed the presence of a ternary complex valinomycin · K^+ · SCN^- kinetically [16]. They analyzed the equilibrium constant K_1 of the reaction; valinomycin + $K^+ \rightleftharpoons$ valinomycin · K^+ and the constant K_2 of the equilibrium; valinomycin · K^+ + $SCN^- \rightleftharpoons$ valinomycin · $K^+ - SCN^-$ and determined K_1 and K_2 as 0.33—1.59 M and 0.0042—0.0173 M, respectively, on the inside surface of the outer phospholipid bilayer of multilayered liposomes. It is interesting to note that the K_1 value of $3.8 \cdot 10^{-1}$ M obtained from measurement of the initial rate of proton uptake (Fig. 10) was close to the reported K_1 vlaue obtained from measurement of K^+ leak.

The constant K_2/A (see Appendix) correlates with the potency of proton conduction by uncoupler in liposomes. Since the extent of binding of anionic form of uncoupler to liposomes is determined by the constant A and formation of a ternary complex is affected by the K_2 value, both values must be determined separately when we compare the structure-activity relationship between different uncoupler molecules. In the following paper [26], we report the K_2 value for SF6847.

Appendix

Calculation of the equilibrium constants for the equilibria:

$$valinomycin_i + K_i^+ \overset{K_1}{\rightleftharpoons} (valinomycin \cdot K^+)_i \text{ and}$$

$$(valinomycin \cdot K^+)_i + SF_i^- \overset{K_2}{\rightleftharpoons} (valinomycin \cdot K^+ \cdot SF^-)_i$$

The following symbols are used and all the values defined below are normalized by the volume of reaction mixture.

$[Valinomycin]_i$ is the amount of uncomplexed valinomycin on the inner surface of the outer liposomal membrane.

$[Valinomycin]_t$ is the total amount of added valinomycin.

$[Valinomycin]_i^t$ is the total amount of valinomycin on the inside surface.

$[Valinomycin \cdot K^+]_i$ is the amount of the complex of valinomycin and K^+ on the inside surface.

$[SF_b^-]_i$ is the amount of the dissociated form of SF6847 on the inside surface.

$[SF]$ is the total amount of uncomplexed SF6847.

$[SF]_t$ is the total amount of added SF6847.

$[Valinomycin \cdot K^+ \cdot SF^-]_i$ is the amount of the ternary complex.

As constant liposome concentration and constant volume of the medium, we can define the equilibrium constants as follows:

$$K_1 = \frac{[valinomycin]_i \cdot [K^+]_i}{[valinomycin \cdot K^+]_i} \tag{7}$$

$$K_2 = \frac{[\text{valinomycin} \cdot K^+]_i \cdot [SF_b^-]_i}{[\text{valinomycin} \cdot K^+ \cdot SF^-]_i} \tag{8}$$

where K_1 and K_2 are equilibrium constants and $[K^+]_i$ is the concentration of the potassium ion inside the liposomes. Multiplying Eqn. 7 by Eqn. 8, we obtain:

$$K_1 \cdot K_2 = \frac{[\text{valinomycin}]_i \cdot [K^+]_i \cdot [SF_b^-]_i}{[\text{valinomycin} \cdot K^+ \cdot SF^-]_i} \tag{9}$$

In the presence of excess valinomycin and a limiting amount of SF6847, we can assume $[\text{valinomycin}]_i >> [SF^-]_i$. Therefore,

$$[\text{valinomycin}]_i = [\text{valinomycin}]_i^t - [\text{valinomycin} \cdot K^+]_i \tag{10}$$

Substituting Eqn. 7 into Eqn. 10 and resolving for $[\text{valinomycin}]_i$ we obtain:

$$[\text{valinomycin}]_i = \frac{K_1 \cdot [\text{valinomycin}]_i^t}{K_1 + [K^+]_i} \tag{11}$$

Substituting Eqn. 11 into Eqn. 9 and resolving for $[\text{valinomycin} \cdot K^+ \cdot SF^-]_i$ gives:

$$[\text{valinomycin} \cdot K^+ \cdot SF^-]_i = \frac{[\text{valinomycin}]_i^t \cdot [K^+]_i \cdot [SF_b^-]_i}{K_2 \cdot (K_1 + [K^+]_i)} \tag{12}$$

Assuming a fast equilibrium of SF_i^- with SFH_i, which is equilibrated with SFH_o (see Fig. 11), we can write:

$$[SF_b^-]_i = A[SF] \tag{13}$$

where A is a constant representing the relative amount of SF_{bi}^-. Neglecting the ternary complex on the outer surface, we can write:

$$[SF] = [SF]_t - [\text{valinomycin} \cdot K^+ \cdot SF^-]_i \tag{14}$$

Substituting Eqn. 14 into Eqn. 13, we obtain:

$$[SF_b^-]_i = A[SF]_t - A[\text{valinomycin} \cdot K^+ \cdot SF^-]_i \tag{15}$$

Substituting Eqn. 15 into Eqn. 12 and resolving for $[\text{valinomycin} \cdot K^+ \cdot SF^-]_i$ gives:

$$[\text{valinomycin} \cdot K^+ \cdot SF^-]_i = \frac{A[\text{valinomycin}]_i^t \cdot [SF]_t}{\frac{K_1 \cdot K_2}{[K^+]_i} + K_2 + A[\text{valinomycin}]_i^t} \tag{16}$$

Taking $[\text{valinomycin}]_i^t$ as $[\text{valinomycin}]_t/2$, Eqn. 16 can be rewritten:

$$[\text{valinomycin} \cdot K^+ \cdot SF^-]_i = \frac{A[\text{valinomycin}]_t \cdot [SF]_t}{2\left(\frac{K_1 \cdot K_2}{[K^+]_i} + K_2\right) + A[\text{valinomycin}]_t} \tag{17}$$

Assuming that the initial rate of proton uptake ($v_{\Delta H^+}$) is directly proportional

to [valinomycin · K^+ · SF^-]$_i$, then:

$$v_{\Delta H^+} = U[\text{valinomycin} \cdot K^+ \cdot SF^-]_i \tag{18}$$

where U is a constant. Substituting Eqn. 18 into Eqn. 17, we obtain:

$$v_{\Delta H^+} = \frac{U[\text{valinomycin}]_t \cdot [SF]_t}{2/A\left(\frac{K_1 \cdot K_2}{[K^+]_i} + K_2\right) + [\text{valinomycin}]_t} \tag{19}$$

Replacing $2/A(K_1 \cdot K_2/[K^+]_i + K_2)$ by K_m and rearranging Eqn. 19, we obtain:

$$\frac{1}{v_{\Delta H^+}} = \frac{K_m}{U[SF]_t} \cdot \frac{1}{[\text{valinomycin}]_t} + \frac{1}{U[SF]_t} \tag{6}$$

Plotting the reciprocal of $v_{\Delta H^+}$ against the reciprocal of [valinomycin]$_t$ and extrapolating the curve to the intercept on the ordinate, we obtain the value of K_m. A plot of K_m against $1/[K^+]_i$ gives a straight line (see Fig. 10).

Acknowledgements

The authors express their gratitude to Drs. S. Nojima, K. Inoue, and S. Ikegami for their suggestions and discussion. They also thank Dr. Y. Nishizawa for kindly supplying SF6847. This work was supported in part by a grant from the Ministry of Education, Science and Culture of Japan.

References

1 Mitchell, P. (1961) Nature 191, 144—148
2 Mitchell, P. and Moyle, J. (1967) Biochem. J. 104, 588—600
3 Bielavsky, J., Thompson, T.E. and Lehninger, A.L. (1968) Biochem. Biophys. Res. Commun. 24, 948—954
4 Skulachev, V.P., Sharaf, A.A. and Lieberman, E.A. (1967) Nature 216, 718—719
5 Hopfer, U., Lehninger, A.L. and Thompson, T.E. (1968) Proc. Natl. Acad. Sci. U.S. 59, 484—490
6 Lieberman, E.A., Mochova, E.N., Skulachev, V.P. and Topaly, V.P. (1968) Biofizika 13, 188—193
7 Lieberman, E.A. and Topaly, V.P. (1968) Biochim. Biophys. Acta 163, 125—136
8 Lieberman, E.A. and Topaly, V.P. (1968) Biofizika 13, 1025—1035
9 Babakov, A.V., Demin, V.V., Sokolov, S.D. and Sotnikov, P.S. (1968) Biofizika 13, 1122—1123
10 Markin, V.S., Krishtalik, L.I., Lieberman, E.A. and Topaly, V.P. (1969) Biofizika 14, 256—264
11 Le Blanc, Jr., O.H. (1971) J. Memb. Biol. 4, 227—251
12 Finkelstein, A. (1970) Biochim. Biophys. Acta 205, 1—6
13 Lea, E.J.A. and Croghan, P.C. (1969) J. Memb. Biol. 1, 225—237
14 Henderson, P.J.F., McGivan, J.D. and Chappell, J.B. (1969) Biochem. J. 111, 521—535
15 Hinkle, P.C. (1973) Fed. Proc. 32, 1988—1992
16 Blok, M.C., de Gier, J. and van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 367, 210—224
17 Ting, H.P., Wilson, D.F. and Chance, B. (1970) Arch. Biochem. Biophys. 141, 141—146
18 Bakker, E.P., van den Heuvel, E.J., Wiechmann, A.H.C.A. and van Dam, K. (1973) Biochim. Biophys. Acta 292, 78—87
19 Cunarro, J. and Weiner, M.W. (1975) Biochim. Biophys. Acta 387, 234—240
20 Muraoka, S. and Terada, H. (1972) Biochim. Biophys. Acta 275, 271—275
21 Bakker, E.P., Arents, J.C., Hoebe, J.P.M. and Terada, H. (1975) Biochim. Biophys. Acta 387, 491—506
22 Bleigh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911—917
23 Gerlach, E. and Deuticke, B. (1963) Biochem. Z. 337, 477—479
24 Scarpa, A. and de Gier, J. (1971) Biochim. Biophys. Acta 241, 789—797
25 Muraoka, S., Terada, H. and Takaya, T. (1975) FEBS Lett. 54, 53—56
26 Yamaguchi, A., Anraku, Y. and Ikegami, S. (1978) Biochim. Biophys. Acta 501, 150—164