ionizes to an imidazolate ion. This is quite conclusive evidence, which was needed after the report that coordinated imidazoles may indeed ionize with low pK_a values,[6] and is in agreement with what was induced from ^1H NMR spectra of the whole diamagnetic zinc enzyme.[39] Another important point in the present research is that the three imidazoles are indeed regularly coordinated, despite the apparent irregularities in bond distances from the X-ray structural data[1] and that they are not replaced by any of the inhibitors investigated.

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Effective Enhancement of Valinomycin-Mediated Potassium Uptake in Organic Phase by Uncouplers of Oxidative Phosphorylation Detected by ^1H NMR

Kenichi Yoshikawa† and Hiroshi Terada*

Contribution from the College of General Education and the Faculty of Pharmaceutical Sciences, University of Tokushima, Tokushima 770, Japan. *Received January 22, 1981*

Abstract: ^1H NMR studies demonstrated the existence of a ternary complex of the electrogenic ionophore valinomycin, K^+, and an anion, such as a protonophoric uncoupler of oxidative phosphorylation, when these components were partitioned between an organic solvent and water. The stoichiometry of the ternary complex was found to be 1:1:1 when the acid was an uncoupler such as SF 6847, FCCP, 2,4-dinitrophenol, or picric acid. However, when 8-anilinonaphthalene-1-sulfonate (ANS) or benzoic acid was used as gegenion, no ternary complex could be detected. Studies on the NMR spectra of valinomycin and organic acids and quantitative determination of potassium showed that potent uncouplers greatly enhance valinomycin-mediated potassium uptake. This effect of uncoupler was well correlated with their uncoupling activity in mitochondria. The synergistic actions of valinomycin and uncoupler in the transport of potassium ion and proton across membranes is suggested.

Uncouplers of oxidative phosphorylation and ionophores, such as valinomycin, are very useful for manipulating the electrochemical potential across biological and model membrane systems. Recently, the synergistic effects of ionophore and uncouplers or other anions have been proposed to be important in the process of alkali-metal cation and proton translocation through the membrane and in the action of uncouplers.[1-6] From the spectral change of the uncoupler[4,5] and kinetic treatment of K^+ or H^+ transport,[1,5,6] the formation of a 1:1:1 complex of valinomycin, K^+, and a monovalent organic anion, such as an uncoupler, is considered to be responsible in these processes. However, there is no direct evidence for the formation of the ternary complex, except for the crystallographic study on the complex of valinomycin-K^+ with an inorganic anion.[26] Further, little is known about the role of anions in valinomycin-mediated potassium transport, though the movement of potassium has been found to depend on the kind of anion acting as gegenion.[7]

This paper reports direct evidence for the existence of the ternary complex obtained by ^1H NMR spectroscopy and describes the molecular basis of the synergistic actions of uncoupler and valinomycin in the transport of K^+ and H^+. This study may shed light on the mechanism of the action of uncoupler on biomembranes.

Experimental Section

Materials. SF 6847[8] and PCP[8] were gifts from Dr. Y. Nishizawa, Sumitomo Chemical Industry, Osaka (Japan), and FCCP[8] was kindly supplied by Dr. P. Heytler, E. I. DuPont de Nemours and Co., Wilmington, DE. Valinomycin was purchased from Sigma Chemical Co., St. Louis MO. Other reagents were commercial products and were used without further purification.

Two-Phase Extraction for NMR Measurement. A volume of 0.5 mL of CCl_4 containing 3 mM of valinomycin was equilibrated with an aqueous solution (1 mL) of 1 N KOH containing an anion (30 mM), such as an uncoupler of ANS,[8] by shaking for 10 min at 20 °C. After equilibrium, the NMR spectrum of the CCl_4 phase was measured with a JEOL FX-100 NMR spectrometer, using the pulsed Fourier transform method. ^1H NMR spectra were usually collected as 200 transients after 45° pulses (10 μs) with the interval of 3.5 s.

Two-Phase Extraction for Measurement by Flame Photometry. One milliliter of chloroform, which had been equilibrated with 10 mM LiOH, was added to 3 mL of an aqueous solution of 10 mM LiOH and 100 μM KCl. Valinomycin was added to the chloroform phase at 300 μM and an uncoupler or other acid was added to the aqueous phase at 0, 100, 300, and 500 μM. The two phases were equilibrated by shaking gently overnight at 4 °C. Then the concentration of K^+ in the aqueous phase was determined by flame photometry in a Hitachi flame photometer, Model 205. LiOH was added to the aqueous phase to maintain a constant pH and also as an internal standard in assay of K^+ by flame photometry. The reference experiment in the absence of valinomycin was carried out in the same way.

Results

The ^1H NMR spectra of valinomycin in CCl_4 under various conditions are shown in Figure 1. The line assignments were made on the basis of the results reported previously.[9-12] Figure 1a is

* Address correspondence to this author at the Faculty of Pharmaceutical Sciences.
† College of General Education.

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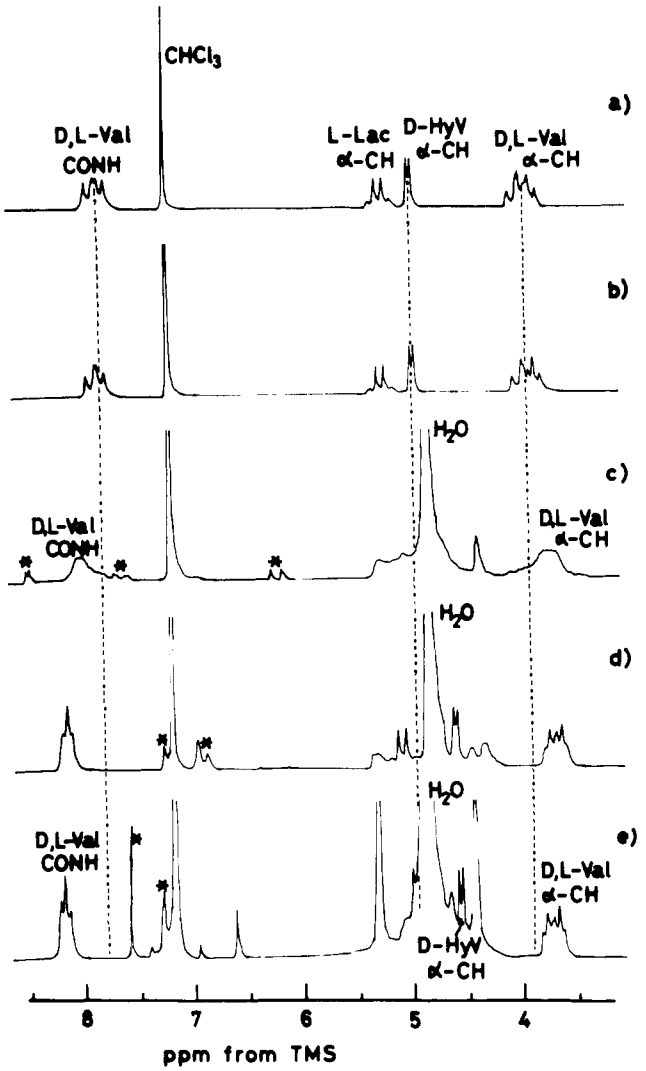

Figure 1. ^{1}H NMR spectra (100 MHz) of valinomycin: (a) in CCl_4, (b) CCl_4 layer after equilibrated with aqueous 1 N KOH solution, (c) b + DNP, (d) b + FCCP, (e) b + SF 6847. $CDCl_3$ (10 v/v %) was added to the sample tube (5 mm outside diameter) as an internal lock. An asterisk indicated absorption due to organic acids (uncouplers).

the NMR spectrum of valinomycin dissolved in CCl_4 and Figure 1b is the spectrum observed after valinomycin in CCl_4 has been equilibrated with an aqueous solution of 1 N KOH. These spectra show that in the presence of potassium the signal of the amide protons of D,L-valine exhibits a very slight downfield shift, and those of α-C–H protons of D,L-valine, D-hydroxyisovalirate, and L-lactate show very small upfield shifts. The degree of these shifts increased moderately in the presence of DNP[8] (Figure 1c), a weak uncoupler of oxidative phosphorylation in mitochondria, and increased greatly in the presence of the potent uncouplers FCCP (Figure 1d) and SF 6847 (Figure 1e).

In Figure 1 it is also interesting to note that uncouplers enhance the uptake of water (^1H_2O) into the organic phase, though accurate concentration of the water in the organic phase was not obtained at present.

From the known ^{1}H NMR spectral change of valinomycin with K^+ concentration,[10-12] it is most probable that the spectral changes reflect increase of K^+-complexed species of valinomycin in the CCl_4 phase, and that almost all the valinomycin molecules in the

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Table I. Relative Concentrations of Acids, K^+, and Valinomycin in CCl_4 Layer Equilibrated with Aqueous 1 N KOH Solution

acid	$[V{\cdot}K^+]/[V_T]^a$	$[acid]/[V_T]^b$
none	0.05 ± 0.05	
SF 6847[8]	0.95 ± 0.05	1.3 ± 0.2
FCCP[8]	0.90 ± 0.05	1.3 ± 0.2
DNP[8]	0.50 ± 0.15	1.3 ± 0.2
picric acid	0.80 ± 0.05	0.8 ± 0.2
phosphoric acid	0.05 ± 0.05	
benzoic acid	0.05 ± 0.05	0.0
ANS[8]	0.05 ± 0.05	0.0

[a] Molar ratio of valinomycin–K^+ complex to total valinomycin in the CCl_4 layer estimated from ^{1}H NMR measurements, shown as the average value for changes of the chemical shifts of the four different protons D,L-Val α-CH, D-Hyv α-CH, L-Lac α-CH, and D,L-Val CONH. [b] Molar ratio of acid to total valinomycin estimated from the integrated intensity of each proton signal in the ^{1}H NMR measurements.

CCl_4 phase complexed with K^+ in the presence of FCCP or SF 6847. It has been established[11,12] that the shifts (ν_{obsd}) of amide N–H and α-H protons in valinomycin are expressed as

$$\nu_{obsd} = (1 - X_{V{\cdot}K^+})(\nu_V) + (X_{V{\cdot}K^+})(\nu_{V{\cdot}K^+})$$

where, $X_{V{\cdot}K^+}$ is the fraction of valinomycin complexed with K^+. The chemical shifts of the protons in the complexed and the free valinomycin molecules are expressed as ν_V and $\nu_{V{\cdot}K^+}$, respectively. We can thus obtain $[V{\cdot}K^+]/[V_T]$, the relative molar ratio of valinomycin–K^+ complex to the total valinomycin, from the ^{1}H NMR spectral changes shown in Figure 1. The calculated values are given in Table I.

Figure 1c–e shows that proton signals of uncouplers appear besides the signals of valinomycin: the aromatic protons (δ 6.2, 7.6, 8.4) of DNP in Figure 1c, the aromatic protons (δ 6.9, 7.2) of FCCP in Figure 1d, and the aromatic protons (δ 7.6) and the vinyl proton (δ 7.3) of SF 6847 in Figure 1e. From the relative area–intensities of these ^{1}H NMR absorptions, the molar ratios of various acids (uncouplers) to the total valinomycin $[V_T]$ were calculated and are shown as $[acid]/[V_T]$ in Table I.

The values of $[V{\cdot}K^+]/[V_T]$ in Table I show that the uptake of potassium ion into the organic phase is greatly enhanced in the presence of potent uncouplers, FCCP and SF 6847, and that this enhancement is less in the presence of picric acid, which is a typical "hydrophobic" anion that extracts cations from the aqueous phase into the organic phase by forming ion-pair complexes. Here, it has been confirmed that in the absence of valinomycin any organic acid listed in Table I was not detected in the CCl_4 layer by ^{1}H NMR, i.e., the transfer of the organic acid to the organic phase is negligibly small.

Table I shows that the values for the relative molar ratio of acid to valinomycin in the organic phase ($[acid]/[V_T]$) of the acids SF 6847, FCCP, and DNP are about unity, indicating that most of the potassium-complexed valinomycin forms 1:1 ion-pair complexes with these anions (uncouplers). On the contrary, in cases of ANS, benzoic acid, and phosphoric acid no enhancement of K^+ uptake was observed.

For confirmation of the above experimental results determined by NMR, partition experiments were carried out to determine the amounts of K^+ in the aqueous KOH and chloroform phases in the presence of valinomycin and various acid molecules, and the extraction constant K_{ext} of K^+ was determined by measuring the amount of K^+ remaining in the aqueous phase by flame photometry (Table II). Table II indicates that SF 6847 and FCCP markedly enhance the transfer of K^+ to the organic (chloroform) phase and that the extraction constants, K_{ext}, were in the following order, SF 6847 > FCCP > PCP > picric acid > DNP. Benzoic acid had no influence on the transfer of K^+. These results correspond well with those obtained by NMR.

Discussion

There is much evidence that valinomycin-mediated potassium uptake is enhanced by anions, and results have suggested that the process of uptake is due to formation of a ternary complex of 1:1:1

Table II. Extraction Constants, K_{ext},[a] in Relation to the Uncoupling Activities

acid	log K_{ext}, $mol^{-2} \cdot L^2$	log (uncoupling activity)[b]
SF 6847	9.04 ± 0.2	8.3
FCCP	8.95 ± 0.2	7.3
PCP	6.87 ± 0.2	5.0
DNP	6.46 ± 0.2	4.3
picric acid	7.42 ± 0.2	NE[c]
benzoic acid	below 5.5	NE[c]

[a] Obtained by flame photometry (see "Experimental Section"). Transfer to the organic phase (denoted as o) of potassium ion is assumed mainly to be by extraction of K^+ and the anion (A^-) in the aqueous phase (denoted as aq) with valinomycin (V), by formation of a 1:1:1 ternary complex of valinomycin, K^+, and the anion ($V\text{-}K^+\text{-}A^-$). The extraction constant (K_{ext}) is thus given by $V_o + K^+_{aq} + A^-_{aq} \rightleftharpoons (V\text{-}K^+\text{-}A^-)_o$, $K_{ext} = [V\text{-}K^+\text{-}A^-]_o / \{[V]_o[K^+]_{aq}[A^-]_{aq}\}$. Where there is a 1:1:1 stoichiometry between valinomycin, K^+, and the anion, the concentration of each species is determined assuming that under the experimental conditions valinomycin is present only in the organic phase and the anion is in the ionized form in the aqueous phase. K_{ext} is determined under the conditions where about 10–80% of K^+ is extracted, except for the case of DNP (7–11%). [b] Determined with rat liver mitochondria, using succinate (plus rotenone) as substrate at pH 7.4 and 25 °C as described in ref 18. [c] Non-effective.

stoichiometry between valinomycin, potassium ion, and an anion.[1-3,7,13,26] In this work we obtained direct evidence for the existence and stoichiometry of this ternary complex in the course of potassium movement by quantitative determination of each of these three species by NMR spectroscopy and flame photometry.

Our results showed that valinomycin in the organic phase takes up K^+ only in the presence of an organic anion as a gegenion, and the potent uncouplers SF 6847 and FCCP were the most effective gegenions. The effects of weakly acidic uncouplers on potassium extraction were in the order of SF 6847 > FCCP > PCP > DNP. This order is in good agreement with their order of uncoupling activity in mitochondria, SF 6847 > FCCP > PCP > DNP, which is directly related to their protonophoretic ability.[14-18] The synergistic actions of uncoupler and valinomycin (as a model of potassium translocator) in the translocation of K^+ and H^+ across the membrane could be important for the action of uncouplers, as proposed by Yamaguchi and Anraku.[5,6] It is of interest to note that picric acid, which is an effective uncoupler in submitochondrial particles but almost ineffective in mitochondria,[19] is more active in the extraction of K^+ than DNP, which is a very weak uncoupler in chloroplasts, but becomes effective on addition of valinomycin.[20,21]

From the finding that in the presence of valinomycin and K^+ the fluorescence of ANS was quenched on addition of FCCP, O'Brien et al.[4] suggested that the mode of interaction of ANS with valinomycin–K^+ in aqueous solution is essentially the same as that of FCCP. However, Haynes and Pressman,[7] and Marinetti et al.[13] concluded, from studies on the extraction of K^+ in a two-phase partition system and on the permeation of ANS through erythrocyte membranes, that this ion does not form an ion-pair complex with valinomycin–K^+. Our results showed that the mechanism of interaction of ANS anion with valinomycin–K^+ is quite different from that of other "hydrophobic" anions (uncouplers), and that the other anions except uncouplers of oxidative phosphorylation have no effect on the formation of valinomycin–K^+ complex in the organic phase.

Furthermore, the partition coefficient of the neutral form of uncouplers between organic solvent and water, as a measure of the hydrophobicity, estimated by the additive rule of the hydrophobic substituent constant (π),[22] is in the order picric acid < DNP < benzoic acid ≪ FCCP < PCP < SF 6847. This order does not correspond to the order of the effectiveness in the valinomycin-mediated K^+ extraction nor the uncoupling activity. The ability of uncoupler anions to form an ion-pair complex with valinomycin–K^+ may be related to the hydrophobicity of the anionic form, not of the neutral form, of uncouplers. The concept of *the hydrophobicity of the organic acid anion* must be established.[23] Thus, the ability of anions to form ion-pair complexes with valinomycin–K^+ does not depend simply on the hydrophobicity in their neutral forms; in other words, some unique molecular structures should be needed to form the ion-pair complex effectively.

At present it is not clear why potent uncouplers easily form ion-pair complexes with valinomycin–K^+ or what molecular structure of the anion is required for formation of an ion-pair complex. Recently we found that the potent uncoupler SF 6847 takes a very unique dynamic structure in solution,[24] and that SF 6847 anion in the ternary complex in solution is more flexible in its intramolecular tumbling motion than the uncomplexed free SF 6847 anion, contrary to expectation.[25] The dynamic behavior of SF 6847[25] is expected to control the hydrophobicity of SF 6847 anion. Studies on the structural requirements of uncouplers and hydrophobic anions for formation of the ternary complex are of importance in obtaining fundamental information on the mechanism of ion translocation and related events in artificial- and biomembrane systems.

In this article, it was also shown that water molecules are transferred into the organic phase accompanied by the formation of the ternary complex. This may be due to the change in the environment of the organic phase because of the presence of the ionic species, i.e., the ternary complex, though the manner of the interaction of water molecules with the ternary complex in the organic phase is not clear at present. Quantitative measurements of the amount of water molecules transferred into the organic phase are now in progress.

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