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Registry No. Indium oxide, 1312-43-2; cytochrome *c*, 9007-43-6.

Restricted Intramolecular Rotation of the Potent Uncoupler of Oxidative Phosphorylation of SF 6847 ((3,5-Di-*tert*-butyl-4-hydroxybenzylidene)malononitrile): Enhanced Motional Freedom of SF 6847 Anion by Formation of a 1:1:1 Complex with Valinomycin and K^+

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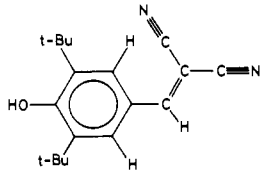
Abstract: The dynamic structure of a potent uncoupler of oxidative phosphorylation, (3,5-di-*tert*-butyl-4-hydroxybenzylidene)malononitrile (SF 6847), was studied by ^1H NMR under various conditions. It was found that the degree of intramolecular motional freedom around the C–C bond between the benzene ring and the malononitrile moiety changes greatly depending on the environment. The freedom of the intramolecular motion (restricted rotation) was greatly reduced by change from the neutral to the anionic form. The freedom of the intramolecular motion in the SF 6847 anion increased on formation of a ternary complex with valinomycin and potassium ion. This unique dynamic structure of SF 6847 is discussed in connection with the acidity and potent biological activity of this uncoupler.

Uncouplers of oxidative phosphorylation abolish the link between substrate oxidation and ATP synthesis in energy-transducing membranes. Various kinds of organic molecules of synthetic fungicides, acaricides, and herbicides exhibit very potent uncoupling activity.[1] The most potent uncoupler known to date is SF 6847 ((3,5-di-*tert*-butyl-4-hydroxybenzylidene)malononitrile),[2,3] which is effective in mitochondria under usual experimental conditions at about 10 nM, while the well-known "classical" uncoupler 2,4-dinitrophenol is effective at about 50 μM. The molecule of SF 6847



is characterized by hydrophobic *tert*-butyl groups, a strong electron-withdrawing malononitrile group, and an acid-dissociable phenolic hydroxyl group.[1] Other potent uncouplers, such as FCCP,[4,5] TTFB,[6] and S-13,[7] have similar structural features. The structural requirements of potent uncouplers for uncoupling activity are still unclear, although extensive studies have been done on this problem from various points of view.[8-15]

These strong uncouplers are thought to be carriers of H^+; the uncoupler cycles in the membrane alone or in the form of a complex with a hypothetical cation translocator, the uncoupler anion picking up H^+ on one side of the membrane–water interface and releasing it on the other side.[1,16-19] Valinomycin is sometimes taken as a model of cation translocators of the latter type.[20,21]

It has been generally thought[19] that the anionic form of potent uncouplers has a planar structure and so is stable in a hydrophobic environment owing to delocalization of the negative charge. Thus, the conformation of the SF 6847 anion is also expected to be planar: the phenol ring and the malononitrile group are thought to be coplanar. However, we found[22,23] that the planar form is quite unstable and that the malononitrile moiety tumbles over the

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Table I. Kinetic Parameters for the Restricted Intramolecular Rotation

molecular form	$\Delta\nu$/Hz[d]	T_c/k[e]	$\Delta G^{\ddagger}(T_c)$/ (kJ/mol)	$\Delta H^{\ddagger}$/ (kJ/mol)	$\Delta S^{\ddagger}$/ (J/(mol K))	k/s^{-1} (at 25 °C)
SFH (neutral form)[a]	148 ± 25[f]	165 ± 2	31.3 ± 1.0	26.3 ± 3.3	−30 ± 20	(1.3–12) × 10^6
SF$^-$ (anionic form)[b]	109[g] (242)[h]	327 ± 2[g]	65.4 ± 0.5	27.8 ± 3.0	−115 ± 10	77–80
Val–K$^+$–SF$^-$ [c]	234[f]	175 ± 5	32.9 ± 1.0	28.2 ± 3.2	−27 ± 18	(1.1–6.6) × 10^6

[a] Solvent diethyl-d_{10} ether. [b] Solvent methanol-d_4–KOH. [c] Solvent CD_2Cl_2. [d] Chemical shift difference between the two aromatic protons. [e] Coalescence temperature. [f] Measured at 200 MHz on a JEOL FX-200 NMR spectrometer. [g] The value is taken from ref 23 in which it was determined at 90 MHz. [h] Converted to the scale of 200-MHz resonance frequencies by a factor of 109 × (200/90).

energy barrier at the right angular conformation between the phenol ring and the malononitrile moiety.

We also found[24] that potent uncouplers greatly enhance valinomycin-mediated potassium uptake from the aqueous into the hydrophobic environment in a two-phase membrane system by forming a ternary complex with valinomycin and potassium. The ability of an uncoupler to transfer potassium to a hydrophobic region is not dependent on the hydrophobicity of the neutral form of uncoupler molecule.[24]

As an extension of these studies, using ^{1}H NMR we determined the kinetic parameters for the intramolecular motion of SF 6847 in different chemical states, i.e., the neutral form (SFH), the anionic form (SF$^-$), and SF$^-$ in the 1:1:1 ternary complex (Val–K$^+$–SF$^-$) with valinomycin and K$^+$. This paper deals with the marked differences in the freedom of the intramolecular tumbling motion (restricted intramolecular rotation) of SF 6847 in these states.

Experimental Section

SF 6847 was a gift from Dr. Y. Nishizawa, Sumitomo Chemical Industry, Osaka (Japan). Valinomycin was purchased from Sigma Chemical Co., St. Louis. Other reagents were commercial products and were used without further purification. For preparation of Val–K$^+$–SF$^-$, a volume of 0.5 mL of CD_2Cl_2 containing 3 mM valinomycin was equilibrated with an aqueous solution (1 mL) of 1 N KOH containing SF 6847 (30 mM). After equilibration, the CD_2Cl_2 phase was transferred to an NMR sample tube (5 mm i.d.). All the NMR spectra (SFH and Val–K$^+$–SF$^-$) were measured at 200 MHz with a JEOL FX-200 NMR spectrometer using the pulsed Fourier-transform mode.

Results

Figure 1 shows the temperature dependence of the ^{1}H NMR spectrum of the neutral form of SF 6847 (SFH) in diethyl-d_{10} ether. As can be seen, the aromatic protons ortho to the malononitrile moiety of SFH show a single resonance down to −101 °C from −50 °C, the shape of the signal being broaded with decrease in the temperature. At −114 °C, the signal separated into two broad resonances. The coalescence of the signal of the aromatic protons was found at −108 °C (165 K). These results indicate that the intramolecular motion of the malononitrile moiety decreases with decrease in the temperature as reflected by the signal of the aromatic protons. In the case of the anionic form of SF 6847 (SF$^-$),[23] we found that the signal of the aromatic protons changed in a similar manner to that of SFH. However, the coalescence temperature was found to be 54 °C (327 K),[23] being much higher than that of SFH.

Next, we measured the ^{1}H NMR spectrum of SF$^-$ in the 1:1:1 ternary complex with the ionophore valinomycin and potassium (Val–K$^+$–SF$^-$) in CD_2Cl_2 at various temperatures. As shown in Figure 2, the aromatic protons of SF$^-$ exhibit a single resonance at room temperature. With decrease in the temperature, the signal becomes broad (at −73 and −81 °C), disappears (at −95 °C, the coalescence temperature), and then separates into two resonances (at −104 and −114 °C). It is interesting to note that the coalescence temperature of SF$^-$ in Val–K$^+$–SF$^-$ is much lower than that of free SF$^-$, but is similar to that of SFH.

The kinetic parameters of the intramolecular motion of the malononitrile moiety can be determined by the line-shape analysis of the temperature dependence of the signal pattern of the aromatic protons in SF 6847. Table I summarizes various parameters characteristic of the intramolecular motion of SFH, SF$^-$, and SF$^-$

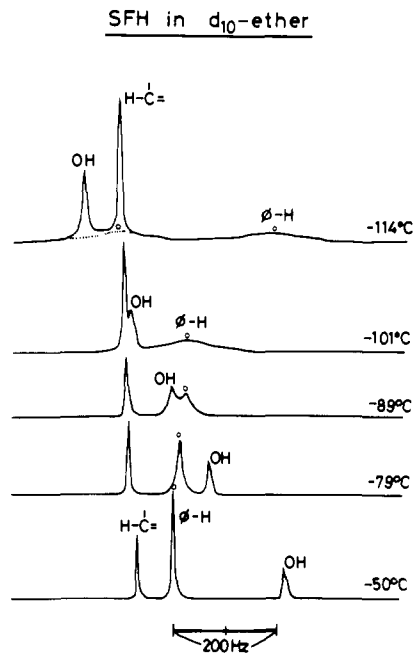

Figure 1. Temperature dependence of 200-MHz ^{1}H NMR spectra of SFH in diethyl-d_{10} ether. At −50 °C, the chemical shifts from tetramethylsilane are δ 8.17 for the aromatic protons and δ 8.34 for the benzylidene proton.

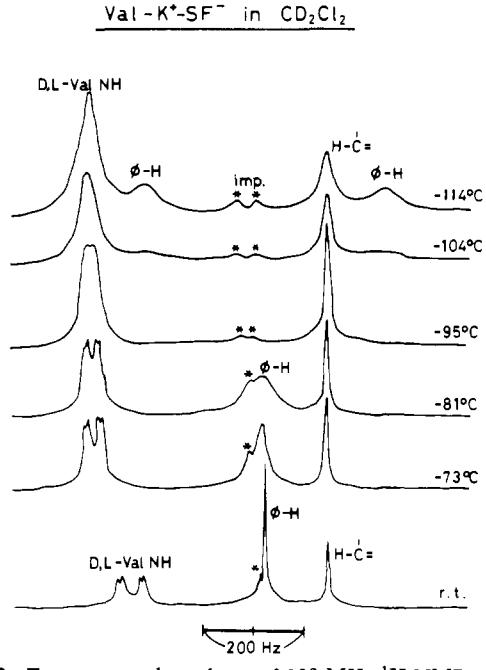

Figure 2. Temperature dependence of 200-MHz ^{1}H NMR spectra of Val–K$^+$–SF$^-$ in CD_2Cl_2. At room temperature (31 °C), the chemical shifts from tetramethylsilane are δ 7.27 for the benzylidene proton and δ 7.58 for the aromatic protons. imp, signal due to impurity.

in Val–K$^+$–SF$^-$. It is of particular interest in the table that the intramolecular motional freedom of the SF 6847 anion is *enhanced*

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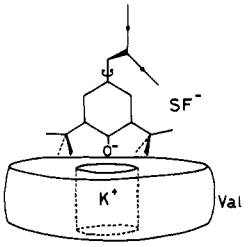

Figure 3. Schematic representation of the manner of interaction between SF^- and Val-K^+ deduced from this study.

upon formation of the ion-pair complex Val-K^+-SF^-. The calculated rate constant, k, of the intramolecular motion at the same temperature (25 °C) is about *10^5 times greater* for SF^- in the ternary complex than for SF^-. On the other hand, in the neutral form of SF 6847 the k value at 25 °C is 1.3×10^6 to 12×10^6 s^{-1}, being the same order of magnitude of the value with the complexed SF^- (1.1×10^6 to 6.6×10^6 s^{-1}). This means that on acid dissociation from SFH to SF^- the intramolecular motion becomes significantly restricted,[22,23] but is again activated on complex formation with valinomycin and K^+.

Discussion

Previously we made molecular orbital studies (CNDO/2 and STO-3G) on the conformation of the derivatives of SF 6847, (4-hydroxybenzylidene)malononitrile (SF-2H), and (3,5-dimethyl-4-hydroxybenzylidene)malononitrile (SF-2Me), in which two *tert*-butyl groups ortho to the OH group of SF 6847 are substituted with H and CH_3, respectively.[22,23] We found that the conformational energy of these compounds in both the neutral and anionic forms is dependent on the dihedral angle (θ) between the aromatic ring and the malononitrile moiety ($\theta = 0°$ and 90° when the malononitrile moiety is coplanar and perpendicular, respectively, to the aromatic ring),[22,23] as shown in the following. In both the neutral and anionic forms, (i) the twisted structure between the malononitrile moeity and the benzene ring is the most stable at about $\theta = 40°$ (according to the CNDO/2 MO calculation), (ii) the energy barrier at $\theta = 0°$ is highest (70–80 kJ/mol for both molecular forms of SF-2Me), i.e., the coplanar structure is quite unstable, (iii) the energy barrier at $\theta = 90°$ is small (being about 20 kJ/mol and less than 10 kJ/mol for the anionic and neutral form, respectively, of SF-2Me), and (iv) the energy barrier at $\theta = 90°$ in the neutral form is smaller than that in the anionic form. From the results of these studies and those of NMR spectrometry reported here and in the previous paper,[22,23] it is concluded that the malononitrile moiety tumbles beyond the energy barrier at $\theta = 90°$ on either side and does not rotate freely.

In the present study the kinetic parameters of the intramolecular restricted rotation of the malononitrile moeity of SFH and SF^- in Val-K^+-SF^- were determined quantitatively through the measurement of ^1H NMR spectra with use of a superconducting magnet below −100 °C. In the previous work[23] these parameters could have been determined only with free SF^-.[23] In this study it is clarified that the intramolecular motion of SF^- changed remarkably on protonation and complex formation with valinomycin and potassium.

The changes of the intramolecular motional freedom of SF 6847 in different environments could be explained as follows. So that the large steric repulsion between the hydrogen atom of the benzene ring (ortho to the malononitrile moiety) and the nitrile group may be avoided, the malononitrile moiety in the neutral form (SFH) is twisted considerably relative to the benzene ring, making the energy barrier in the rectangular conformation rather small. Upon proton dissociation, the negative charge on the oxygen atom is delocalized due to enhancement of the conjugation between the benzene ring and the π-electron-withdrawing group of the malononitrile moeity, and as a result the nonplanarity is reduced in the anionic form (SF^-). This effect is related to the fact[23] that SF 6847 still keeps a moderate pK_a value ($pK_a = 6.83$), overcoming the steric hindrance of the bulky, *tert*-butyl groups that usually weakens the acidity of phenols. It is noteworthy that the pK_a of the corresponding unhindered phenol, SF-2H, is 7.25.[23]

Nonplanarity is enhanced in Val-K^+-SF^-, which implies that the negative charge tends to be localized on the oxygen atom and the benzene ring due to reduction of the π conjugation between the benzene ring and the malononitrile group. Thus it is probable that SF^- interacts with Val-K^+ in the manner shown schematically in Figure 3; the localization of the negative charge, induced by the increase in the dihedral angle θ, is energetically favorable for electrostatic attraction between SF^- and Val-K^+.

Here it should be remembered that potent uncouplers greatly enhance valinomycin-mediated potassium uptake into a hydrophobic enviroment by forming a 1:1:1 complex.[24] Enhanced freedom of the intramolecular restricted rotation, therefore, increases the stability of the complex of Val-K^+-SF^- in a hydrophobic environment. Since the effects of various uncouplers of oxidative phosphorylation, including SF 6847, on the valinomycin-mediated potassium transfer to the hydrophobic region correlated well[24] with the order of their uncoupling activities in mitochondria, the unique dynamic structure of SF 6847 would be directly related to the exhibition of its potent activity. In this connection the uncoupling mechanism in concert with the intrinsic ionophore should particularly be noted.

Contrary to our findings, results in X-ray diffraction studies showed that the benzene ring and the malononitrile moiety of SF 6847 are coplanar both in SFH[25] and in SF^- in an ion-pair complex with the ionophore tetranactin and K^+.[26] The marked difference between the conformation of SF 6847 in crystals and in solution is noteworthy. In crystals, the SF 6847 molecule is forced to be packed tightly, and this is why in crystals SF 6847 takes the planar form. Quite recently, *X*-ray analysis of the crystal structure of the complex of valinomycin, K^+, and the picrate anion showed[27] that the interaction between picrate anion and valinomycin-K^+ is weak. Our result also showed that the intramolecular motion of SF^- is not restricted in the complex of Val-K^+-SF^-, and thus the electrostatic interaction between SF^- and K^+ complexed with valinomycin is probably not so strong as to make a rigid complex.

In conclusion the molecule of SF 6847 is characterized with a very unique dynamic structure, in which motional freedom is enhanced upon formation with the cationic site in the hydrophobic region. It is generally believed that the motional freedom of substrates, hormones, and other bioactive compounds becomes reduced on interaction with their site of action on biopolymers, such as enzymes and receptors. However, the present study indicates that this is not always the case. Fluctuation of molecular structure of bioactive compounds is expected to play a key role for exhibition of their activities. Thus the dynamic structure of various bioactive compounds is very important in understanding the molecular mechanism of their actions.

Registry No. SFH, 10537-47-0; SF^-, 83747-00-6; Val-K^+-SF^-, 83746-99-0.

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