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MOLECULAR BASIS OF THE PROTONOPHORIC AND UNCOUPLING ACTIVITIES OF THE POTENT UNCOUPLER SF-6847 ((3,5-DI-*tert*-BUTYL-4-HYDROXYBENZYLIDENE)MALONONITRILE) AND DERIVATIVES

REGULATION OF THEIR ELECTRONIC STRUCTURES BY RESTRICTED INTRAMOLECULAR ROTATION

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We reported recently (Yoshikawa, K. and Terada, H. (1982) J. Am. Chem. Soc. 104, 7644–7646) that the potent uncoupler of oxidative phosphorylation SF-6847 ((3,5-di-*tert*-butyl-4-hydroxybenzylidene)malononitrile) shows unique intramolecular restricted rotation of the malononitrile moiety. In this study, values for the activation energy E_a of the restricted rotation of SF-6847 derivatives with the same alkyl chain R in both *ortho* positions of the phenolic hydroxyl group were determined from the temperature-dependent change in the ^{1}H-NMR signals of their aromatic protons. The E_a values of the neutral forms of these derivatives were found to be the same irrespective of R, but those of the anionic forms increased with increase in the alkyl chain length of R. It was found that the restricted rotation of the malononitrile moiety regulates its electron-withdrawing ability in such a way as to keep the acid dissociability of these derivatives similar, overcoming the effect of steric hindrance by R. The protonophoric activity of these derivatives, in a phospholipid bilayer membrane and their uncoupling activity in rat-liver mitochondria were both found to depend on E_a of their anionic forms. The stability of the uncoupler anions regulated by the restricted rotation of the malononitrile group in a nonpolar membrane environment was found to be important for exhibition of these activities. The hydrophobicity of the anionic forms of these derivatives was suggested also to be affected by the intramolecular rotation.

Introduction

There are many reports that uncoupling of the link between substrate oxidation and ATP synthesis by weakly acidic uncouplers is due to their action as protonophores in proton-impermeable energy synthesizing membranes (cf. reviews in Refs. 1–4), although other mechanisms are also possible for the actions of versatile uncouplers [3,5,6]. The induction of increased electrical conductivity across a phospholipid bilayer membrane (black lipid membrane) by uncouplers [7–11], and the swelling of liposomes and mitochondria associated with proton transfer [12–15] have been regarded as indications that the uncoupling is due to the protonophoric action of uncouplers.

For exhibition of the protonophoric activity of weakly acidic uncouplers, it has been considered that their anionic form takes a flat structure in the membrane, allowing as effective withdrawal as

Abbreviations: SF-6847, (3,5-di-*tert*-butyl-4-hydroxybenzylidene)malononitrile; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone.

possible of the polar negative charge by the strong electron-withdrawing group, which is situated at a certain distance from the acid-dissociable group in the uncoupler molecule. Thus, the polarity of the anionic form of uncouplers is minimized by delocalization of their negative charge [1].

However, we found recently that the flat structure of the most potent uncoupler SF-6847 is energetically most unstable [16,17], and that SF-6847 exhibits a unique dynamic structure, as depicted schematically in Fig. 1: the strong electron-withdrawing malononitrile group at the *para* position of the phenolic hydroxyl group tumbles to both sides over the energy barrier at the position where the phenol ring and malononitrile moiety are at right angles ($\theta = 90°$, $\theta = 0°$ in the flat structure) [16,17]. This restricted intramolecular rotation regulates the electronic structure of the SF-6847 anion, and owing to this, SF-6847 anion is able to extract K^+ very effectively from an aqueous to an oil phase in the presence of valinomycin by forming a 1 : 1 : 1 ternary complex with valinomycin and K^+ [18,19].

It seems of great importance for elucidation of the molecular mechanism of the protonophoric action and related biological actions, such as uncoupling, to examine whether such a dynamic structure is directly related to these activities. Thus, in this study, we determined the activation energy E_a of the intramolecular restricted rotation of the anionic and neutral forms of SF-6847 and its derivatives. The relations of E_a with the protonophoric activity, determined as induction of increase in electrical conductance of a phospholipid bilayer membrane, and with the uncoupling activity in rat-liver mitochondria were examined. The effects of the restricted rotation of a series of SF-6847 derivatives on their electrical and hydrophobic properties were also investigated.

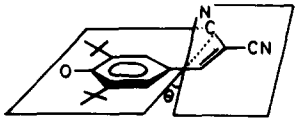


Fig. 1. Restricted intramolecular rotation of the malononitrile moiety of SF-6847. θ, angle between the benzene ring and the malononitrile moiety.

Materials and Methods

SF-6847 was obtained from Wako Pure Chemical Industries, Osaka (Japan). All the other 3,5-dialkylbenzylidene malononitriles were prepared by the procedure of Horiuchi et al. [20] and purified by column chromatography and repeated recrystallization. Soybean phosphatidylcholine and cholesterol were obtained from Nakarai Chemical Company, Kyoto (Japan) and Sigma Chemical Company, St. Louis (USA), respectively.

Fourier-transformed ^{1}H-NMR spectra of the neutral and anionic forms of SF-6847 derivatives were recorded at 90 MHz with a JEOL FX-90Q NMR spectrometer or at 200 MHz with a JEOL FX-200 NMR spectrometer.

Molecular orbital calculations were performed by the complete neglect of differential overlap (CNDO)/2 method as described previously [16]. Since the energy of the anionic form of SF-6847 derivatives did not converge in the usual CNDO/2 program, we adopted a modified program using the density matrix method proposed by McWeeny [21]. Bond angles and bond lengths used in calculations were deduced from the crystal geometry of SF-6847 determined by X-ray diffractometry [22].

The acid dissociation constants pK_a of SF-6847 derivatives were determined spectrophotometrically with a Shimadzu recording spectrophotometer UV 3000 as reported previously [23].

A solution of 10 mg soybean phosphatidylcholine/5 mg cholesterol/1 ml *n*-decane, which had been distilled and passed through a column of activated alumina, was used to form a bilayer membrane across a hole of about 1 mm diameter in the wall of a Teflon cell. The electrical conductivity of the bilayer membrane was measured at 25°C, as described previously [24]. The bathing solution comprised 100 mM KCl/25 mM Tris-HCl buffer (pH 7.0).

Uncoupling activity was determined as the minimum concentration of uncoupler required for the maximum release of State 4 respiration of rat-liver mitochondria with succinate as substrate in incubation medium comprising 200 mM sucrose/2 mM $MgCl_2$/1 mM EDTA/10 mM potassium phosphate buffer (pH 7.4) [15,23].

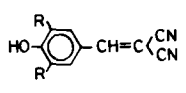
Results

Physical properties of SF-6847 derivatives

In this study, we used the uncoupler SF-6847 and derivatives of SF-6847 with the same alkyl groups, R, at both *ortho* positions of the phenolic hydroxyl group, as shown in Scheme I. For simplicity, we used abbreviations for these derivatives such as SF-2H and SF-2*tert*Bu (= SF-6847) for those in which both R groups were hydrogen and *tert*-butyl groups, respectively. The neutral and anionic forms of SF-6847 derivatives are referred to as SFH and SF^-, respectively.

Fig. 2A shows the temperature dependence of the ^{1}H-NMR spectrum of the neutral form of SF-2Me. As in the spectrum of SF-6847 [19], the signals of aromatic protons *ortho* to the malononitrile moiety are temperature-dependent; the signal shows a single resonance, but this becomes broader as the temperature decreases, and at -112°C, it separates into two peaks, indicating that the malononitrile moiety of SF-2Me does not remain in the same plane as the aromatic ring, but exhibits restricted rotation [19]. Other derivatives showed similar temperature-dependent ^{1}H-NMR spectra. The activation energies E_a of the restricted intramolecular motions of the neutral and anionic forms of these compounds were determined from the lifetimes (τ) at various temperatures of the restricted rotations, which were determined by simulation of the spectra (cf. Fig. 2B). These values, together with those reported previously [17], are listed in Table I.

The values of E_a of the neutral forms of this series of compounds were found to be the same, irrespective of R. On the other hand, the values of E_a of the anionic forms increased with increase in the alkyl chain length. Thus, the intramolecular tumbling motion of SF^- becomes less and its coplanarity becomes greater with increase in the alkyl chain length of R. It is noteworthy that the increase in E_a per CH_2-group in R was 4 kJ · mol^{-1} in the series of anions with R of hydrogen to an



Scheme I. Structure of SF-6847 derivatives.

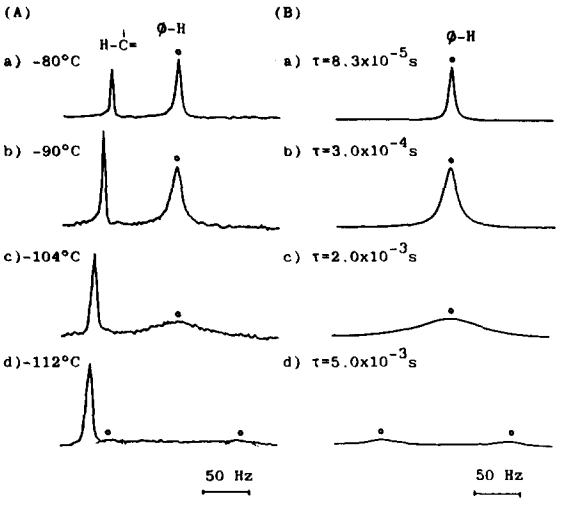


Fig. 2. ^{1}H-NMR spectra of the neutral form of SF-2Me at various temperature in diethyl-d_{10} ether measured at 200 MHz (A) and their simulated spectra (B).

iso-propyl group. However, this linear increase of E_a was not observed with more bulky groups, such as secondary butyl and tertiary butyl groups: the increase in E_a from SF-2isoPr anion to SF-2*sec*Bu anion was very small, while that from SF-2isoPr anion to SF-2*tert*Bu anion was very large. Furthermore, the fact that E_a of the SF^- form is consistently greater than that of the SFH form indicates that the rotation of the malononitrile moiety of the anionic forms of SF-6847 derivatives is more restricted than that of their neutral forms.

The λ_{max} of the absorption spectrum of the neutral form of SF-6847 is at 362 nm and that of the anionic form is at 455 nm, as reported previously [23]. From the pH-dependence of the absorbance at these two wavelengths, the acid dissociation constant pK_a was determined as 6.80. The pK_a values of the SF-6847 derivatives and the values of λ_{max} of their neutral and anionic forms were determined similarly and are listed in Table I.

As seen in Table I, the introduction of methyl (SF-2Me), ethyl (SF-2Et) and iso-propyl (SF-2isoPr) groups at both positions *ortho* to the phenolic OH of SF-2H had little effect on the acid dissociation of the phenolic OH, whereas the introduction of secondary butyl groups (SF-2*sec*Bu)

TABLE I

PHYSICOCHEMICAL PROPERTIES AND PROTONOPHORIC AND UNCOUPLING ACTIVITIES OF SF-6847 DERIVATIVES

Uncoupler	E_a (kJ·mol^{-1})		λ_{max} (nm)		$\Delta(1/\lambda_{max})$ [a] (nm^{-1})	pK_a	Π_R [b]	log $1/C_{blm}$ [c]	log $1/C_{unc}$ [d]
	SFH	SF$^-$	SFH	SF$^-$					
SF-2H	31	42	350	415	$4.48 \cdot 10^{-2}$	7.25	0	5.20	4.51
SF-2Me	32	46	362	437	$4.74 \cdot 10^{-2}$	7.31	0.56	5.75	5.57
SF-2Et	31	50	362	440	$4.90 \cdot 10^{-2}$	7.20	1.02	6.90	6.32
SF-2isoPr	31	54	362	443	$5.05 \cdot 10^{-2}$	7.34	1.53	7.46	6.58
SF-2*sec*Bu	31	55	361	445	$5.23 \cdot 10^{-2}$	7.60	2.07	7.50	6.74
SF-2*tert*Bu	31	64	362	455	$5.65 \cdot 10^{-2}$	6.80	1.98	8.50	7.60

[a] $\Delta(1/\lambda_{max}) = (1/\lambda_{max(SFH)} - 1/\lambda_{max(SF^-)})$.

[b] Hydrophobic substituent coefficients of substituent R groups are cited from Ref. 31, except that of the secondary butyl group. $\Pi_{sec\text{-}butyl} = \Pi_{isopropyl} + \Pi_{\text{-}CH_2\text{-}} = 1.53 + 0.54$.

[c] C_{blm} is the minimum concentration of uncoupler required to increase the electrical conductance of the phospholipid membrane.

[d] C_{unc} is the minimum concentration of uncoupler required for inducing maximal uncoupling.

caused an increase in pK_a. Conversely, introduction of two *tert*-butyl groups (SF-2*tert*Bu) resulted in a considerable decrease in the pK_a value. Thus, the hindered phenol SF-6847 is a stronger acid than SF-2H.

Except for that of SF-2H, the values of λ_{max} of the neutral forms of SF-6847 derivatives listed in Table I are almost the same, whereas those of the anionic forms show a red-shift as increase in length of the alkyl chain. The λ_{max} of SF$^-$ should reflect the degree of electrical conjugation governed by the coplanarity between the benzene ring and the malononitrile moiety, which is represented by the angle, θ, of the compounds (cf. Fig. 1). Thus, the difference between the reciprocal values of the λ_{max} of the neutral form and the anionic form ($\Delta(1/\lambda_{max}) = 1/\lambda_{max(SFH)} - 1/\lambda_{max(SF^-)}$) may be directly related to θ and also to the difference in the orbital energy between the highest occupied and lowest unoccupied orbitals ($\Delta E_{ho,lu}$). Indeed,

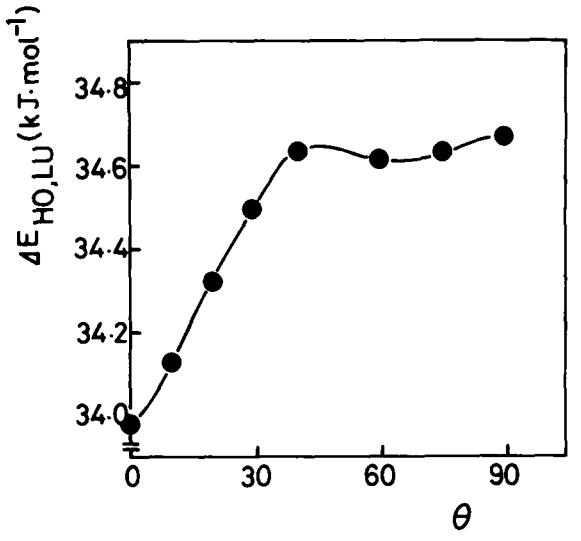


Fig. 3. Dependence of the difference in the orbital energy ($\Delta E_{ho,lu}$) of the SF-2H anion on the angle θ between the benzene ring and malononitrile moiety.

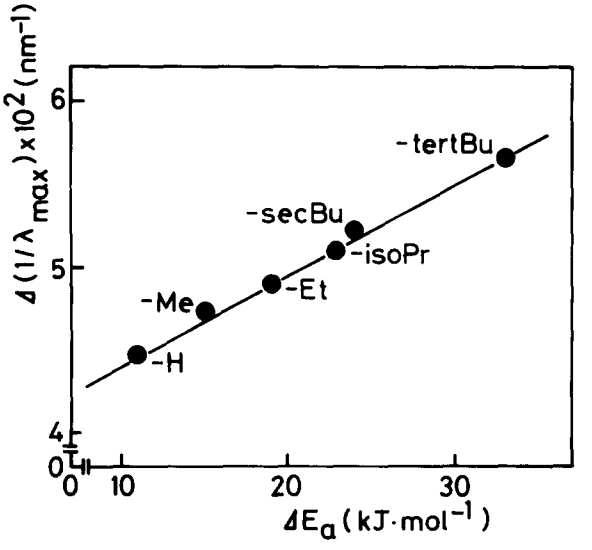


Fig. 4. Linear relation between $\Delta(1/\lambda_{max})$ and ΔE_a. For explanation of $\Delta(1/\lambda_{max})$ and ΔE_a, see text.

$\Delta E_{ho,lu}$ of SF-2H anion tends to increase with increase in θ up to $\theta = 40°$, as shown in Fig. 3. Furthermore, it is expected that $\Delta(1/\lambda_{max})$ is correlated with the difference in the values of activation energy E_a of the anionic and neutral forms (ΔE_a). This expectation is clearly confirmed by the good correlation between $\Delta(1/\lambda_{max})$ and ΔE_a shown in Fig. 4.

Protonophoric and uncoupling activities

The effects of SF-6847 derivatives on the electrical conductance of a lipid bilayer membrane composed of soybean phosphatidylcholine and cholesterol wree studied at pH 7.0. As shown in Fig. 5, SF-6847 derivatives began to cause an increase in the electrical conductance of the lipid bilayer at a certain concentration depending on the compound, and at above this concentration the conductance increased almost linearly with increase in the concentration of uncoupler with a slope of about unity. This increase has been interpreted as due to the proton transport mediated by the uncouplers [7–11]. The minimum concentration at which the increase in the electrical conductance took place (C_{blm}) became smaller as the length of the alkyl chain R increased. Values of C_{blm} are summarized in Table I as logs of their reciprocal values, log $1/C_{blm}$.

Fig. 6 shows the relation bteween log $1/C_{blm}$ and E_a of the SF^- forms of the compounds. The linear relation indicates that the protonophoric activity of SF-6847 derivatives depends on the intramolecular rotational motion of the malononitrile moiety in their anionic forms. Thus,

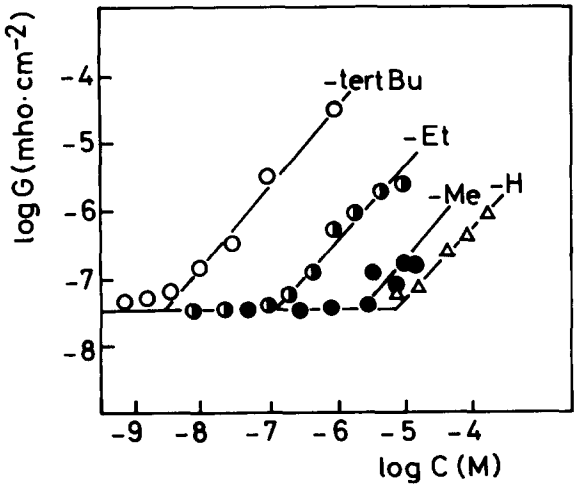


Fig. 5. Induction of electrical conductance of lipid bilayer membranes by SF-6847 derivatives. C, concentration of uncoupler.

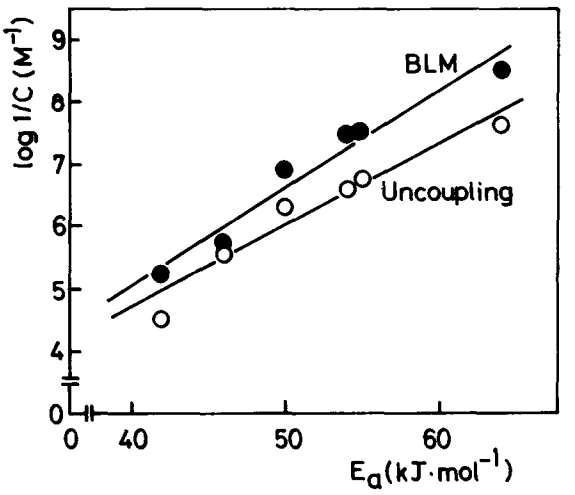


Fig. 6. Correlations between E_a and the protonophoric activity in a lipid bilayer membranes (BLM) and the uncoupling activity in rat-liver mitochondria (Uncoupling). These activities are expressed as logs of the reciprocal uncoupler concentrations (C) required for inducing these activities.

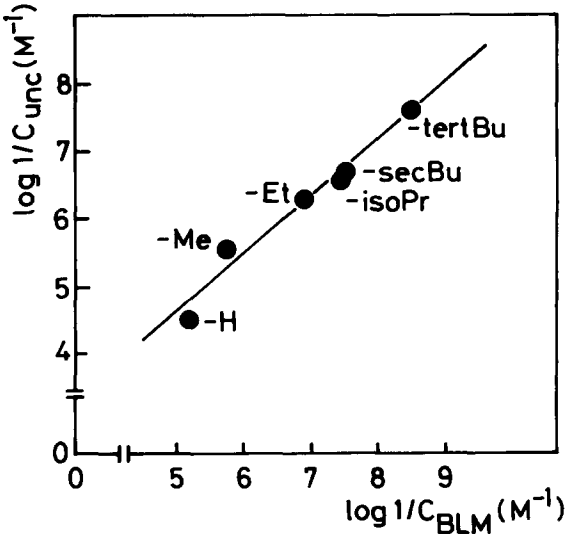


Fig. 7. Correlation between the effect of uncouplers on lipid bilayer membrane (C_{blm}) and that on mitochondria (C_{unc}). For details, see Fig. 6.

the stability of the anionic species of uncouplers in the membrane is suggested to play a decisive role in induction of proton transfer.

The uncoupling activities of SF-6847 derivatives in rat-liver mitochondria were determined as the minimum concentrations that induced maximum release of State 4 respiration (C_{unc}). These values are listed in Table I as logs of their reciprocal values, $\log 1/C_{unc}$. The role of the restricted intramolecular rotation of the malononitrile moiety in the uncoupling was examined by plotting $\log 1/C_{unc}$ vs. E_a for the SF^- forms (Fig. 6). Like the protonophoric activities, the uncoupling activities are linearly dependent on E_a of the SF^- forms, indicating that the intramolecular rotational motion of the malononitrile moiety is very important for exhibition of uncoupling activity. Accordingly, a close correlation between the protonophoric activity and the uncoupling activity was demonstrated, as shown in Fig. 7.

Discussion

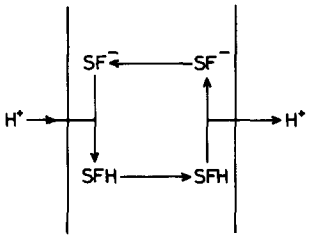
In this study, derivatives of the most potent uncoupler, SF-6847, in which the same alkyl groups, R, were introduced into both *ortho* positions of the phenolic hydroxyl group of 4-hydroxy-benzylidenemalononitrile (SF-2H) were found to exhibit restricted intramolecular rotation of the malononitrile moiety, as observed with SF-6847 (SF-2*tert*Bu) [17,19]. The rotational freedoms of the neutral forms of these derivatives are the same irrespective of R, and much greater than those of the anionic forms, which decrease with increase in the alkyl chain length of R. Thus, the unique restricted intramolecular rotation is concluded to regulate the electron-withdrawing ability of the malononitrile moiety of anionic forms of SF-6847 derivatives. This electron-withdrawing ability is reflected as a red-shift of the absorption spectra of the anionic forms from those of the neutral forms.

It was of interest to examine the effects of various alkyl groups, introduced at both *ortho* positions of SF-2H, on the intramolecular tumbling motion of the malononitrile group at the *para* position of the phenolic OH. These alkyl groups are considered to have two effects on the acid dissociation: (i) occlusion of the acid-dissociable group from the environment and (ii) reduction of the tumbling motion of the malononitrile group. The first effect, which is due to steric hindrance, reduces the acid dissociation, and the second effect, which is due to increase in coplanarity of the uncoupler molecule, enhances the acid dissociation. Thus, these effects influence the acid dissociation in opposite ways. The similar pK_a values of SF-2H, SF-2Me, SF-2Et and SF-2isoPr indicate that in these compounds the two opposite effects are well-balanced. However, in the case of SF-2*sec*Bu, the first effect is greater than the second, because the addition of a methylene group to the isopropyl group of SF-2isoPr anion only caused a slight increase in E_a, but the secondary butyl group is much more bulky than the isopropyl group. Conversely, in the case of SF-2*tert*Bu anion, the second effect is dominant due to the much greater E_a than that of SF-2*sec*Bu (cf. Table I).

This study showed that all the SF-6847 derivatives exhibit protonophoric actions in a phospholipid bilayer membrane, but to different degrees, as observed with other weakly acidic uncouplers [7–11]. Like their effects on a phospholipid bilayer membrane, the uncoupling activities of the SF-6847 derivatives are dependent on E_a of their SF^- forms. The close correlation between the uncoupling and protonophoric activities suggests that their uncoupling is based on the same type of action as their effect on a model phospholipid membrane.

The simplest model for the protonophoric action of uncouplers is a shuttle-type mechanism, as depicted schematically in Scheme II, where the anionic form of the uncoupler SF^- combines with a proton at the water/membrane interface to become the neutral form SFH, and then SFH moves to the other membrane surface, releases the proton to become SF^- and returns to the original membrane surface. This cycle of the uncoupler, causing proton transfer across the membrane and thus dissipating the proton motive force generated across the 'energized' membranes, results in uncoupling [1,3]. The availability of the mechanism shown in Scheme II was reported by Benz and McLaughlin [11] for the uncoupler FCCP.

According to the shuttle-type mechanism shown in Scheme II, the anionic form SF^- should exist stably in the nonpolar membrane environment.



Scheme II. Schematic representation of the shuttle-type protonophoric action of the uncoupler SF. SFH and SF^- are the neutral and anionic forms of SF, respectively.

The demonstration that the protonophoric activity of SF-6847 derivatives is governed by the E_a of their SF^- forms indicates that the stability of SF^- is regulated by the restricted intramolecular rotation of the malononitrile moiety. Furthermore, high hydrophobicities of both the SFH and SF^- forms are required to ensure that they remain in the membrane during their protonophoric and uncoupling actions. Usually, the hydrophobicity of a bioactive compound is expressed as the partition coefficient P of its neutral form between 1-octanol and water, and Π_R ($= \log P_R - \log P_H$, where P_R is the partition coefficient of the compound with substituent R and P_H is that of the parent compound) is denoted as the hydrophobic substituent coefficient of the substituent R [25,26]. The Π_R values of the derivatives are listed in Table I.

Since the partition coefficient P, or Π, of compounds determined between 1-octanol and water have been shown to be well correlated with their biological activities in biomembrane systems including mitochondria and phospholipid bilayer membranes [27–30], 1-octanol is regarded as similar to biomembranes in hydrophobic property. Thus, Π_R could be a good index for evaluating whether the uncoupling and protonophoric activities of SF-6847 derivatives are governed by the hydrophobicities of compounds.

These Π_R values show that the hydrophobicity of SF-6847 derivatives increases with increase in their alkyl chain length, and that the hydrophobicity of SF-2*sec*Bu is slightly greater than that of SF-2*tert*Bu. The protonophoric and uncoupling activities are governed by the hydrophobicities of uncouplers with R = H to R = isopropyl, while this relationship is reversed with SF-2*sec*Bu and SF-2*tert*Bu. From these facts and the fact that both activities are well-expressed in terms of the E_a values of their SF^- forms, the intramolecular rotational freedom of the malononitrile moiety of SF^- is suggested to regulate the hydrophobicity of SF^- as well as its stability.

Examination of the effect of restricted intramolecular rotation on the hydrophobicity of SF^- is worth further study, since although the hydrophobicity of ionic forms of bioactive compounds is expected to play a significant role in exhibition of their biological activities, it has as yet received little attention.

Acknowledgements

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