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Quantitative analyses of uncoupling activity of SF6847 (2,6-di-*t*-butyl-4-(2,2-dicyanovinyl)phenol) and its analogs with spinach chloroplasts

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Uncoupling activity with spinach thylakoids was measured for a series of acidic uncouplers related to the most potent uncoupler known until now, SF6847 (2,6-di-*t*-butyl-4-(2,2-dicyanovinyl)phenol). The activity of each compound with thylakoids was lower than that measured previously with rat-liver mitochondria. The two kinds of uncoupling activity were both linearly related to protonophoric potency across the liposomal membrane if the effect of the stability of the ionized form of the uncouplers in the membrane phase was considered. The difference in the apparent uncoupling activity between the two systems may be attributed to a difference in the intrinsic uncoupling activities at the phosphorylation assemblies. The results provided further evidence for the shuttle-type mechanism of weakly acidic uncouplers.

Introduction

We have quantitatively analyzed physicochemical factors governing both protonophoric potency across the phosphatidylcholine-liposomal membrane and the uncoupling activity with oxidative phosphorylation of rat-liver mitochondria for a number of weakly acidic uncouplers [1,2]. Uncoupling activity is linearly related with protonophoric potency, when the difference in the stability of the ionized form of the uncoupler molecules in the membrane phase and the difference in the extramembranous pH conditions are taken into account between liposomes and mitochondria. We took these findings to be evidence for the shuttle-type mechanism of uncoupling action in which the uncoupler molecules work as a protonophore and discharge a transmembrane proton gradient.

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Several investigations [1–4] have provided evidence of the shuttle-type mechanism, but the possibility that the uncoupler molecules specifically interact with functional membrane protein, probably with ATPase, could not be ruled out [5,6]. To establish a generally acceptable mode of action of weakly acidic uncouplers, it would be worthwhile to compare uncoupling activities between different energy-transducing membranes. In this study, we measured the uncoupling activity of a series of analogs of a very potent uncoupler, SF6847, with the photophosphorylation of spinach chloroplasts. We examined quantitatively the possible relationship between the uncoupling activity of the analogs with chloroplasts and their protonophoric potency across the liposomal membrane as well as that between their uncoupling activities with chloroplasts and rat-liver mitochondria. We also measured the stoichiometry of uncoupling molecules relative to a unit phosphorylation assembly in chloroplasts and compared it with that in the mitochondria. We found that the uncoupling ac-

tivity of SF6847 and related compounds with chloroplasts is related linearly to that with mitochondria, further supporting the shuttle-type mechanism.

Materials and Methods

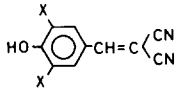
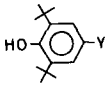
The compounds used here are listed in Table I. 2,6-Dialkyl-4-(2,2-dicyanovinyl)phenols (**1–6**) were kindly supplied by Professor H. Terada (Tokushima University, Japan). 2,6-Di-*t*-butyl-4-substituted phenols (**7–13**) were the generous gift of Dr. K. Watanabe of Kanegafuchi Chemical Co. [7]. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and carbonyl cyanide *p*-trifluoromethylphenylhydrazone (FCCP) of reagent grade were purchased from Sigma Chemical Co.

Class II chloroplasts were isolated from spinach from a market by the method of Nelson [8] with a medium comprising 20 mM Tris-KOH (pH 7.4)/0.40 M sucrose/30 mM NaCl. We measured the rate of electron transport under light of the intensity 2300 $\mu E/m^2$ per s by monitoring oxygen generation with a Clark-type oxygen electrode at 25 °C in a mixture containing 20 mM Tricine-KOH (pH 7.4), 0.10 M sucrose, 5 mM $MgCl_2$, 1 mM $K_3Fe(CN)_6$, and chloroplasts equivalent to 28 μg chlorophyll/ml. The total amount of chlorophyll was measured by the method of MacKinney [9]. Uncoupling activity was evaluated as the concentration, C_{200}^{chl}, needed to increase the rate of electron transport to twice that of the control. As the index for the quantitative analysis, log $(1/C_{200}^{Chl})$ was used to represent the potency.

The transthylakoid pH difference, ΔpH, was measured by the degree of quenching of the fluo-

TABLE I

PHYSICOCHEMICAL CONSTANTS AND UNCOUPLING AND PROTONOPHORIC ACTIVITIES OF UNCOUPLERS

No.	Compound	log K_A [a]	log P_p [a]	log $1/C_{200}^{mit}$ [a]	log $1/C_{200}^{Chl}$	
					obs.	calc. [b]
						
1	X = H	−7.04	−5.77	5.37	3.68	4.25
2	Me	−6.91	−5.02	6.39	4.79	5.32
3	Et	−6.98	−4.68	6.76	5.60	5.68
4	*i*-Pr	−7.06	−4.48	7.35	6.00	5.86
5	*s*-Bu	−7.22	−4.01	7.66	6.22	6.30
6	*t*-Bu	−6.84	−4.06	8.44	6.86	6.60
						
7	Y = CH=C(CN)COOEt	−7.51	−4.48	7.14	5.42	5.43
8	CH=C(CN)$CONH_2$	−7.73	−5.47	5.92	4.64	3.98
9	$CH=CHNO_2$	−6.89	−5.22	7.16	5.44	5.09
10	CH=C(CN)SO_2Me	−6.77	−4.62	7.73	5.79	5.96
11	CH=C(CN)COOMe	−7.45	−4.51	7.60	5.62	5.45
12	CN	−8.89	−4.85	5.42	3.24	3.64
13	CHO	−7.93	−5.98	5.12	3.36	3.13
14	FCCP	−6.20	−4.99	7.49	6.10 [c]	6.04
15	CCCP	−5.95	−4.30	7.30	5.58 [c]	7.15

[a] From Ref. 2.
[b] By Eqn. 1.
[c] Not included in the analyses of Eqn. 1.

rescence of 9-aminoacridine caused by the formation of the corresponding ammonium ion followed by the uptake of the uncharged molecule into the intrathylakoid spaces [10]. For the calculation of ΔpH, a volume of the intrathylakoid spaces of $7.5 \cdot 10^{-3}$ m^3/mol chlorophyll, taken from the literature [11], was used.

The uncoupling potency with rat-liver mitochondria was expressed as log $1/C_{200}^{mit}$, where C_{200}^{mit} is the concentration needed to double the state-4 respiration rate. As the index of protonophoric potency across the liposomal membrane, log P_p, in which P_p is the increment of the proton permeability per unit molar concentration of uncouplers, was used. The values of log $1/C_{200}^{mit}$ and log P_p were from our earlier study [2]. The substrate of the oxidative phosphorylation in mitochondria was sodium succinate (10 mM). The amount of mitochondrial protein was measured by the biuret method [12]. The acid dissociation constant, K_A, was also taken from our previous paper [2].

For the stoichiometric study, the amount of phosphorylation assemblies in thylakoids and mitochondria in the reaction medium were calculated with the assumption of a values of 300 molecules of chlorophyll per Photosystem I and II [13] and 0.10 nmol respiratory chain per mg mitochondrial protein [14].

Results

Transthylakoid pH difference

The mean value of the transthylakoid pH difference, ΔpH, in the absence of an uncoupler was 3.3 ± 0.2 under our experimental conditions. This valule was close to the 3.2–3.5 observed by Pick et al. [15]. With increasing concentrations of uncouplers in the medium, the ΔpH was markedly reduced, as shown in Fig. 1 for SF6847. At the concentration needed to double the rate of oxygen generation of the control, C_{200}^{Chl}, the ΔpH was between 1.0 and 1.5 for SF6847 and FCCP, which corresponds to the intrathylakoid pH of 5.9–6.4.

Analysis of uncoupling activity with thylakoids

The uncoupling activity of the compounds studied here measured with thylakoids at pH 7.4 in terms of log $1/C_{200}^{Chl}$ is listed in Table I. The uncoupling activity with rat-liver mitochondria at

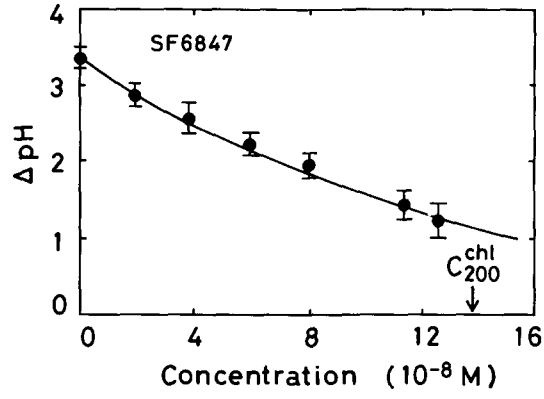


Fig. 1. Effect of SF6847 on the transthylakoid pH difference, ΔpH.

pH 7.4, log $1/C_{200}^{mit}$, and the protonophoric potency across the liposomal membrane, log P_p, of the corresponding compounds are also shown in Table I. The variation in log $1/C_{200}^{Chl}$ was between 3.5 and 7 and that in log $1/C_{200}^{mit}$ was between 5.5 and 8.5. SF6847 (**6**) had the highest uncoupling activity in both thylakoids and mitochondria.

The relationship between the uncoupling activity with thylakoids and protonophoric potency was examined by regression analysis, giving Eqn. 1:

$$\log 1/C_{200}^{Chl} = \underset{(0.429)}{1.263} \log P_p + \underset{(0.442)}{0.957} \log K_A + \underset{(3.391)}{18.273} \quad (1)$$

($n = 13$, $s = 0.394$, $r = 0.947$)

In this and the following equations, n is the number of compounds included in the correlation, s is the standard deviation, and r is the correlation coefficient. The figures in parentheses are the 95% confidence intervals. The log K_A term is important. Without the addition of this term, the correlation was much worse. Eqn. 1 shows that variations in the uncoupling activity are governed by both the protonophoric potency and the dissociation constant in a way very similar to that observed previously for the uncoupling activity with mitochondria [2]. The correlation for mitochondria is shown in Eqn. 2 for the 13 compounds used here plus six other uncouplers (data

not shown).

$$\log 1/C_{200}^{mit} = 1.174 \log P_p + 0.748 \log K_A + 17.889 \quad (2)$$
$$(0.431) \qquad (0.148) \qquad (2.465)$$

$(n = 19,\ s = 0.447,\ r = 0.948)$

The effect of acidity on the uncoupling activity with thylakoids is slightly higher than that on the uncoupling activity with mitochondria, but Eqn. 1 is practically equivalent to Eqn. 2. This shows that the physicochemical factors governing the variations in the uncoupling activity are almost identical for thylakoids and mitochondria. The two activity indices were correlated by Eqn. 3, and the relationship is shown in Fig. 2.

$$\log 1/C_{200}^{Chl} = 1.037 \log 1/C_{200}^{mit} - 1.896 \quad (3)$$
$$(0.183) \qquad (1.250)$$

$(n = 13,\ s = 0.301,\ r = 0.967)$

The addition of the log K_A term to Eqn. 3 was not justified over the 95% level.

At pH 7.4, the uncouplers used here were present as an equilibrium mixture of ionized and non-ionized species. Therefore, the values of log $1/C_{200}^{Chl}$ were the apparent ones and the ultimate uncoupling potency could be estimated by consideration the effect of ionization. As shown before [1], however, the two species showed almost the same order of uncoupling activity with mitochondria. Uncoupling activity was attributable to both species, so the C_{200}^{Chl} values observed for the 'equilibrated mixture' at pH 7.4 were used in the analysis with thylakoids also. With potency in terms of either one of the ionized and non-ionized forms contained in the overall C_{200}^{Chl} concentration at pH 7.4, the correlation was much worse (data not shown).

Stoichiometry in uncoupling action

That the intercept in Eqn. 3 is highly significant means that the uncoupling activity with chloroplasts is almost universally lower than that with mitochondria for each uncoupler by a factor of $1/77$ ($\log^{-1} 1.89 = 77$). The amount of phosphorylation assemblies does not differ much between these two experimental systems, being about $7.0 \cdot 10^{-11}$ mol of phosphorylation assemblies per ml of medium for mitochondria and about $10.0 \cdot 10^{-11}$ mol for thylakoids. Thus, we examined the stoichiometric relationship between uncoupler molecules and the phosphorylation assemblies under complete uncoupling conditions. We measured the rate of respiration with different amounts of thylakoids and mitochondria using SF6847 and FCCP. As shown in Fig. 3 with SF6847 and mitochondria as an example, the minimum concentration of uncouplers needed for the maximum stimulation of the electron-transport rate (titration point) was found for each concentration of mitochondria from the point of intersection between the straight line of the initial ascending and the final horizontal parts of the titration curve. The concentration of uncouplers corresponding to the titration point was taken to be that for complete uncoupling with the given amount of mitochondria. The concentration of uncouplers at the titration point increased as the amount of mitochondria increased. Plotting the moles of uncouplers per unit amount of mitochondrial protein at the titration point against the reciprocal of the protein concentration gave a straight line (Fig. 4). The number of moles of the uncoupler needed for the complete uncoupling per 1 milligram of pro-

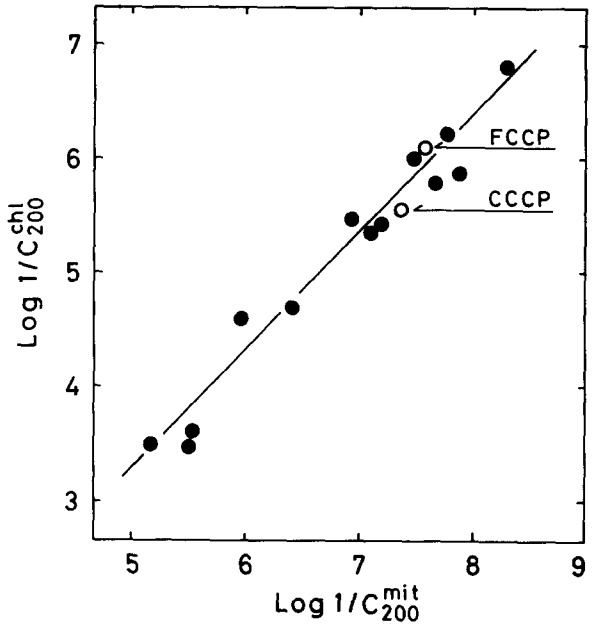


Fig. 2. Plot of uncoupling activity with thylakoids (log $1/C_{200}^{Chl}$) vs. that with mitochondria (log $1/C_{200}^{mit}$). FCCP and CCCP were not phenolic, but behaved like phenolic uncouplers.

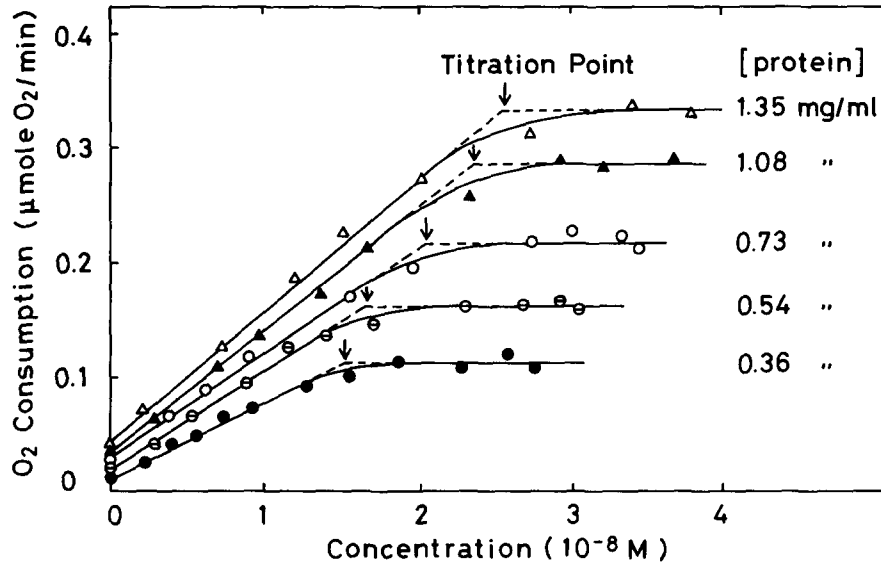


Fig. 3. Acceleration of the respiration rate by SF6847 with various concentrations of mitochondria. The concentration of the mitochondria is expressed as the amount of protein.

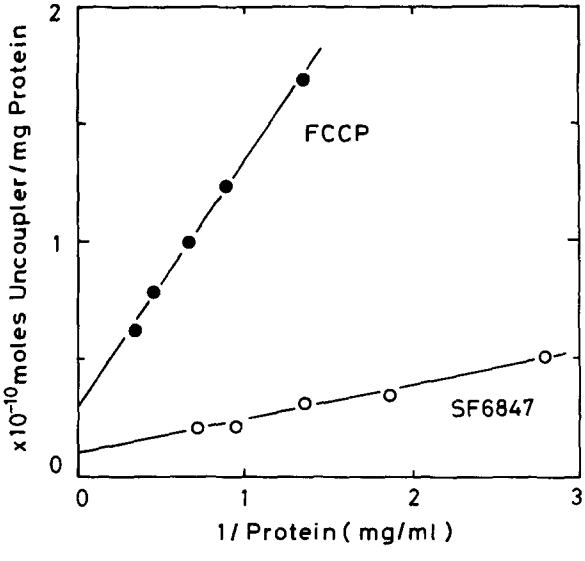


Fig. 4. Relationship between the moles of uncoupler per unit amount of mitochondria at the titration point and the reciprocal of the mitochondrial concentration.

tein at an infinite concentration of mitochondria was estimated from the intercept of the straight line. The value of the intercept may be called the intrinsic uncoupling concentration of uncouplers, i.e., the concentration needed for complete uncoupling when all of the added uncoupler molecules are bound to mitochondria [16]. The intrinsic uncoupling activity of SF6847 and FCCP with mitochondria and chloroplasts is listed in Table II. The difference in the slope of the straight line between SF6847 and FCCP in Fig. 4 reflects the difference in the affinity of the uncoupler with mitochondria. The affinity of FCCP seems to be lower than that of SF6847.

Discussion

The mechanism of action of phenolic uncouplers with thylakoids has not been investigated in detail, since most simply substituted phenols

TABLE II

INTRINSIC AND APPARENT UNCOUPLING ACTIVITIES

	Molecules uncoupler/phosphorylation assembly			C_{200} (M)		
	mitochondria	chloroplasts	chloroplasts / mitochondria	mitochondria	chloroplasts	chloroplasts / mitochondria
SF6847	0.09	2.7	30	$3.63 \cdot 10^{-9}$	$1.38 \cdot 10^{-7}$	38
FCCP	0.30	8.7	29	$3.24 \cdot 10^{-8}$	$7.94 \cdot 10^{-7}$	25

strongly inhibit Photosystem II electron transport at a concentration lower than that needed for uncoupling action [17,18]. The compounds used here were very potent uncouplers and had uncoupling action, while inhibiting electron transport little if at all.

The ability of uncouplers to work as a protonophore depends strongly on the dielectric constant of the membrane [4]. The dielectric constant of thylakoids seems to be higher than that of pure lipid membranes. The appreciable amount of protein in thylakoids would increase the dielectric constant [4,19]. As reported elsewhere [1,2], the log K_A term in Eqn. 2 reflects the fact that the ionized form of uncouplers is more stable in the mitochondrial membrane with a higher dielectric constant than in pure lipid membranes as liposomes. Since the log K_A term in Eqn. 1 for thylakoids is similar to that in Eqn. 2, the physicochemical characteristics, notably the dielectric constant, of membranes would not be very different for thylakoids and mitochondria.

We have reported that [2], besides the stability of the ionized form in the membrane phase, the effect of the difference in the extramembranous pH between mitochondrial and liposomal membranes under the experimental conditions used is important when the uncoupling activity of various weakly acidic uncouplers with mitochondria is correlated with the protonophoric potency across the liposomal membrane. This effect was formulated as P_f in Eqn. 4.

$$P_f = \frac{Pr(\text{mitochondria})}{Pr(\text{liposome})} \tag{4}$$

The Pr in Eqn. 4 is defined by Eqn. 5,

$$Pr = \frac{[H^+] \cdot K_A}{(K_A + [H^+])(K_A + 200[H^+])} \tag{5}$$

where $[H^+]$ is the extramembranous H^+ concentration in either mitochondrial or liposomal measurements, and K_A is the dissociation constant of uncouplers. In the correlation analyses, log P_f was used. This type of effect of the difference in pH conditions is probably important also when the uncoupling activity with thylakoids is correlated with the protonophoric potency, as was done in Eqn. 1. For instance, the pH value of the intrathylakoid spaces is about 6, but that of the internal aqueous phase of liposomes is from 4.5 to 7.0 [2]. However, the log P_f term defined for the thylakoidal experiments was not significant in Eqn. 1. The range of variation in the log K_A value was small (-6.77 to about -8.89) for the series of compounds used here, so the range of the log P_f value was not wide either, being from 0 to 0.2 (data not shown). Moreover, significant collinearity existed between log K_A and log P_f for the uncouplers used, so that the two factors would not be completely separate in regression analysis.

A one-to-one stoichiometric relationship was not found for the intrinsic interaction between uncoupler molecules and phosphorylation assemblies of mitochondria for either SF6847 or FCCP (Table II). The value of 0.09 molecule of SF6847/electron-transport chain in mitochondria was close to the value of 0.12 observed by Terada et al. [16]. The variation in the 'apparent' uncoupling activities of SF6847 and FCCP between thylakoid ($1/C_{200}^{Chl}$) and mitochondria ($1/C_{200}^{mit}$) corresponds well to that in the 'intrinsic' uncoupling activities at phosphorylation assemblies. This observation suggests that the lower activity of uncouplers measured with thylakoids as represented as the negative intercept in Eqn. 3 arises from the lower intrinsic uncoupling activity at the phosphorylation sites.

Although FCCP and CCCP are not phenolic, their uncoupling activity with thylakoids was also related with that with mitochondria in a way that resembled the correlation for phenols that was shown in Fig. 2. Along with this observation, the fact that two kinds of uncoupling activity of a wide range of weakly acidic uncouplers were correlated in a manner similar to each other with protonophoric potency across a lipid bilayer and the acidity of compounds is further evidence for the shuttle-type mechanism of weakly acidic uncouplers.

The results reported here seem to show that the 'direct-interaction' hypothesis of uncouplers [5,6,20] that assumes a specific site(s) for the uncoupler binding, probably ATPase, is unlikely. The orientation of ATPase in membranes is opposite in mitochondria and thylakoids, so the location of the 'specific' site(s) is also different for the two systems. The good correlation of Eqn. 3,

showing almost identical modes of action regardless of the experimental system, would not have been observed if the direct-interaction hypothesis were true.

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