Biochimica et Biophysica Acta 933 (1988) 193–199
Elsevier

BBA 42729

Unique action of a modified weakly acidic uncoupler without an acidic group, methylated SF 6847, as an inhibitor of oxidative phosphorylation with no uncoupling activity: possible identity of uncoupler binding protein

Hiroshi Terada [a], Yousuke Fukui [a], Yasuo Shinohara [a] and Motoharu Ju-ichi [b]

[a] *Faculty of Pharmaceutical Sciences, University of Tokushima, Tokushima, and* [b] *Faculty of Pharmaceutical Sciences, Mukogawa Women's University, Hyogo (Japan)*

(Received 14 October 1987)

Key words: Uncoupler (weakly acidic, modified); SF 6847; Protonophoric action; Oxidative phosphorylation inhibitor; Uncoupler binding protein; (Mitochondria)

The potent weakly acidic uncoupler SF 6847 was modified by methylation of its phenolic OH group, and the effect of the resulting derivative, with no acid-dissociable group, on oxidative phosphorylation in rat liver mitochondria was examined. The methylated SF 6847 did not induce uncoupling at up to 40 μM, while SF 6847 uncoupled oxidative phosphorylation completely at about 20 nM, indicating that the acid-dissociable group is essential for uncoupling. The *O*-methylated SF 6847 at 20 μM did, however, inhibit state 3 respiration of mitochondria, although it did not inhibit electron-flow through the respiratory chain, ATPase activated by weakly acidic uncouplers or P_i–ATP exchange. At the same concentration, it also inhibited ATP synthesis in submitochondrial particles. These features are different from those of known inhibitors of oxidative phosphorylation. Thus, *O*-methylated SF 6847 is a unique inhibitor of oxidative phosphorylation. The possible identity of the uncoupler binding protein is discussed on the basis of these results.

Introduction

Almost all potent uncouplers of oxidative phosphorylation, such as SF 6847, CCCP and 2,4-dinitrophenol, are weak acids, and their uncoupling actions are thought to be due to their protonophoric actions, enabling short-circuit transfer of protons across proton-impermeable energy-transducing membranes, as described in a review [1]. Their protonophoric action is supposed to be based on the shuttle-type circulation of the weakly acidic uncoupling in the membrane. At the membrane surface, the anionic form of uncoupler combines with H^+ in the aqueous phase, and consequently becomes neutral. The neutral form of the uncoupler traverses the membrane to the opposite membrane interface, where it releases H^+ to become the anionic form again, and the uncoupler anion returns to the original membrane interface. According to this model, both neutral and anionic molecular species of the compound are necessary,

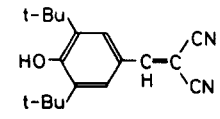
Abbreviations: SF 6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidene malononitrile; MeO-SF, 3,5-di-*tert*-butyl-4-methoxybenzylidene malononitrile (methoxy SF 6847, or methylated SF 6847); CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone; N_3CCP, carbonyl cyanide 4-azido-2-nitrophenylhydrazone; P_i, inorganic phosphate.

Correspondence: H. Terada, Faculty of Pharmaceutical Sciences, University of Tokushima, Shomachi-1, Tokushima 770, Japan.

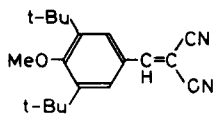
and their interconversion is essential for exhibition of uncoupling activity. Such a protonophoric action has been observed in model membrane systems, such as liposomes and black lipid membranes [2–7], and in non-respiring mitochondria [8,9].

Although the protonophoric action of uncouplers is widely accepted [1,10,11], it is still unknown whether the acid-dissociable group of a weakly acidic uncoupler is essential for induction of uncoupling activity in energy-transducing membranes, or whether the uncoupling action of a weakly acidic uncoupler is based solely on its shuttle-type cycling in phospholipid membranes without its binding to some protein site(s). In fact, proteins that bind to uncouplers have been isolated and the alternative mechanism that the specific binding of these proteins to uncouplers is a trigger for induction of uncoupling has been proposed [12–18]. Furthermore, organic acids in which the acidic group is modified, and compounds devoid of acidic groups are reported to show uncoupling activity in mitochondria [19–21]. However, in these studies, little attention seems to have been paid to whether the derivatives were contaminated with the original weak acid, or whether the chemical conversion to the acidic compound took place in the stock solution [1,22].

In this study, we examined the effect of mitochondrial function of methoxy SF 6847, MeO-SF, in which the phenolic OH group of the powerful weakly acidic uncoupler SF 6847 was methylated (Chart 1). MeO-SF was found not to induce uncoupling, even at 1000-times the concentration required for uncoupling by SF 6847. This finding indicated that the acid-dissociable group of SF 6847 was essential for uncoupling activity. Furthermore, MeO-SF was found to be a unique inhibitor of oxidative phosphorylation.



SF 6847



MeO-SF

Chart 1.

Materials and Methods

A novel compound MeO-SF, was prepared from 2,6-di-*tert*-butylanisole by a reported procedure [23]. The product at each step of the synthesis was purified by repeated column chromatography, distillation and recrystallization. The purity of MeO-SF was checked mainly by absorption spectrometry, because this method is very sensitive for detecting more than a few thousandth parts of contaminating SF 6847 due to the characteristic and strong absorption band of the SF 6847 anion at 445 nm [24]. The purity of MeO-SF was also examined by NMR, mass and infrared spectrometries. No contaminating SF 6847 was detected in the MeO-SF preparation by these spectrometries. Thus, the synthesized MeO-SF was concluded to be very pure, and contamination with SF 6847 was negligible.

SF 6847 was purchased from Wako Chemical Industries, Co., Osaka. The other reagents used were of the highest grade available commercially.

Mitochondria were isolated from the liver of adult male Wistar rats as described by Myers and Slater [25]. Submitochondrial particles were prepared by the method of Thayer and Rubin [26] from fresh rat liver mitochondria. The protein contents of mitochondria and submitochondrial particles were determined by the biuret method [27] with bovine serum albumin as standard.

Mitochondrial respiration with 5 mM succinate (plus 1 μg of rotenone/ml) as respiratory substrate was monitored with a Clark oxygen electrode at 25 °C. The incubation medium consisted of 200 mM sucrose/20 mM KCl/3 mM $MgCl_2$/3 mM potassium phosphate buffer (pH 7.4), and the total volume was 2.53 ml.

The P_i-ATP exchange reaction was measured by the method of Pullman [28] at 25 °C in medium consisting of 50 mM sucrose/20 mM KCl/0.1 mM Na_2EDTA/3 mM $MgCl_2$/5 mM Tris-HCl buffer (pH 7.4)/3 mM potassium phosphate buffer

(pH 7.4) containing 1 μCi [^{32}P]P_i (pH 7.4). A known amount of MeO-SF was added to the mitochondrial suspension (0.7 mg protein/ml in a final volume of 1.0 ml), and the reaction was initiated by addition of 1 mM ATP. After 4 min, the reaction was stopped by adding perchlorate, and the amount of [^{32}P]ATP formed was determined in an Aloka liquid scintillation counter, LSC-700.

The ATPase activity of mitochondria was determined by the method of Takahashi [29] at 25 °C in medium consisting of 200 mM sucrose/20 mM KCl/3 mM $MgCl_2$/5 mM Tris-HCl buffer (pH 7.4). Mitochondria (0.7 mg protein/ml in a total volume of 1.0 ml) were incubated for 1 min with either MeO-SF or SF 6847, or both. Then 2.5 mM ATP was added, and after 2 min, ATP hydrolysis by ATPase was stopped by adding perchlorate. The P_i released by ATP hydrolysis was trapped with molybdate and measured spectrophotometrically at 770 nm.

ATP synthesis in submitochondrial particles was determined at 25 °C by the method of Nishimura et al. [30]. Submitochondrial particles (0.7 mg protein per ml) were suspended in medium consisting of 200 mM sucrose/20 mM KCl/3 mM $MgCl_2$/3 mM potassium phosphate buffer (pH 7.4), in a total volume of 4.75 ml. The particles were energized with 5 mM succinate (plus 1 μg of rotenone per ml). ATP synthesis was started by addition of 200 μM ADP, and the pH change of the medium due to the synthesis of ATP was monitored with a Toko TP 1000-GP pH-meter.

The protonophoric activity of SF 6847 in non-respiring mitochondria (0.7 mg/ml) was examined as described by Hendenson et al. [2], in 145 mM potassium acetate/5 mM Tris-HCl buffer (pH 7.4) in a total volume of 3.0 ml. Extrusion of H^+ from the matrix space in exchange with K^+ was started with valinomycin and SF 6847. The pH-dependent swelling of mitochondria accompanied by movements of H^+ and K^+ was monitored as decrease in the optical absorbance at 500 nm.

Partition of SF 6847 with or without MeO-SF between an organic phase consisting of *n*-octanol and *n*-hexane (7 : 3) and an aqueous phase (10 mM NaOH) was examined at 25 °C. In this partition experiment, 2.5 ml of organic phase with and without 50 μmol MeO-SF and 5.0 ml of aqueous phase containing 200 μmol of SF 6847 were used, and the amount of SF 6847 partitioned into the organic phase from the aqueous phase at equilibrium was determined spectrophotometrically at 455 nm.

Results

Many hydrophobic weak acids are reported to be uncouplers of oxidative phosphorylation in mitochondria [1]. Of these, SF 6847 is one of the most potent uncouplers known [19]. The main purpose of this study was to determine whether the acid-dissociable group of a weakly acidic uncoupler is essential for exhibition of its uncoupling activity. To this purpose, we synthesized *O*-methylated SF 6847 (methoxy SF 6847) in which acid dissociation of the phenolic OH group is blocked, and confirmed that the final preparation was not contaminated with any appreciable amount of SF 6847 (see Materials and Methods). We also confirmed that MeO-SF was not converted to SF 6847 during experiments. Thus, the effects observed in the presence of added MeO-SF were due entirely to MeO-SF.

Generally, release of state 4 respiration, inhibition of P_i-ATP exchange and activation of ATPase are taken as indices of uncoupling [1]. Thus, we examined the effects of MeO-SF on these activities in rat liver mitochondria. As shown in Fig. 1,

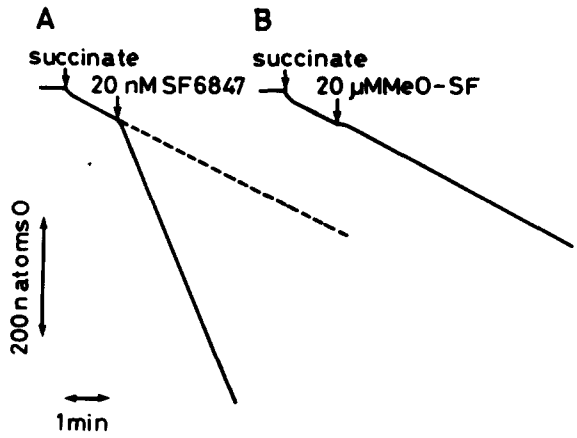


Fig. 1. Effects of SF 6847 (A) and MeO-SF (B) on state 4 respiration of mitochondria with 5 mM succinate (plus rotenone) as repiratory substrate. The broken line in trace A is the record without added SF 6847.

TABLE I

EFFECTS OF MeO-SF AND SF 6847 ON MITOCHONDRIAL FUNCTIONS RELATED TO UNCOUPLING ACTIVITY

	Control	MeO-SF (μM)			SF 6847 (nM)		
		10	20	40	10	20	40
State 4 respiration (natom O/mg per min)	32.5	32.0	32.8	41.3	114.3	222.1	220.8
ATPase activity (nmol P_i/mg per min)	19.2	21.3	20.8	27.1	61.2	106.8	105.3
P_i-ATP exchange (nmol ATP/mg per min)	91.3	90.5	96.3	95.8 [a]	–	7.8	–

[a] The value at 30 μM.

the weakly acidic uncoupler SF 6847 at 20 nM caused about 7-fold release of respiratory rate of state 4 mitochondria. In contrast its *O*-methylated derivative MeO-SF did not have any effect at the 1000-times higher concentration of 20 μM. Even at 40 μM, MeO-SF had little effect on state 4 respiration (cf. Table I). Furthermore, MeO-SF had little effect on ATPase activity or P_i–ATP exchange at concentrations of up to 40 μM, as shown in Fig. 2 and Table I. These results indicated that MeO-SF itself is not an uncoupler in mitochondria.

Next, we examined the effect of MeO-SF on the uncoupling effect of SF 6847. Fig. 3 shows the effects of the various concentrations of MeO-SF on the respiration in partially uncoupled mitochondria by 6 nM SF 6847. Addition of MeO-SF either before or after SF 6847 enhanced the uncoupling effect of SF 6847: 20 μM MeO-SF increased the uncoupling effect of SF 6847 about 2-fold. MeO-SF also enhanced the activation of ATPase by SF 6847, as shown in Fig. 2, where the effects of various concentrations of MeO-SF on the action of 10 nM SF 6847 are depicted. However, 20 μM MeO-SF did not enhance the actions of other weakly acidic uncouplers, such as CCCP and 2,4-dinitrophenol, at their effective concentrations for uncoupling (data not shown).

This enhancement of the uncoupling action of Sf 6847 by MeO-SF might be due to the enhancement of its protonophoric activity. To study this possibility, we examined the effect of MeO-SF on

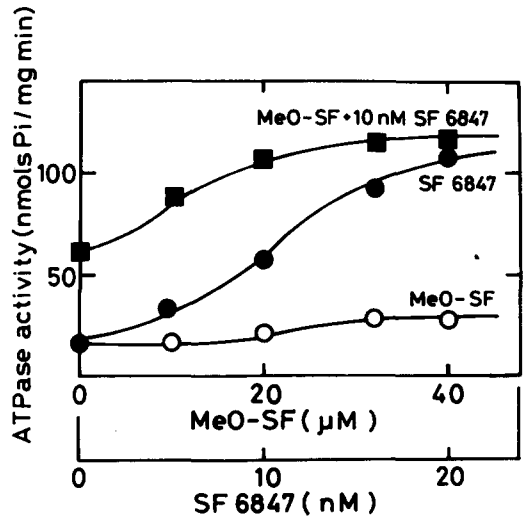


Fig. 2. Effects of SF 6847 and MeO-SF on latent ATPase activity in mitochondria. The effects of MeO-SF (○) and SF 6847 (●) singly, and of MeO-SF on ATPase partially activated by 10 nM SF 6847 (●) are depicted.

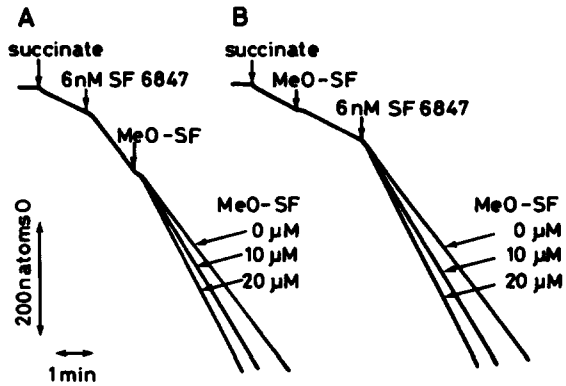


Fig. 3. Combined effects of MeO-SF and SF 6847 on uncoupling. Mitochondrial respiration was measured as in Fig. 1, but 6 nM SF 6847 was added to induce partial uncoupling, 2 min before (A) or after (B) addition of MeO-SF.

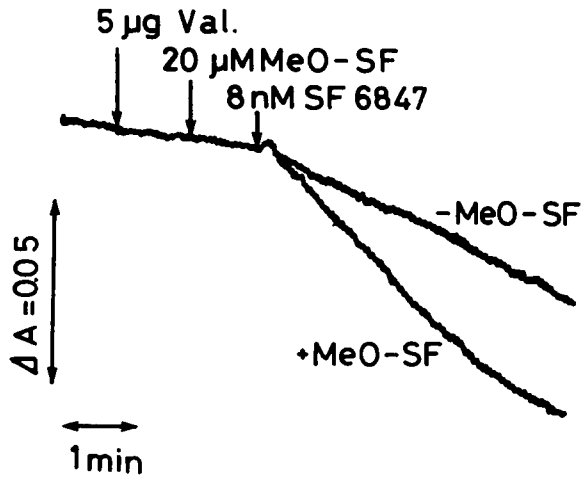


Fig. 4. Acceleration by MeO-SF of swelling of non-respiring mitochondria in response to H^+ transfer in exchange with K^+ induced by SF 6847 and valinomycin. For experimental conditions, see Materials and Methods. Swelling was measured as decrease in optical absorbance at 500 nm. Rotenone (1 μg/ml) and antimycin (1 μg/ml) were added to the incubation medium. Val, valinomycin.

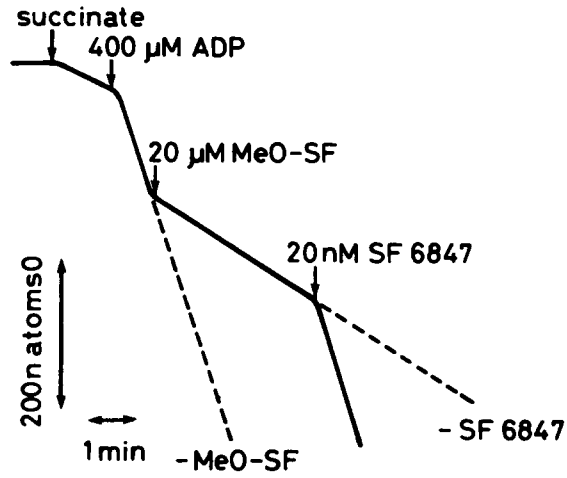


Fig. 5. Inhibition of state 3 respiration in mitochondria by 20 μM MeO-SF. Experimental conditions were as for Fig. 1, except that 400 μM ADP was added.

the swelling of non-respiring mitochondria induced by valinomycin and SF 6847 in the presence of potassium acetate [2]. As shown in Fig. 4, MeO-SF increased the swelling induced by SF 6847, monitored as decrease in the optical absorbance at 500 nm. This swelling is the results of extrusion of H^+ mediated by the uncoupler in exchange with K^+ entry mediated by valinomycin [2]. Thus, MeO-SF enhanced the protonophoric activity of SF 6847.

MeO-SF was found to increase the transfer of SF 6847 anion into an organic phase consisting of *n*-octanol and *n*-hexane (7 : 3) from an alkaline aqueous phase. The aqueous phase initially contained 200 μmol of SF 6847. In the absence and presence of 50 μmol of MeO-SF in the organic phase, about 50% and 90%, respectively, of the SF 6847 anions were partitioned into the organic phase. Thus, MeO-SF seems to enhance the effect of the weakly acidic uncoupler SF 6847 on mitochondria by forming a complex with it in the membrane, and thus stabilizes the anionic form of SF 6847.

To obtain further information on the effect of MeO-SF on oxidative phosphorylation, we next examined its effect on state 3 respiration. As shown in Fig. 5, MeO-SF at 20 μM inhibited state 3 respiration almost completely. This inhibition was released by SF 6847, indicating that it was not caused by an action of MeO-SF on the respiratory chain. Thus, MeO-SF itself acts as an inhibitor of oxidative phosphorylation.

Next, the effect of MeO-SF on the ATP synthe-

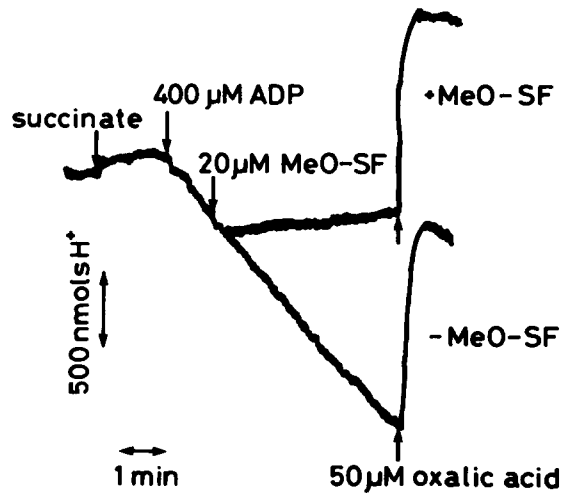


Fig. 6. Inhibition by MeO-SF of ATP synthesis in submitochondrial particles. ATP synthesis by submitochondrial particles supported by succinate (plus rotenone) was monitored as decrease in the pH of the medium (4.75 ml). In the figure, a downward pH change indicates the increase in the ATP synthesis. The pH change was corrected by addition of 50 μM oxalic acid.

sis in submitochondrial particles was examined to determine whether the inhibition of state 3 mitochondria could be due to inhibition of uptake of substrates for ATP synthesis into the mitochondria. As shown in Fig. 6, MeO-SF at 20 μM completely inhibited ATP synthesis in submitochondrial particles, indicating that the inhibition of state 3 respiration in mitochondria by MeO-SF was not due to its inhibition of the transport of ADP and of P_i into mitochondria.

Discussion

MeO-SF, the *O*-methylated derivative of SF 6847, has no acid-dissociable group. Thus, this compound is a good model of the neutral form of the potent, weakly acidic, phenolic-type uncoupler, SF 6847. The present study showed that the neutral form did not induce uncoupling in mitochondria, and thus that the acid-dissociable group is essential for uncoupling. Strong acids such as picrate are known not to induce uncoupling in mitochondria and other energy transducing membranes that have the same orientation of membrane proteins related to ATP synthesis as that of mitochondria [31–33]. Thus, the anionic form of a weakly acidic uncoupler is not alone effective for inducing uncoupling in mitochondria. Consequently, the protonophoric activity resulting from repeated interconversion of the anionic and neutral forms of an uncoupler molecule in the membrane is concluded to be responsible to the uncoupling action [1].

A non-acid-dissociable derivative of SF 6847 was reported to be capable of inducing uncoupling [19]. However, the observed uncoupling was probably due to contamination of the preparation with its mother compound, having an acid-dissociable group. The finding that the uncoupling activity of a weak acidic uncoupler was reduced, but not lost completely, when its acid-dissociable group was esterified [34], can also be explained as due to contamination of the preparation with the original weakly acidic uncoupler.

MeO-SF was found to enhance the uncoupling and protonophoric action of SF 6847. Since a partition experiment showed that the transfer of SF 6847 into the organic phase was increased in the presence of MeO-SF, the enhancing effect of MeO-SF on the uncoupling was probably due to its formation of a complex with the SF 6847 anion, thus stabilizing the SF 6847 anion in the membrane. The importance of the stability of an uncoupler anion in the membrane, and of the association of the anionic form with the neutral form of uncoupler for uncoupling activity have been reported [6,7,35]. However, the affinity of MeO-SF for the SF 6847 anion for association with MeO-SF (or the neutral form of SF 6847) can not be great, because its concentration for 2-fold enhancement of the uncoupling action of SF 6847 was about 1000-times that of SF 6847 (Figs. 2 and 3). Our observation that MeO-SF did not enhance the uncoupling activities of CCCP and 2,4-dinitrophenol can be explained by the fact that the relative concentration of MeO-SF to these uncouplers was too low, because higher concentrations for these compounds than SF 6847 were required for uncoupling.

Although MeO-SF itself did not induce uncoupling, it was found to inhibit oxidative phosphorylation. It inhibited state 3 respiration, but did not inhibit the electron-flow in the respiratory chain (Fig. 5), ATPase activity (Fig. 2), or P_i–ATP exchange (Table I). The finding that MeO-SF inhibited ATP synthesis in submitochondrial particles also suggests that its inhibition of state 3 respiration in mitochondria was not due to inhibition of the entry of P_i or ADP into mitochondria (Fig. 6).

The energy transfer inhibitor oligomycin inhibits both state 3 respiration and uncoupler-activated ATPase, whereas MeO-SF inhibited state 3 respiration but had no effect on ATPase. Thus, MeO-SF is a unique inhibitor of oxidative phosphorylation. The action mechanism of MeO-SF is not clear at present. Possibly MeO-SF exerts its effect by interacting with some site in mitochondrial protein. The mechanism of the unique inhibitory action of MeO-SF on ATP synthesis will be reported subsequently.

Finally the identity of the uncoupler binding protein should be considered. Uncoupler binding proteins in mitochondria and submitochondrial particles from various sources have been isolated by use of photoaffinity labeling uncouplers, such as 2-azido-4-nitrophenol and N_3CCP [12–18]. Various molecular masses have been reported for

these proteins, depending on the source of mitochondria, but a major component seems to be a 30 kDa protein. From these studies, the binding of uncouplers to the uncoupler binding protein(s) is thought to be a trigger of uncoupling. As suggested from this study, besides circulating in the phospholipid bilayer by Brownian motion, and thus rendering the membrane permeable to H^+, SF 6847 molecules (possibly the anionic form as well as the neutral form) may bind to a certain site in the membrane protein.

This binding may induce inhibition of ATP synthesis, although higher concentrations may, in general, be required for this inhibition than for inducing uncoupling. Even though the inhibition is concealed by uncoupling and cannot be detected, because state 3 and 4 respirations are both accelerated, and ATPase is activated by the uncoupling. It should be noted that photoaffinity labeling of uncouplers to isolate uncoupler binding proteins has usually been carried out at concentrations of uncoupler higher than those used for uncoupling. Furthermore, though the actions of free photoaffinity labeling uncouplers on mitochondrial functions have been examined, the action of photoaffinity-labeled uncouplers on proteins have not been reported [1]. In view of these results, the uncoupler binding protein(s) could be a site for induction of state 3 inhibition, but not for exerting uncoupling activity. We are now studying the function of uncoupler binding proteins in relation to the inhibition of ATP synthesis rather than the uncoupling action.

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