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Uncoupling activity of a newly developed fungicide, fluazinam [3-chloro-*N*-(3-chloro-2,6-dinitro-4-trifluoromethylphenyl)-5-trifluoromethyl-2-pyridinamine]

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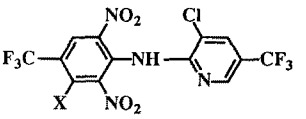
A very unusual uncoupling activity was found in a newly developed phenylpyridylamine fungicide for agricultural use, fluazinam. The compound had extraordinarily strong uncoupling activity, but the activity rapidly disappeared with rat-liver mitochondria isolated by the usual method with centrifugation. Treatment that lowered the concentration of glutathione (GSH), in the mitochondrial matrix prevented the disappearance of the uncoupling activity. The activity of an analog of fluazinam in which the 3-chloro substituent on the phenyl moiety, probably works as a leaving substituent, was replaced by the *i*-propoxy group lacking such a function, did not disappear. These results suggest that fluazinam was metabolically transformed on the mitochondrial level, probably by a GSH conjugation mechanism. When GSH was completely eliminated, fluazinam had powerful uncoupling potency, greater than that of SF6847, the most potent acidic uncoupler known until now.

According to the chemiosmotic hypothesis of oxidative phosphorylation [1], acidic uncouplers work as protonophores across bioenergy-transducing membranes impermeable to protons. This mode of action is called the shuttle-type mechanism [2]. Because this mechanism does not involve a specific binding site of the uncoupler molecules, it has been thought difficult to develop an acidic uncoupler with high selectivity among bioenergy-transducing membranes of different biological origins. In fact, the most potent uncoupler known to date, SF6847, does not have practical value as a pesticide because of its high toxicity to fishes and warm-blood animals [3].

Weakly acidic uncouplers, which could be inactivated in mitochondria to different extents in different biological species, may be of interest in the design and synthesis of selective acidic uncouplers. Various kinds of potent acidic uncoupler have been synthesized, but those that can be metabolized in isolated mitochondria have not so far been reported. In this study, we describe a very potent uncoupler, fluazinam (structure I: X = Cl), which is readily metabolized in mitochondria.



(I)

Fluazinam (m.p. = 100–102°C) and its *i*-propoxy analog (X = *i*-OPr, m.p. = 138–139°C) were prepared by the method reported [4]. Mitochondria were isolated from the liver of adult male Wistar rats in medium A containing sucrose (0.25 M) and Tris-HCl (2 mM) (pH 7.4) as described by Myers and Slater [5]. For the isolation of mitochondria used for the preparation of mitoplasts, medium B containing mannitol (220 mM), sucrose (70 mM), Tris-HCl (2 mM) (pH 7.4), and bovine serum albumin (0.5 mg/ml) was used. Mitoplasts were prepared in medium B according to the usual method [6,7] and washed twice in medium B without bovine serum albumin.

Abbreviations: fluazinam, 3-chloro-*N*-(3-chloro-2,6-dinitro-4-trifluoromethyl)phenyl-5-trifluoromethyl-2-pyridinamine; SF6847, 3,5-di-(*t*-butyl)-4-hydroxybenzylidenemalononitrile; GSH, reduced glutathione.

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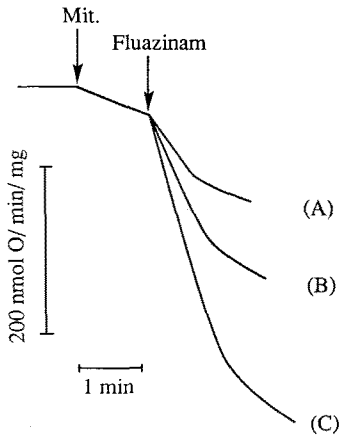


Fig. 1. Effect of fluazinam on state 4 respiration of mitochondria with 10 mM succinate as the respiration substrate. The incubation medium consisted of sucrose (200 mM), $MgCl_2$ (2 mM), EDTA (1 mM), rotenone (0.4 μg/ml), and potassium phosphate buffer (pH 7.4, 2.5 mM), in a total volume of 2.5 ml. The concentrations of fluazinam were $5 \cdot 10^{-9}$ (A), $1.0 \cdot 10^{-8}$ (B), and $2.0 \cdot 10^{-8}$ M (C).

The respiration rate of mitochondria and mitoplasts with succinate (10 mM) as the repiration substrate was monitored with a Clark oxygen electrode at 25 °C. The transmembrane electrical potential was monitored by measurement of the uptake of the tetraphenylphosphonium (TPP^+) from the incubation medium into the mitochondrial matrix using a TPP^+-sensitive membrane electrode prepared as described by Kamo et al. [8]. The glutathione (GSH) concentration in the mitochondrial matrix was measured by the method of Rapuano and Maddaiah [9]. The intrinsic uncoupling activity of fluazinam and SF6847 in terms of the number of uncoupler molecules needed for maximum respiration release per respiration assembly was evaluated by the method of Terada and Van Dam [10] on the assumption that the respiration assembly is 0.10 nmol/mg mitochondrial protein [11]. The acid dissociation constants (K_A) of fluazinam and its *i*-propoxy analog were measured in 50% EtOH-H_2O (v/v) medium as described previously [12] at 25 °C.

The uncoupling activity of fluazinam ($pK_A = 7.11$) evaluated from the acceleration of the state 4 respiration rate disappeared with time. The duration of the activity was longer when there was more fluazinam in the incubation medium (Fig. 1). Disappearance of the uncoupling activity of fluazinam was also seen in the change in the transmembrane electrical potential (Fig. 2). The transmembrane electrical potential established by the addition of the succinate was reduced by the addition of fluazinam, but the potential returned to its state 4 level with time.

The ultraviolet and visible spectra of fluazinam in the incubation medium (pH 7.4) at the concentrations of 1–10 μM decreased very slowly, with a half-time of about 40 min. The change over a few minutes was almost undetectable. Therefore, spontaneous decomposition of the uncoupler molecules during measurements of respiration was unlikely. Thus, the disappearance of the uncoupling activity was rather due to some biochemical transformation mechanism such as the metabolism in the isolated mitochondria.

The microsomal fraction contains various drug-metabolizing enzyme systems [13]. It is therefore important to examine isolated mitochondria for the possible contaminating presence of other organelles when drug metabolism is to be studied. The activity of NADPH–cytochrome *c* reductase, a marker enzyme of the microsomal fraction [14], was not detected at all in the mitochondrial fraction isolated. To exclude the possible contributions of other organelles, which may be tightly bound to the outer membrane of mitochondria, the uncoupling activity of fluazinam was also measured with mitoplasts at the concentration of 0.7 mg of protein/ml. The uncoupling effect disappeared after a few minutes even with mitoplasts in a manner similar to that shown in Fig. 1 for intact mitochondria, although the absolute rate of respiration was lower.

The *i*-propoxy analog ($pK_A = 8.48$) of fluazinam also had potent uncoupling activity. The activity of the analog did not disappear; that is, the respiration rate brought about by a certain amount of this compound was constant (data not shown). These results suggest that the chlorine atom on the benzene ring was important in the metabolic decomposition of fluazinam. This atom might undergo attack by nucleophilic reagents because the benzene ring is activated by strong electron-withdrawing groups such as double NO_2 and CF_3 substituents. Thus, the mechanism of metabolic transformation would be GSH conjugation, in which there is nucleophilic substitution of Cl by GS (glutath-

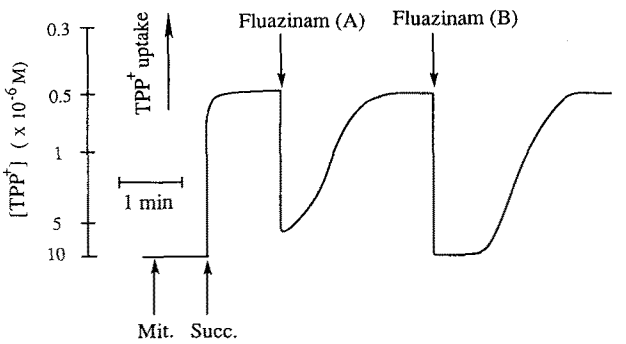


Fig. 2. Effect of fluazinam on the transmembrane electrical potential of mitochondria, monitored by the uptake of TPP^+ in the reaction medium. The incubation medium (15 ml) was the same as that used for the respiration experiments except that succinate was not included. The final protein concentration was 0.7 mg/ml. Succ. indicates the addition of 10 mM succinate. The upward change in the TPP^+ concentration reflects the uptake of TPP^+ from the incubation medium into the mitochondrial matrix, that is, the increase in the transmembrane electrical potential. Fluazinam was added at the time indicated by the arrow at the concentrations of $1.0 \cdot 10^{-8}$ (A) and $2.0 \cdot 10^{-8}$ M (B).

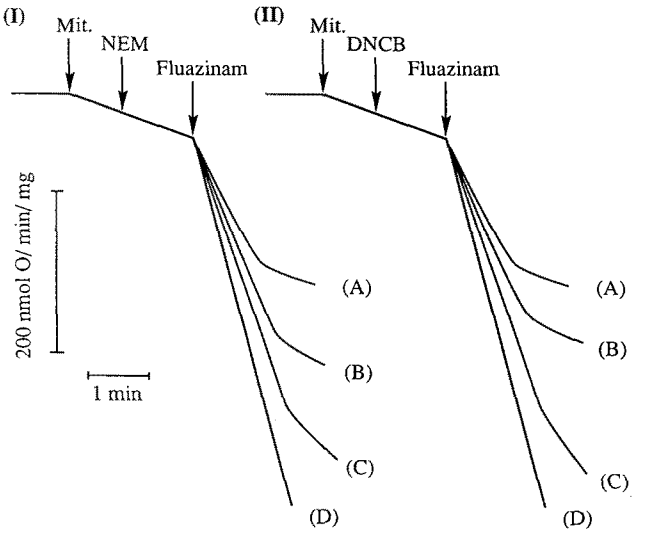
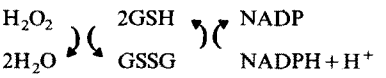


Fig. 3. Effects of NEM (I) and DNCB (II) on the uncoupling activity of fluazinam ($8 \cdot 10^{-9}$ M) monitored by the acceleration of the respiration rate of mitochondria (0.7 mg protein/ml). The concentrations of NEM in the incubation medium were 0 (A), 0.005 (B), 0.01 (C), and 0.05 mM (D), and those of DNCB were 0 (A), 0.1 (B), 0.5 (C), and 5 μM (D).

ione residue), with the chloride working as the leaving anion.

Much GSH is present in the mitochondrial matrix. The mean concentration of GSH is 9 mM, with the assumption that the matrix volume is 1.0 μl/mg of protein [15]. This value was close to the 11 mM observed by Wahlländer et al. [16]. To confirm the possibility of the participation of GSH conjugation in the transformation of the fluazinam molecule, we examined the effects of such SH-reagents as *N*-ethyl maleimide (NEM) and *p*-chloromercuriphenyl sulfate (CMS), which react easily with GSH, and the effect of 2,4-dinitrochlorobenzene (DNCB), which is a typical substrate of GSH conjugation. The results with NEM and DNCB are shown in Fig. 3. Fig. 3 indicates that the disappearance of the activity of fluazinam was prevented by the incubation of mitochondria with NEM and DNCB, and the extent of prevention depended upon the amount of these reagents in the medium. The amount of free GSH maintained in the mitochondrial matrix after the incubation with sufficient NEM (0.1 mM), CMS (0.2 mM), or DNCB (30 μM) was measured, and we found that these treatments resulted in a loss of about 90–95% of the level of initial mitochondrial GSH. Therefore, prevention of the disappearance of the uncoupling activity was attributable to the decreased GSH level in the mitochondrial matrix.

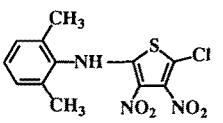
It is widely known that rat-liver mitochondria have much GSH in the matrix space, detoxifying hydroperoxides generated from the O_2 consumption in respiration chains according to the scheme shown here [17].



Olafsdottir and Reed [18] have reported that exogenous peroxides such as *t*-butylhydroperoxide also lower the GSH level in the mitochondrial matrix. We measured the uncoupling activity of fluazinam with mitochondria (0.7 mg protein/ml) that were preincubated with *t*-butylhydroperoxide (5 mM) for 10 min. As we expected, the uncoupling activity of fluazinam did not disappear. This observation was further evidence for the involvement of the GSH conjugation mechanism. Fluazinam, which is hydrophobic, seems to be transformed to reduce the hydrophobicity by the GSH conjugation. The glutathione-conjugated fluazinam molecule would be no longer able to remain in the inner mitochondrial membrane, leading to the disappearance of its activity.

The uncoupling potency of fluazinam under conditions with 30 μM DNCB was measured at various concentrations of fluazinam. With this concentration of DNCB, the transformation of the fluazinam molecule was completely prevented and mitochondrial functions such as the respiration rate and transmembrane electrical potential at state 4 were not affected. The minimum concentration of fluazinam inducing the maximum respiration rate with DNCB was $(1.0–1.5) \cdot 10^{-8}$ M. Under conditions without DNCB, the minimum concentration estimated from the initial respiration rate was $(1.5–2.5) \cdot 10^{-8}$ M. The concentration of the *i*-OPr analog inducing the maximum respiration rate was $(1.0–1.5) \cdot 10^{-7}$ M regardless of the presence or absence of DNCB. The concentration of SF6847 inducing the maximum respiration rate is reported to be $(2.5–3.5) \cdot 10^{-8}$ M [19]. Fluazinam is slightly more potent than SF6847. The intrinsic uncoupling activity of fluazinam in terms of the mean number of molecules needed for the maximum respiration release per respiration assembly was 0.07 in the presence of 30 μM DNCB. That of SF6847 is 0.09 [20]. Thus, fluazinam is slightly more potent than SF6847, even in the inner mitochondrial membrane phase.

Schäfer and Büchel [21] have observed a similar disappearance of the uncoupling activity of an acidic uncoupler with an NH bridge as the acidic dissociable group, 2-(2,6-dimethylanilino)-3,4-dinitro-5-chlorothiophene (DDCT, II), with rat-liver mitochondria.



(II)

They have suggested, without any experimental evidence, that the disappearance of the uncoupling effect may arise from the accumulation of the hydrophobic neutral form of the uncoupler molecule in the inner mitochondrial membrane phase because of its low mobility through the inner membrane. Their suggestion seems to be incorrect, because Kasianowicz et al. [22] have shown that the permeation by the neutral form of the uncoupler molecule across the lipid bilayer membrane is much greater than that by the ionized form. The DDCT also seems to have a suitable leaving chlorine atom on its thiophene ring. The disappearance of the uncoupling activity of DDCT is probably due to its metabolic transformation by GSH conjugation.

Acidic uncouplers have been widely used in bioenergetic studies. The potent acidic uncouplers such as SF6847, S-13 (2′,5-dichloro-3-(*t*-butyl)-4-nitrosalicylanilide), FCCP (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone), and CCCP (carbonylcyanido *m*-chlorophenylhydrazone) have their own stable uncoupling effect on mitochondria, that is, the extent of the acceleration of the respiration rate and the extent of the decrease in the transmembrane electrical potential are governed by the amount of each uncoupler incorporated into the mitochondria. Fluazinam, however, was able to change the mitochondrial state continuously from the uncoupling condition to state 4 at a very low concentration. This unusual characteristic of fluazinam may make new procedures in bioenergetic research possible. Moreover, this compound could be a selective toxic agent usable as a pesticide if the target and non-target organisms have different metabolic detoxication mechanisms.

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