Respiratory Inhibition of Acaricide AKD-2023 and Its Deacetyl Metabolite

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To define acaricidal mechanism of a new acaricidal AKD-2023 (2-acetoxy-3-n-dodecyl-1,4-naphthoquinone) and its metabolite, DHN (2-hydroxy-3-n-dodecyl-1,4-naphthoquinone), the effects of these compounds on the respiration of flight-muscle mitochondria isolated from house flies were investigated. The inhibitory potency of AKD-2023 for succinate and pyruvate oxidation activity was much less than that of DHN. This result suggested that the hydrolyzed metabolite, DHN is virtually biologically active and inhibits the respiration of mitochondria. It was also proved that the inhibition occurs at the complex III in mitochondrial electron transfer chain. On the basis of reduction kinetics of cytochromes b and c, the binding site was shown to be ubiquinol oxidation site (Q$_0$ center) of complex III, being different from that of other acaricides developed recently.

INTRODUCTION

AKD-2023 (2-acetoxy-3-n-dodecyl-1, 4-naphthoquinone, Fig. 1) is an acaricidal compound under product development for the control of broad species of mites in agricultural crops such as pone fruits, teas and vegetables. Recently, there have been observations in the field that some commercial synthesized acaricides tend to loose their efficacy due to the built mite resistance to the chemicals. AKD-2023, featured in its chemical structure and mode of action, is therefore to be an acaricide possibly producing less cross-resistance with other commercial on the present markets.

When the mode of action of AKD-2023 is considered, it is suggestive that DHN (2-hydroxy-3-n-dodecyl-1,4-naphthoquinone), a hydrolyzed metabolite of AKD-2023, is the specific inhibitor of bovine heart mitochondrial complex III. It is therefore likely that after uptake by mite, AKD-2023 is hydrolyzed to DHN, and this metabolite inhibits mitochondrial electron-transfer of mite. To confirm this possibility, we investigated the effect of DHN as well as AKD-2023 on the respiration of flight-muscle mitochondria isolated from house flies. Although it is ideal that their effects are examined with mites mitochondria, the preparation of sufficient amount of intact mitochondria is experimentally difficult from this biological source. We therefore used flight-muscle mitochondria as the second best policy in the study. It is possible to premise a similar inhibitor sensitivity of electron-transfer system between fly and mite mitochondria since a high homology has been claimed for mitochondrially encoded subunits consisting electron-transfer complexes.2,3

MATERIALS AND METHODS

1. Chemicals

AKD-2023 and DHN were synthesized at Lab. of Kawasaki Kasei Chemicals, Ltd. Antimycin A and cytochrome c were purchased from Sigma. Pyridaben was purchased from Kanto Chemical Co., Inc. Myxothiazol was obtained from Boehringer. 2,3-Dimethoxy-5-methyl-6-n-decyl-1,4-benzoquinol (DBH$_2$) was synthesized according to Sakamoto et al. Other reagents were of the purest grade commercially available.

2. Preparation of Mitochondria from House Flies

Mitochondria were isolated from the flight-muscle of house flies by a procedure of Ilivecky et al. The final mitochondrial pellet was suspended in the medium containing 0.25 M sucrose, 5 mM EDTA and 10 mM Tris-HCl (pH 7.4). The amount of mitochondrial protein was measured by the method of Bradford with bovine serum albumin as the standard.
3. Measurement of Mitochondrial Respiration

Mitochondrial respiration was measured with a Clark-type oxygen electrode fixed with a thermostatic reaction cell at 25°C. The reaction medium was 0.15 M KCl, 2.5 mM MgCl₂, and 30 mM phosphate buffer (pH 7.4). Various respiration substrates supplying electrons at different sites were used as described in the figures legends.

4. Identification of Metabolite DHN in Mitochondrial Medium

HPLC analysis was conducted with a Shimadzu Model LC-6A instrument. AKD-2023 (2 × 10⁻⁵ M) was incubated at 25°C with mitochondria (0.12 mg/ml) and the same incubation medium as that for the oxygen consumption experiments. After centrifugation at 10,000 rpm for 5 min, the supernatant (50 μl) was applied to a Senshu-Pak VP-304 column (250 × 4.6 mm i.d., Senshu Science Co.). Elution was performed with a linear gradient of 50–90% acetonitrile in 0.1% phosphoric acid at a flow rate of 1 ml/min and UV absorbance at 250 nm was detected.

S. Effects of DHN on Reduction Kinetics of the Hemes b and c₁ in Intact Mitochondria by Succinate

The relative extent of reduction of cytochromes b and c₁ was determined using intact mitochondria in a Shimadzu UV-3000 spectrophotometer in the dual-wavelength mode; the wavelength pairs 562–575 nm for cytochrome b and 553–540 nm for cytochrome c₁ were used. The incubation medium was the same as that used for the oxygen consumption experiment, except that 1 μM rotenone and 2 mM KCN was added. The final mitochondrial protein was 0.88 mg/ml. Complex III was energized by adding 10 mM succinate.

RESULTS AND DISCUSSION

1. Effects of AKD-2023 and DHN on Flight-Muscle Mitochondrial Respiration by Succinate

Effects of AKD-2023 and its hydrolyzed product DHN on flight-muscle mitochondrial respiration was examined using succinate as a respiration substrate with Clark-type oxygen electrode. DHN elicited rapid and complete inhibition of the respiration (Fig. 2A). Effect of the inhibitors on the respiration was similar when pyruvate was used as a respiration substrate in place of succinate (data not shown). In contrast, the inhibition by AKD-2023 appeared with time, and the extent of inhibition was not so much as that by DHN (Fig. 2A). These results suggest that AKD-2023 is hydrolyzed even by incubating with mitochondrial fraction, as described later, and that the metabolite DHN is an active form for the respiratory inhibition. Considering that carboxylic esters of primary alcohols are readily hydrolyzed by the metabolic system in insect and mite, AKD-2023 might be hydrolyzed to its active form (DHN) after uptake by mites. Similar activation has been proposed for esterified dinitrophenol-type acidic uncouplers. The inhibitory potency of DHN in terms of I₅₀ value was compared with that of the potent, specific inhibitor of complexes I (pyridaben) and III (antimycin A) (Table 1). DHN was less potent than the two inhibitors at least at organelle level.

![Fig. 1 Structures of AKD-2023 and DHN.](image)

![Fig. 2 Effects of DHN (A) and AKD-2023 (B) on mitochondrial respiration.](image)

### Table 1 Inhibitory potency of inhibitors with flight-muscle mitochondria of house flies.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>I₅₀ (nmol/mg of protein)</th>
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<tbody>
<tr>
<td>AKD-2023</td>
<td>45.1</td>
</tr>
<tr>
<td>DHN</td>
<td>43.6</td>
</tr>
<tr>
<td>Pyridaben</td>
<td>0.94</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.45</td>
</tr>
</tbody>
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N.A: Not active at 100 nmol/mg of protein.
2. Analysis of AKD-2023 and DHN Contents after Incubation with Intact Mitochondria

As described above, there was a considerable lag in appearance of the respiratory inhibition by AKD-2023, suggesting that this compound can be hydrolyzed during the incubation with mitochondrial fraction. To confirm this possibility, we analyzed degradation and production of AKD-2023 and DHN, respectively, by HPLC as shown in Fig. 3. AKD-2023 decomposed gradually with the production of DHN, indicating that AKD-2023 is hydrolyzed even by isolated mitochondria. This notion is supported by the fact that esterified dinitrophenol-type uncouplers of mitochondrial oxidative phosphorylation gradually elicit uncoupling activity during the incubation with isolated mitochondria (Miyoshi et al., unpublished work). In this context, it is notable that isolated mitochondria are also capable of metabolizing some pesticides which possess an adequate leaving group by glutathion conjugation.\textsuperscript{14}

3. Identification of the Inhibition Site of DHN

DHN is a specific inhibitor of complex III in bovine heart mitochondria.\textsuperscript{1} It is needed to determine experimentally whether this is also the case for insect mitochondria. As shown in Table 1, the inhibitory potencies of DHN were almost identical irrespective of respiration substrates used. Considering that NADH produced by pyruvate oxidation and succinate donate electrons complexes I and II, respectively, the inhibition site of DHN would be a crossover point of the two respiratory pathways, namely complex III. The inhibition by pyridaben\textsuperscript{13} (complex I inhibitor) of the respiration energized by pyruvate was completely released by adding succinate (Fig. 4A). The inhibition by DHN was not released by adding succinate, but was done by the addition of ascorbate plus TMPD (\(N,N,N',N'-\text{tetramethyl-p-}
\text{phenylenediamine}\)) electron donor to complex IV (Fig. 4B). These results show that DHN is also a specific inhibitor of complex III in flight-muscle mitochondria. In fact, spectrophotometrically determined DBH\textsubscript{2}-cytochrome c oxidoreductase activity of intact mitochondria was completely inhibited in the presence of 0.36 \(\mu\text{M}\) DHN (data not shown).

4. Effect of DHN on Reduction Kinetics of the Hemes b and c\textsubscript{1} by Succinate in Intact Mitochondria

In the protonmotive Q-cycle model of complex III,\textsuperscript{15} ubiquinol is oxidized at \(Q_0\) center, and ubiquinone is reduced at \(Q_1\) center of the enzyme. On the basis of a difference in the effect on reduction kinetics of cytochromes b and c\textsubscript{1}, \(Q_0\) and \(Q_1\) center inhibitors are defined.\textsuperscript{15} To identify the binding site of DHN, we examined the effect of DHN on the reduction kinetics of cytochromes b and c\textsubscript{1} at the concentration of the inhibitor sufficient to exhibit full inhibition (Fig. 5). As a standard inhibitor, antimycin A and myxothiazol were used as \(Q_1\) and \(Q_0\) center inhibitors, respectively. In the absence of inhibitors, respectively about 50 and 70% of cytochromes b (Fig. 5A) and c\textsubscript{1} (Fig. 5F) were reduced by the addition of succinate. The extent of rapidly reduced cytochrome b in the presence of inhibitor was higher than that without inhibitor (Figs. 5B and 5C). The inhibition of cytochrome b reduction was almost complete in the presence of antimycin A and myxothiazol (Fig. 5D), \textit{i.e.,} under double-kill conditions. The cytochrome b reduction was also remarkably inhibited by a
pair of antimycin A and DHN (Fig. 5E). The extent of reduction of cytochrome c₁ was slightly decreased by antimycin A (Fig. 5G). Myxothiazol and DHN blocked the reduction of cytochrome c₁ (Figs. 5H and 5I). These results showed that DHN binds to Qo center of complex III. However, since the effect on cytochrome c₁ reduction was slightly different between myxothiazol and DHN, the binding manner of DHN may be somewhat different from that of myxothiazol.

In conclusion, DHN appeared to be a potent inhibitor of flight-muscle mitochondrial complex III, acting at Q₀ center of the enzyme. An active form of AKD-2023 for respiration inhibition was its hydrolyzed metabolite DHN.

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