Biochemical studies of pigments from a pathogenic fungus; *Microsporum cookei* VI. Formation of a xanthomegnin-bypass to the mitochondrial electron transport system

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Summary. Enzymatic redox response of xanthomegnin was studied by means of rat liver mitochondria and the possibility of a xanthomegnin-bypass to mitochondrial electron transport system is suggested.

A binaphthoquinone pigment, xanthomegnin, has been isolated from the pathogenic fungi *Microsporum cookei*[1], *Trichophyton rubrum*[2], *T. megnini*[3], and *T. violaceum*[4], and some species of saprophytic fungi such as *Aspergillus melleus*[5], *A. sulphreus*[5], and *Penicillium viridicatum*[6] as a mycotoxin. Toxicity of this pigment to mice and rat has been suggested[7,8]. Therefore it is of importance to study its biological properties. Xanthomegnin has been shown to uncouple the oxidative phosphorylation of isolated rat liver mitochondria[9,10]. The inhibitory effect of the pigment on ATP synthesis may be responsible for its cytotoxicity to animal liver and kidney. It has been demonstrated that the lipophilic and acid dissociable properties of this pigment may be involved in its strong uncoupling effect on mitochondrial respiration[8]. On the other hand, the naphthoquinone structure of xanthomegnin suggests the possibility of an electron transport bypass to the mitochondrial respiratory chain as reported for menadione-bypass[11]. The existence of such a bypass may lead to inhibition of ATP synthesis. This communication deals with the enzymatic reduction of xanthomegnin by the mitochondrial electron transport system and a tentative model for the xanthomegnin-bypass is presented.

Materials and methods. Xanthomegnin was isolated from dried mycelium of *Microsporum cookei* according to the procedure previously reported[1] and was used as a solution of N,N-dimethylformamide. Rat liver mitochondria were prepared according to the method[12] using 250 mM sucrose solution which contained 0.5 mM EDTA and 10 mM Tris-HCl (pH 7.4) as an isolation medium. Reductions of xanthomegnin and cytochrome c were monitored spectroscopically by absorbance change at 412 and 550 nm, respectively, using Hitachi Recording Spectrophotometer EPS-3T. Protein in reaction mixture was measured by the method of Lowry et al.[13], using bovine serum albumin as a standard protein. NADH, Tris, and cytochrome c (horse heart cytochrome c) were products of Sigma Chemical Co. Antimycin A and p-chloromercuribenzoate (PCMB) were purchased from Kyowa Hakko Co. and Nakarai Chemicals Co., respectively. Other reagents were of the purest grade commercially available.

Results and discussion. Figure 1 shows the enzymatic reduction of xanthomegnin by intact mitochondria. Addition of NADH caused a rapid increase in absorbance at 412 nm, suggesting the enzymatic reduction of xanthomegnin by mitochondrial electron transport system. When added NADH was completely oxidized, an immediate decrease in the absorbance was observed suggesting the oxidation of the reduced xanthomegnin. Subsequent addition of NADH was also followed by the reduction of the pigment (curve a). However, in the presence of the SH inhibitor PCMB, the enzymatic reduction of xanthomegnin was completely inhibited (curve b). A specific electron transport inhibitor antimycin A caused no inhibition (data not shown). These results suggest that xanthomegnin was reduced by NADH-cytochrome c reductase present in the outer membranes of mitochondria[14,15], since the inner membranes are impermeable to externally added NADH[14,15].

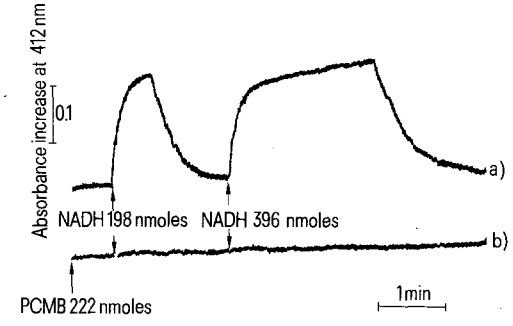

Fig. 1. Enzymatic reduction of xanthomegnin by NADH-cytochrome c reductase of outer membranes of mitochondria. Reaction medium contained 225 mM sucrose, 10 mM $MgCl_2$, 10 mM KCl, 0.5 mM EDTA, 10 mM Tris-HCl (pH 7.4), and 1.88 mg/ml of mitochondrial protein in a final volume of 3.0 ml. Incubations were carried out at 25 °C.

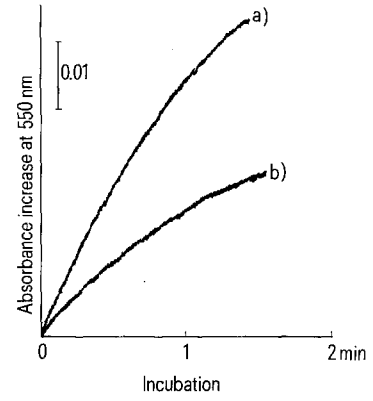

Fig. 2. Reduction of externally added cytochrome c. Reaction medium contained 225 mM sucrose, 10 mM $MgCl_2$, 10 mM KCl, 0.5 mM EDTA, 10 mM Tris-HCl (pH 7.4), 0.5 mg/ml of cytochrome c, and 0.2 mg/ml of mitochondrial protein in a final volume of 2.7 ml. Incubations were carried out at 25 °C.

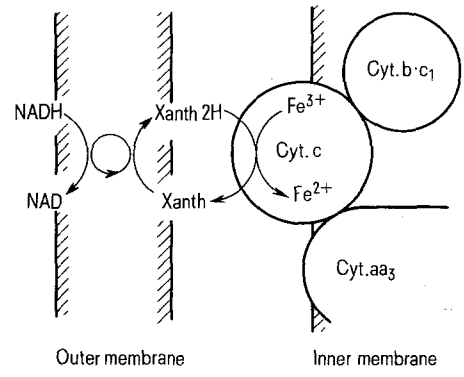

Fig. 3. Tentatively proposed model for xanthomegnin-bypass.

Figure 2 shows the reduction of externally added cytochrome c in the presence or absence of xanthomegnin. Addition of NADH produced an increase in the absorbance at 550 nm suggesting that the externally added cytochrome c was reduced by NADH-cytochrome c reductase of mitochondrial outer membranes (curve b). In the presence of xanthomegnin, cytochrome c was reduced at a higher rate than that in the control experiment (curve a). This suggests that the redox response of xanthomegnin increased the reduction rate of cytochrome c.

The reduction of cytochrome c by reduced xanthomegnin has already been demonstrated by us using ascorbate-loaded liposomes[18]. These data suggest that xanthomegnin, reduced by NADH-cytochrome c reductase present in the outer membranes was oxidized by cytochrome c. Cytochrome c, localized on the cytoplasmic-side of the mitochondrial inner membranes, may be reduced by xanthomegnin in a similar fashion as described above. This may give rise to a xanthomegnin-bypass as presented in figure 3.

As demonstrated in our previous communication[19], xanthomegnin was also reduced by mitochondria respiring succinate or L-glutamate as a substrate. But the reduction was initiated when the dissolved oxygen of reaction mixture was completely consumed. The reduction was completely inhibited by antimycin A or cyanide. This evidence indicates that xanthomegnin can form a bypass only in the presence of NADH. Thus xanthomegnin may oxidize cytoplasmic NADH by means of mitochondrial electron transport system.

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The inability of nuclear dehydrogenating clostridia to oxidize bile salt hydroxyl groups[1]

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Summary. In a survey of the intracellular bile salt oxidoreductase activity in fecal bacteria, 16 strains of nuclear dehydrogenating clostridia and 2 strains of non-nuclear dehydrogenating *C. paraputrificum* were demonstrated unable to oxidize cholate at any of the 3 OH groups. Since nuclear dehydrogenation at the Δ-1 and Δ-4 position requires a 3-oxo precursor steroid, it appears that these organisms require the presence of a 3α-hydroxysteroid dehydrogenating organism for nuclear dehydrogenation.

In the intestinal flora, the primary bacteria capable of introducing double bonds into the steroid skeleton are the lecethinase negative clostridia: predominantly *C. paraputrificum, C. indolis,* and *C. tertium*[2]. These organisms contain nuclear dehydrogenases (NDH) active at the Δ-1 and Δ-4 positions of the A ring of 3 oxo-bile salts and 3 oxo-steroids[3,4]. High fecal populations of nuclear dehydrogenating clostridia (NDC) and high fecal concentrations of bile salts have been associated with bowel cancer patients and may be thereby implicated in the aetiology of colon cancer. Putative aromatisation of bile salts and steroids by gut bacteria has been a proposed mechanism of carcinogen formation in the human bowel.

The requirement of NDC for 3 oxo-bile salts or 3 oxo-steroids for the introduction of unsaturations into the A ring has prompted a search in these organisms for intracellular 3α-hydroxysteroid dehydrogenase (HSDH) activity which could then provide substrates for the NDH.

Methods and materials. Nuclear dehydrogenating and non-nuclear dehydrogenating clostridia were isolated from clinical sources[5] and prescreened for the presence of NDH as demonstrated by the formation of Δ1 and Δ4 unsaturations into 5β-androstan-3, 17 dione and 3 oxo-5β-cholanoate as described before[3]. These organisms were maintained in cooked meat medium at 4 °C and grown in 10 ml Brain Heart Infusion medium containing 0.1% sodium thioglycolate and 1.0×10^{-3} M sodium cholate for 48 h.

Cultures were centrifuged at 6000 × g for 20 min at 4 °C in an International B-20 centrifuge. The pellet was resuspended in 1.5 ml of 0.1 M sodium phosphate containing 10^{-3} M