原 著

Biochemical Studies of Pigments from a Pathogenic
Fungus *Microsporium cookei* IV. Enzymatic
Reduction of Xanthomegnin by
Mitochondria and Microsomes

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The enzymatic oxido-reduction of xanthomegnin has been investigated spectroscopically using rat liver mitochondria and microsomes. Xanthomegnin, a binaphthoquinone pigment isolated from a dermatophyte *Microsporium cookei*, has been shown to strongly uncouple mitochondrial respiration and has been suggested to exert a cyto-toxic effect in animals. The redox response of this pigment has also been suggested to participate in uncoupling of oxidative phosphorylation in mitochondria, by forming a bypass to the electron transport system. In the presence of succinate or L-glutamate as substrate, xanthomegnin was reduced by mitochondria after dissolved oxygen was completely consumed. This suggests that complex IV of the respiratory chain of mitochondria is concerned with enzymatic reduction of xanthomegnin and that its redox response is not involved in the uncoupling effect on mitochondrial respiration. On the other hand, when NADH was added, xanthomegnin was immediately reduced by mitochondria, submitochondrial particles, and microsomes, indicating the xanthomegnin is reduced by NAD-linked electron transport system of mitochondria and microsomes. Xanthomegnin reduced by ascorbate was auto-oxidized by dissolved oxygen.

These results would indicate that xanthomegnin forms an electron transport bypass to the NAD-linked respiratory chain and readily oxidize cytosol NADH. This may cause the cyto-toxic effect to animals.

Many kinds of quinone pigments have been isolated from various kinds of fungi¹⁻³⁾. But their biological significance of pigment production is still unknown. It has been shown that dermatophytes also produce various pigments⁴⁻⁸⁾. We have investigated the biological properties and chemical structures of pigments isolated from a dermatophyte *Microsporium cookei*⁹⁻¹⁵⁾, which produces naphthoquinone pigments; xanthomegnin, aurosporin, violosporin, luteosporin, rubrosporin, and iridosporin⁹⁾. Some of these pigments have been shown to be

produced by the other kinds of dermatophytes⁴⁻⁷⁾. Recently two kinds of pigments with the identical chemical structures to aurosporin and violosporin have been isolated from *Aspergillus melleus* and are called viomellein and viopurpurin, respectively¹⁶⁾. This fungus has also been shown to produce xanthomegnin¹⁸⁾. This suggests that these naphthoquinone pigments would be involved in a certain biochemical reaction in the fungal mycelia. Therefore it is of interest to study their biochemical properties, biological functions, and metabolic relationship. One

could speculate various biological actions of pigments; antibiotic or toxic effects on other microorganisms, protection from UV radiation, or involvement in the intracellular metabolism such as oxido-reduction due to their quinone structures. However, at the present time we have too small quantity of evidence at hand to clarify them.

In our earlier studies, xanthomegnin, a hydrophobic pigment isolated from the dried mycelium of *M. cookei*, has been observed to show a hydrophobic binding to bovine serum albumin and a strong uncoupling effect on the oxidative phosphorylation of rat liver mitochondria⁹⁻¹²). But lutesporin, a hydrophilic pigment isolated from the culture medium of *M. cookei*, showed no uncoupling effect on the mitochondrial respiration, although this pigment is very similar in chemical structure to xanthomegnin¹¹). The uncoupling effect of xanthomegnin on mitochondria respiration may bring about an antibiotic effect or toxic effect on the other organisms or host cells. The hydrophilic pigment lutesporin has been supposed to be one of water soluble metabolites of hydrophobic pigments¹¹). On the other hand, naphthoquinone pigments such as juglone (5-hydroxy naphthoquinone) and menadione (2-methyl naphthoquinone) have been shown to be reduced by mitochondrial electron transport system^{17,18}). Therefore xanthomegnin also was expected to be reduced by enzymes. This communication deals with the enzymatic reduction of xanthomegnin by rat liver mitochondria, sub-mitochondrial particles, and microsomes.

MATERIALS AND METHODS

Xanthomegnin was isolated from dried mycelia of *M. cookei* by the procedure described previously¹¹). Mitochondrial fraction was prepared from rat liver homogenate in 0.25 M sucrose containing 0.5 mM EDTA and 10 mM Tris-HCl, pH 7.4. Rat liver homogenate was centrifuged at $1,000\times g$ for 10 min and the supernatant fraction was recentrifuged at $10,000\times g$ for 10 min. The resulting pellet was resuspended in the same buffer and centrifuged at $1,000\times g$ for 5 min. The supernatant frac-

tion was further centrifuged at $5,000\times g$ for 10 min to pellet mitochondria. Mitochondria thus obtained were washed by two additional centrifugation under the same condition ($5,000\times g$ for 10 min). For preparation of microsomal fraction, rat liver homogenate was centrifuged at $12,000\times g$ for 20 min and its supernatant was spun down at $105,000\times g$ for 60 min. The pellet obtained was resuspended in the SET solution and centrifuged at $105,000\times g$ for 60 min. The final pellet was used as a microsomal fraction. Submitochondrial particles (EPT) were prepared by the method of Ruzicka and Carne¹⁷) from rat liver mitochondria. The reduction of xanthomegnin was observed by tracing the changes of the absorbance at 412 nm by means of Hitachi Recording Spectrophotometer EPS-3T. The oxygen uptake of mitochondria was measured by using Galvani-type oxygen electrode (Kjusui Kagaku Kenkyusho), which was attached to a potentiometric recorder (Toa Denpa Custom Demand Recorder 81A). Protein in reaction medium was determined by the method of Lowry et al.¹⁹) using bovine serum albumin as a standard protein. NADH and Tris were purchased from Sigma Pharmaceutical Co. Other reagents were of the purest grade commercially available.

RESULTS

Enzymatic Reduction of Xanthomegnin by Mitochondria

Fig. 1 shows the enzymatic reduction of xanthomegnin by rat liver mitochondria. As shown in curve (a), NADH itself caused no reduction of xanthomegnin but the addition of mitochondria caused abrupt increase of the absorbance at 412 nm, indicating the enzymatic reduction of xanthomegnin by mitochondria. After the complete consumption of NADH added, a decrease of the absorbance to the original level was observed, which is due to oxidation of reduced xanthomegnin. In the presence of mitochondria the increase and subsequent decrease of absorbance at 412 nm was observed by repeating addition of NADH as shown in curve (a). The reduction of xanthomegnin by mitochondria in the presence of

Fig. 1. Enzymatic reduction of xanthomegnin by mitochondria respiring NADH as a substrate. Reaction medium was composed of 0.225 M sucrose, 10 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, and 20 mM Tris-HCl (pH 7.4) in a final volume of 3.0. The concentrations of mitochondrial protein were 2.0 mg/ml (curve a) and 1.8 mg/ml (curve b), respectively. Incubations were carried out at 25°C.

Fig. 2. Enzymatic reduction of xanthomegnin by mitochondria respiring succinate as a substrate. The composition of the reaction medium was essentially the same as in Fig. 1. The concentrations of succinate and xanthomegnin in reaction medium were 5.0 mM and 20 μ M, respectively. The mitochondrial protein in reaction medium was 1.2 mg/ml.

Fig. 3. Enzymatic reduction of xanthomegnin by ETP respiring NADH as a substrate. The conditions were the same as in Fig. 1. Curve (a) shows the enzymatic reduction of xanthomegnin by ETP (1.0 mg/ml) and curve (b) shows the effect of matrix enzymes of mitochondria on the redox response of xanthomegnin. The concentration of protein in curve (b) was 4.8 mg/ml.

NADH was not inhibited by antimycin A which was known to inhibit the electron transport system of mitochondria between cytochrome b and c (data not shown). This suggest that NADH dehydrogenase or the NADH-CoQ reductase (complex I) of the electron transport system of mitochondria may be involved in the enzymatic reduction of xanthomegnin.

On the other hand, mitochondria respiring succinate as a substrate showed a delayed reduction of the pigment. As seen in Fig. 2, a small scale decrease of the absorbance at 412 nm was preceded, then a marked increase of the absorbance occurred indicating the reduction of xanthomegnin. Similar result was obtained by using mitochondria respiring L-glutamate as a substrate (data not shown). These results suggest that neither of complex I nor complex II of the electron transport system are involved in the enzymatic reduction of xanthomegnin.

The Enzymatic Reduction of Xanthomegnin by ETP Respiring NADH as a Substrate

Fig. 3 shows the enzymatic reduction of xanthomegnin by ETP prepared by freeze-thawing and sonic disruption of mitochondria. The addition of NADH also caused the immediate increase of the absorbance at 412 nm as observed in the case of intact mitochondria. The complete consumption of oxygen in reaction medium caused a further increase in the absorbance and the decrease of the absorbance was no longer observed as shown in curve (a). The supernatant fraction after ultracentrifugation at $105,000 \times g$ for 60 min of the sonically disrupted mitochondria showed no reduction of xanthomegnin, indicating that the enzymes capable of reducing xanthomegnin are bound to the mitochondrial membranes (curve (b)).

The Enzymatic Reduction of Xanthomegnin by ETP Respiring Succinate as a Substrate

Fig. 4 shows the enzymatic reduction of xanthomegnin by ETP in the presence of succinate. The reduction of xanthomegnin was initiated after a lag time as observed in the case of intact mitochondria respiring succinate as a substrate. As seen in curve (b) and (c), xanthomegnin was reduced simultaneously with reduction of methylene blue which has been well

Fig. 4. Enzymatic reduction of xanthomegnin by ETP respiring succinate as a substrate. The composition of the incubation medium was the same as in Fig. 1. Curve (a) shows the reduction of xanthomegnin by ETP which was pre-incubated with succinate for 15 min. Curve (b) and (c) show the changes in the absorbance at 412 and 600 nm as a function of incubation time in the presence of xanthomegnin and methylene blue, respectively.

known to be reduced by cytochrome oxidase of mitochondria under anaerobic condition. EPT preincubated with succinate for 15 min initiated the pigment reduction without any lag time as shown in curve (a). These results show apparently that xanthomegnin can be reduced by mitochondria or submitochondrial particles respiring succinate as a substrate when the incubation medium became anaerobic.

Non-enzymatic Oxido-Reduction of Xanthomegnin

Non-enzymatic redox response of xanthomegnin was observed by using oxygen electrode (Fig. 5). Mitochondria can utilize ascorbate as a substrate in the presence of tetramethyl p-phenylenediamine (TMPD). Addition of TMPD lead to state 4 respiration of mitochondria and subsequent addition of ADP

Fig. 5. Non-enzymatic oxido-reduction response of xanthomegnin. The reaction medium was composed of 0.225 M sucrose, 10 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA and 20 mM Tris-HCl (pH 7.4) in a final volume of 3.3 ml. protein was 1.2 mg/ml.

Fig. 6. Enzymatic reduction of xanthomegnin by microsomes. The incubation conditions were the same as in Fig. 1. The microsomal proteins were 8.9 mg/ml (curve a) and 2.6 mg/ml (curve b), respectively.

accelerated markedly the oxygen consumption as shown in curve (a). The ADP-driven respiration (state 3 respiration) was inhibited by NaN_3 which had been well known to inhibit cytochrome oxidase of mitochondria. On the

contrary, addition of xanthomegnin in stead of TMPD caused immediate oxygen consumption in a maximal velocity and the induction of state 3 respiration was not observed as seen in curve (b). The oxygen consumption

stimulated by xanthomegnin was not inhibited by NaN_3 . This shows that xanthomegnin was reduced non-enzymatically by ascorbate and also auto-oxidized by oxygen. The direct reduction of xanthomegnin by ascorbate has also been demonstrated spectrophotometrically¹⁶. These results suggest a possibility that xanthomegnin provides a bypass in the electron transport system of mitochondria.

Xanthomegnin was reduced by rat liver microsomes in the presence of NADH. As shown in Fig. 6, addition of NADH caused the immediate reduction of xanthomegnin and reduced xanthomegnin was oxidized when NADH added was completely consumed, indicating the enzymatic redox response of xanthomegnin by NAD-linked electron transport system of microsomes. In the reaction medium containing lower concentration of microsome protein, a slight but prolonged reduction of xanthomegnin was observed.

DISCUSSION

The enzymatic reduction of xanthomegnin by mitochondria, submitochondrial particles (ETP), and microsomes has been investigated by means of spectrophotometric techniques. Xanthomegnin was reduced various kinds of reducing agents such as ascorbate, sodium dithionite, potassium hydroborate, and dithiothreitol. And as demonstrated above, xanthomegnin shows a marked redox response in the presence of mitochondria, submitochondrial particles, and microsomes. Such enzymatic reduction of xanthomegnin may suggest that the biological significance of the pigment is related to the oxido-reduction metabolism in the fungal cell. Ubiquinones are well known to be a member of an obligatory component in the electron transport system of mitochondria, and are exclusively localized in mitochondria. However, xanthomegnin is not distributed so ubiquitously as ubiquinones. It would be therefore expectable that xanthomegnin shows a characteristic property in the redox response according to its binaphthoquinone structure.

On the other hand, the enzymatic reduction of xanthomegnin by mitochondria or microsomes

may result in a formation of a bypass in the electron transport system, thereby disorder in the oxidative phosphorylation or normal oxido-reduction in the cell to result in causing toxic effect on the host cells.

REFERENCES

- 1) Thomson, R.H.: *In*; Naturally Occurring Quinones, Academic Press, London and New York, 1971
- 2) Turner, W.B.: Polyketides. *In*; Fungal Metabolites, p. 74—213, Academic Press, London and New York, 1971.
- 3) Shibata, S., Natori, S. and Udagawa, S.: Quinones. *In*; List of Fungal Products, p. 65—86, University of Tokyo Press, 1964.
- 4) Ito, Y., Fujii, T. and Nozawa, Y.: The pigments of *Trichophyton* species. *J. Invest. Dermatol.* **42**, 175—176, 1964.
- 5) Ito, Y., Nozawa, Y. and Yoneya, T.: Pigment patterns of dermatophytes on thin-layer chromatography. *J. Chromatog.* **35**, 573—575, 1968.
- 6) Ito, Y., Fujii, T., Nozawa, Y. and Takeyama, A.: Thin-layer chromatography of the pigments isolated from some *Trichophyton* species. *J. Invest. Dermatol.* **44**, 345—437, 1965.
- 7) Nozawa, Y.: Studies on the pigments of the dermatophytes III. Differences in the distribution of the pigments among the genera and species using thin-layer chromatography. *Acta School Med. Univ. Gifu* **15**, 15—20, 1967.
- 8) Nozawa, Y. and Ito, Y.: Separation of quinone pigments from *Microsporum cookei* by thin-layer chromatography. *Experientia* **26**, 803—804, 1970.
- 9) Ito, Y., Nozawa, Y. and Kawai, K.: Effect of naphthoquinone pigment, xanthomegnin from *Microsporum cookei* on the respiration of rat liver mitochondria. *Experientia* **25**, 826—827, 1969.
- 10) Ito, Y., Kawai, K. and Nozawa, Y.: Biochemical studies of pigments from the pathogenic fungus, *Microsporum cookei*. Effect of the 1, 4-naphthoquinone pigment xanthomegnin on oxidative phosphorylation in rat liver mitochondria. *J. Biochem.* **74**, 805—810, 1973.
- 11) Akita, T., Kawai, K., Shimonaka, H., Nozawa, Y., Ito, Y., Nishibe, S. and Ogihara, Y.: Biochemical studies on pigments from the dermatophyte *Microsporum cookei* II. Effect of luteosporin on the oxidative phosphorylation

- of rat liver mitochondria. Japan. J. Med. Mycol. **16**, 177—184, 1975.
- 12) Kawai, K., Akita, T., Nishibe, S., Nozawa, Y., Ogihara, Y. and Ito, Y.: Biochemical studies of pigments from a pathogenic fungus *Microsporium cookei* III. Comparison of the effects of xanthomegnin and O-methylxanthomegnin of the oxidative phosphorylation of rat liver mitochondria. J. Biochem. **79**, 145—152, 1976.
 - 13) Kawai, K., Akita, T. and Shimonaka, H.: Interaction of xanthomegnin, a pigment from a dermatophyte *Microsporium cookei*, with bovine serum albumin. Medicine and Biology **94**, 507—510, 1977.
 - 14) Kawai, K., Akita, T. and Shimonaka, H.: Interaction of xanthomegnin, a pigment from a dermatophyte *Microsporium cookei*, with hydrophobic and basic amino acids. Medicine and Biology **95**, 5—8, 1977.
 - 15) Kawai, K., Akita, T. and Nozawa, Y.: On a transmembrane permeability of xanthomegnin, isolated from a pathogenic fungus *Microsporium cookei* across phospholipid bilayer membranes. Proc. Jap. Assoc. Mycotoxicol.
 - 16) Richard, C.D., MacMillan, J., Thomas, J.S., Glen, A.T. and Turner, W.B.: Fungal products. Part XIII. Xanthomegnin, viomellin, rubrosulphin, and viopurpurin, pigments from *Aspergillus sulphureus* and *Aspergillus melleus*. J. Chem. Soc. Perkin I 163—169, 1975.
 - 17) Ruzicka, F.J. and Crane, F.L.: Quinone interaction with the respiratory chain-linked NADH dehydrogenase of beef heart mitochondria I. Juglone reductase activity. Biochim. Biophys. Acta **223**, 71—85, 1970.
 - 18) Brodie, A.F.: The role of naphthoquinones in oxidative metabolism. In; Biochemistry of quinones (ed. Morton, R.A.) p. 355—404, 1965.
 - 19) Rowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J.: Protein measurement with Folin Phenol reagent. J. Biol. Chem. **193**, 265—275, 1951.
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