ENZYMATIC STUDIES OF AVIAN CYTOCHROME P450 1A4 AND 1A5 ISOZYMES HETEROLOGOUSLY EXPRESSED IN YEAST CELLS: ANALYSIS OF INTERACTION WITH ALKOXYRESORUFINS AND 2,3,7,8-TCDF

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Abstract
The present study addresses the enzymatic properties of avian cytochrome P450 (CYP) 1A4 and 1A5 using a yeast-based vector system. Recombinant CYP1A4 and 1A5 proteins from common cormorant (*Phalacrocorax carbo*) were expressed in yeast cells, and showed typical reduced CO-difference spectra with a peak at 446 nm. Kinetic analyses of *O*-dealkylase of methoxy-, ethoxy-, pentoxy- and benzyloxyresorufins catalyzed by the CYP1A enzymes revealed that V_{max} value for ethoxyresorufin *O*-deethylase (EROD) activity was the highest for both isozymes. Remarkable substrate specificity was observed for *O*-dealkylation of benzyloxyresorufin and methoxyresorufin; CYP1A4 was highly specific for catalyzing benzyloxyresorufin *O*-debenzylase activity, whereas CYP1A5 was more efficient in catalyzing methoxyresorufin *O*-demethylase activity. In this study, chicken (*Gallus gallus*) CYP1A5 protein was also expressed in yeast cells. Interestingly, recombinant chicken CYP1A5 showed no catalytic potential of AROD activities. We further measured cormorant CYP1A-dependent EROD activity in the presence of 2,3,7,8-tetrachlorodibenzofuran (TCDF) to evaluate the ability and mode of this dioxin-like congener to interact with CYP1A isozymes. One hundred nM TCDF noncompetitively inhibited CYP1A5-dependent EROD activity (K_i = 74 nM), whereas no inhibitory effect was detected for CYP1A4-dependent EROD activity. These results indicate that avian CYP1A4 and 1A5 have distinct catalytic efficiencies and specificities of substrate oxidation and binding.

Introduction
Cytochrome P450 (CYP) enzymes are composed of a large and growing superfamily of heme-proteins that play key roles in the oxidative metabolism of a wide variety of endogenous and xenobiotic compounds. Members of CYP1A subfamily are of toxicological significance due to their involvement in mediating the biological effects of polycyclic and halogenated aromatic hydrocarbons.[1-3] It is well known that CYP1A is induced by environmental contaminants such as dioxins and related compounds (DRCs), and thus, the expression level and enzymatic activities of CYP1A can be regarded as markers for evaluating exposure of animals to these contaminants and their effects.
Our recent study has revealed that common cormorant (*Phalacrocorax carbo*) possesses at least two paralogous CYP1A genes, ccCYP1A4 and ccCYP1A5 that are orthologous to chicken (*Gallus gallus*) CYP1A4 and 1A5, respectively.[4] Furthermore, we have clarified that common cormorants collected from Lake Biwa in Japan accumulate high levels of DRCs (12–1900 pg/g wet weight as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxic equivalents) in the liver and ccCYP1A4 and 1A5 are induced by DRCs at transcriptional and translational levels.[4-6] However, enzymatic properties of CYP1A4 and 1A5 isozymes in avian species including cormorant are not yet clarified, while extensive data have been available on mammalian CYP1A1 and 1A2 that may be orthologous to avian CYP1A4 and 1A5, respectively.[7]
The objective of this study is to characterize the catalytic function of avian CYP1A4 and 1A5 isozymes. We initially constructed an *in vitro* yeast expression system for avian CYP1A isozymes, and then investigated their enzymatic properties. Particular focus is placed on the catalytic profiles of alkoxyresorufin *O*-dealkylation by avian CYP1A isozymes heterologously expressed, and the interaction potential of CYP1As with a DRC, 2,3,7,8-tetrachlorodibenzofuran (TCDF).

Materials and Methods
Common cormorants were collected from Lake Biwa in May 2001 under license from Shiga Prefecture. Liver samples were immediately excised onboard and subsamples of liver were flash frozen in liquid nitrogen and stored at –80° C until RNA isolation. Total RNA was isolated from the liver with TRIzol reagent (Invitrogen) and RNeasy® (QIAGEN). The full-length cDNAs of cormorant CYP1A4 and 1A5 were isolated from the hepatic cDNA library.[4] The CYP1A4 and 1A5 were expressed with plasmids containing cDNA of each isozyme by an *in*

vitro yeast expression system following the method described previously.[8] Expression of recombinant CYP1A proteins in yeast microsomes was confirmed by the reduced CO-difference spectrum and detected by western blotting.[6,9] Kinetics and specificity of the CYP1A enzymes toward *O*-dealkylation of methoxy-, ethoxy-, pentoxy-, and benzyloxyresorufin (MROD, EROD, PROD, and BROD, respectively) were measured as described previously with some modifications.[6] Furthermore, EROD activity was measured in the absence or presence of TCDF to investigate the specificity and mode of interaction of avian CYP1As with TCDF. The present study also measured AROD activities and mRNA expression levels of CYP1A4 and 1A5 in the liver of common cormorants (n = 7) collected from Lake Biwa, according to the methods described previously.[6,10] The AROD profiles from the wild cormorant livers were compared with those of individual CYP1As heterologously expressed.

Results and Discussion

Yeast microsomes containing CYP1A4 or 1A5 isozyme exhibited typical reduced-CO difference spectra with a peak at 446 nm. The expression levels of CYP1A4 and 1A5 proteins were estimated to be 351 and 275 pmol/mg protein, respectively. By immunoblotting using an anti-rat CYP1A1 polyclonal antibody, a single cross-reactive band with a molecular weight similar to that of a rat CYP1A1 standard was detected in the yeast microsomes containing CYP1A4 or 1A5. With regard to chicken, although the CYP1A4 protein appeared to be less expressed, expression of CYP1A5 was detected by spectral analysis at a level of 185 pmol/mg protein, and further confirmed by western blotting.

Kinetic studies of AROD activity catalyzed by the recombinant cormorant CYP1A4 and 1A5 enzymes revealed that each AROD reaction followed a typical Michaelis Menten equation. Among all the AROD activities, V_{max} value of EROD activity was the highest for both isozymes (V_{max} = 1.0 and 1.5 nmol/min/nmol CYP for CYP1A4 and 1A5, respectively). With regard to BROD activity, CYP1A4 (V_{max} = 0.98 nmol/min/nmol CYP) was more specific than CYP1A5 (V_{max} = 0.028 nmol/min/nmol CYP). As for MROD activity, CYP1A5 (0.89 nmol/min/nmol CYP) exhibited a greater capacity than CYP1A4 (0.095 nmol/min/nmol CYP). On the other hand, both CYP1A4 and 1A5 showed weak activities toward PROD reaction. Taken together, AROD profiles were different between CYP1A4 and 1A5 (Fig. 1). From these profiles, we suggest that cormorant BROD and MROD activities may be useful and specific markers for evaluating expressions of CYP1A4 and 1A5, respectively.

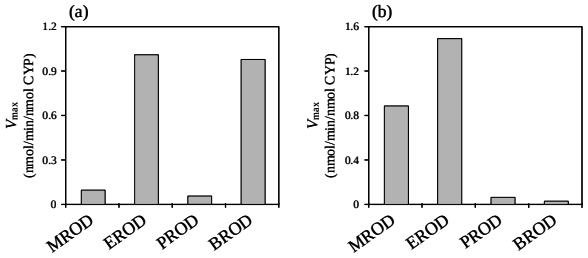

Fig. 1. AROD specificities of recombinant common cormorant CYP1A4 (a) and 1A5 (b). The V_{max} values calculated from the nonlinear regression analyses were compared among AROD activities.

In order to evaluate whether AROD profile in the hepatic microsomes of wild cormorants can be reconstituted by the AROD activities from the recombinant CYP1A4 and 1A5, we measured the hepatic microsomal AROD activities and the profile was compared with that of recombinant CYP1A4 and 1A5. Prior to the comparison, mRNA expression levels of CYP1A4 and 1A5 in the cormorant livers were measured by quantitative real time RT-PCR. As a result, CYP1A5 occupied 76 ± 14 % and the remainder was CYP1A4, indicating that CYP1A5 may contribute more to the AROD profile than CYP1A4 in the cormorant liver. Indeed, AROD profile estimated from the hepatic mRNA abundance and AROD activities from individual ccCYP1A isozymes heterologously expressed is quite similar to that in the

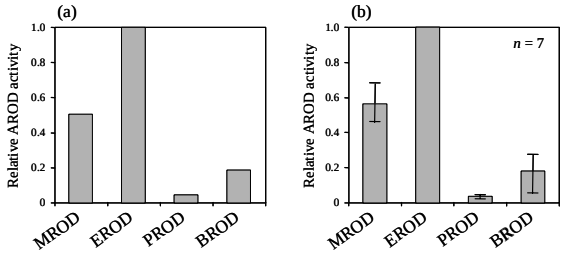

Fig. 2. AROD profile estimated from CYP1A mRNA abundance and AROD activities in individual recombinant CYP1A isozymes (a). The AROD activities were estimated by the following equation: AROD activity = 0.24 x CYP1A4 V_{max} + 0.76 x CYP1A5 V_{max}, where 0.24 and 0.76 were derived from the mRNA abundance of CYP1A4 and 1A5 in the cormorant livers, respectively. AROD profile in the hepatic microsomes of wild cormorants (b). Each AROD activity was expressed as a value relative to EROD activity.

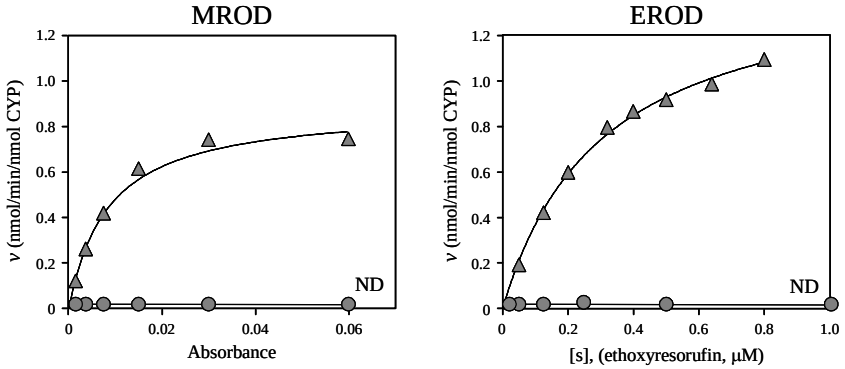

Fig. 3. Comparison of kinetics of AROD activities between recombinant chicken and common cormorant CYP1A5 isozymes. Circles and triangles indicate initial velocities of chicken and cormorant CYP1A5 isozymes, respectively. Each assay was conducted in triplicate.

hepatic microsomes of wild cormorants (Fig. 2). These results clearly suggest that our CYP1A proteins expressed in yeast system enzymatically work well as they actually do in cormorant liver.

To further investigate species difference in catalytic profile of CYP1A isozymes, the AROD activities were determined for chicken CYP1A5, and CYP1A5-dependent AROD activities were compared between cormorant and chicken. The result showed no detectable AROD activity by chicken CYP1A5 even at relatively high concentration of the alkoxyresorufins tested (Fig. 3), indicating no potential of chicken CYP1A5 to catalyze AROD activities. Considering that cormorant CYP1A5 contributes to the catalytic reactions for both EROD and MROD, this result strongly suggests that CYP1A-dependent catalytic function is different even within the avian species.

Recombinant cormorant CYP1A4- or 1A5-dependent EROD activity was measured in the presence or absence of TCDF to examine the inhibitory effect of this DRC congener toward EROD activity. The results showed that CYP1A5-dependent EROD activity was specifically inhibited by TCDF, whereas no inhibition was observed for CYP1A4-dependent EROD activity (Fig. 4). Since the treatment with TCDF lowered the maximum CYP1A5 activity (V_{max}), but less affected the K_m, the mode of inhibition by TCDF is regarded to be noncompetitive. Therefore, the inhibitor constant (K_i) was calculated by the following equation:

$$V_{max}^{app} = V_{max} / (1 + [I]_0/K_i)$$

where V_{max}^{app} is maximum velocity in the presence of inhibitor, V_{max} is maximum velocity in the absence of inhibitor, and $[I]_0$ is concentration of inhibitor (TCDF). The K_i was estimated to be 74 nM. The noncompetitive inhibition indicates that TCDF does not affect binding of ethoxyresorufin to CYP1A5, and binds CYP1A5 at a site distinct from binding site of ethoxyresorufin. Several previous investigations have reported that certain DRC congeners have potential to bind rodent CYP1A2 and inhibit its enzymatic function, and only CYP1A2, not but

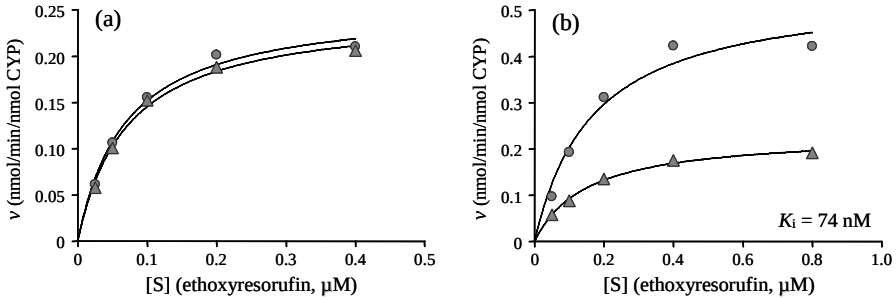

Fig. 4. Effects of TCDF (100 nM) on EROD activities catalyzed by recombinant common cormorant CYP1A4 (a) and 1A5 (b). Inhibitor constant (K_i) was calculated by the equation, $V_{max}^{app} = V_{max}/(1 + [I]_0/K_i)$. Nonlinear regression was used for the determination of kinetic parameters. Circles and triangles indicate initial velocities in the absence and presence of TCDF, respectively. Data are represented by means of duplicate assays. Each assay was conducted in triplicate.

CYP1A1, is involved in the hepatic sequestration of DRCs in mammal.[11-16] These results clearly indicate that TCDF inhibit the enzymatic activity of avian CYP1A5 as well as mammalian CYP1A2, which shows no conflict with our previous hypothesis indicating that the avian CYP1A5 is orthologous to mammalian CYP1A2.[4,7] On the other hand, as the K_i (74 nM) estimated for TCDF is much higher than the residue level in the liver of wild cormorants, it is unlikely that accumulated TCDF affected EROD activities in the hepatic microsome of wild cormorants that have reported in our previous study.[5,6]

Further study is necessary to understand the underlying mechanism accounting for the inter-species and -isoform differences in catalytic function. The approach using recombinant CYP proteins may provide a novel insight into the physiological and toxicological roles of avian CYP1A4 and 1A5.

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