BIOLOGICAL REACTIVE INTERMEDIATES VI

Chemical and Biological Mechanisms in Susceptibility to and Prevention of Environmental Diseases

Edited by

Patrick M. Dansette
Université René Descartes
Paris, France

Robert Snyder
Rutgers University and The Environmental & Occupational Health Sciences Institute
Piscataway, New Jersey

Marcel Delaforge
CNRS
Paris, France

G. Gordon Gibson
University of Surrey
Guildford, Surrey, England

Helmut Greim
Institute of Toxicology and Environmental Health
The Technical University of Munich
Munich, Germany

David J. Jollow
Medical University of South Carolina
Charleston, South Carolina

Terrence J. Monks
University of Texas
Austin, Texas

I. Glenn Sipes
University of Arizona
Tucson, Arizona

Kluwer Academic / Plenum Publishers
New York, Boston, Dordrecht, London, Moscow

Library of Congress Cataloging-in-Publication Data

Biological reactive intermediates VI: chemical and biological mechanisms in susceptibility to and prevention fo environmental disease/edited by Patrick M. Dansette ... [et al.].

p. ; cm. — (Advances in experimental medicine and biology; v. 500)
Includes bibliographical references and index.
ISBN 0-306-46659-7

1. Biochemical toxicology—Congresses. 2. Environmental toxicology—Congresses. 3. Pollutants—Structure-activity relationship—Congresses. I. Title: Biological reactive intermediates six. II. Title: Biological reactive intermediates 6. III. Dansette, Patrick M. IV. International Symposium on Biological Reactive Intermediates (6th: 2000: Université René Descartes) V. Series.

[DNLM: 1. Toxicology—methods—Congresses. 2. Biotransformation—Congresses. 3. Environmental Illness—etiology—Congresses. 4. Environmental Illness—prevention & control—Congresses. 5. Environmental Pollutants—toxicity—Congresses. 6. Structure-Activity Relationship—Congresses. QV 602 B6155 2001]
RA1219.5 .B576 2001
615.9'02—dc21

2001038407

Proceedings of the International Symposia on Biological Reactive Intermediates VI, held July 16–20, 2000 held at the Université René Descartes, Paris, France

ISBN 0-306-46659-7

233 Spring Street, New York, N.Y. 10013

http://www.wkap.nl/

10 9 8 7 6 5 4 3 2 1

A C.I.P. record for this book is available from the Library of Congress

Printed in the United States of America

ASSOCIATION OF CYTOCHROMES P450 1A2 AND 2B4: ARE THE INTERACTIONS BETWEEN DIFFERENT P450 SPECIES INVOLVED IN THE CONTROL OF THE MONOOXYGENASE ACTIVITY AND COUPLING?

D. R. Davydov,[1] N. A. Petushkova,[1] E. V. Bobrovnikova,[1] T. V. Knyushko,[1] and P. Dansette[2]

[1]Institute of Biomedical Chemistry RAMS
10 Pogodinskaya, 119832, Moscow, Russia
[2]Universite Rene Descartes, CNRS UMR 8601
45 Rue des Saints Peres 75270, Paris Cedex 06, France

INTRODUCTION

The membranes of endoplasmic reticulum contain a number of co-existing isoforms of cytochrome P450. These multiple P450 species compete for the partners, namely NADPH-cytochrome P450 reductase (CPR) and cytochrome b_5, and hence have to be considered as a members of a single ensemble. Moreover, these different P450 isozimes also appear to interact with each other. Despite of numerous evidences on the oligomerization of P450s both in solution (Dean and Gray, 1982; Wendel et al., 1983; Tsuprun et al., 1986) and in the membranes (Greinert et al., 1982; Kawato et al., 1982; Schwartz et al., 1990, Alston et al., 1991), the functional significance of this phenomenon remains obscure. However, it is likely to cause several perplexing features of microsomal P450s, such as biphasic kinetics of their reduction by NADPH and dithionite (Karyakin and Davydov, 1985), multiphasic kinetics of interactions with carbon monoxide (Davydov et al., 1986) and notable allosteric behavior of several P450 isoforms (Korzekwa et al., 1998). A remarkable observation in this context we made studying the barotropic behavior of P450 2B4 (CYP2B4) (Davydov et al., 1992,1995). We have found that only about 65-70% of the ferrous carbonyl complex of this oligomeric protein in solution is exposed to pressure-induced P450→P420 inactivation. The same non-uniform barotropic behavior was also observed for the oligomers of ferric CYP2B4, where only about 30-35% of the hemoprotein participates in the substrate binding and related spin transitions, being, at the same time, insensitive to the pressure-induced inactivation (Davydov et al., 1995). As these irregularities disappear at the P450 monomerization in the presence of detergent, we suggest them to reflect some peculiarities of the oligomer architecture resulting in inequality of the subunits in conformation and/or orientation. This peculiarity might be related to the asymmetry of the dimeric crystallization unit of the heme-containing domain of the cytochrome P450BM-3 (BMP), where two constituting BMP molecules have significantly different conformations, one with a more open substrate- and water-access to the heme moiety than the other (Ravichandran et al., 1993). Although P450BM-3 is believed to be monomeric in solution, the organization of its dimeric crystallization unit is likely to be related to the architecture of the oligomers of eukaryotic P450s. We suppose that this apparent inequality of the P450 subunits may serve in the control of the activity, degree of coupling and production of reactive oxygen species (ROS) in microsomal monooxygenase (MMO). This apparent mechanism may function through the substrate-modulated formation of the mixed oligomers of several P450 species, where the isozymes lacking the substrate are hidden from the interactions with their red/ox partners.

The present study of the mutual effects of CYP1A2 and CYP2B4 on their activity and interactions with CPR was designed to probe this hypothesis.

Biological Reactive Intermediates VI, Edited by Dansette *et al.*
Kluwer Academic / Plenum Publishers, 2001

MATERIALS AND METHODS

Electrophoretically homogeneous cytochromes P450 1A2, P450 2B4 and NADPH-cytochrome P450 reductase (CPR) were purified from rabbit liver by published procedures (Imai and Sato, 1974, Alterman, e.a., 1990, Kanaeva, et al., 1992). The procedure used to introduce the 7-ethylamino-3-(4'-malcimidilphenyl)-4-methylcoumarin maleimide (CPM) probe into CPR was detaily described earlier (Davydov et al., 2000). Essentially the same technique was employed to attach the fluorescent malcimide probes, either CPM or N-(1-pyrenyl)maleimide (PM), to cytochromes P450. The detailed procedure of this modification will be described elsewhere. Labeling of P450 by fluorescein isothiocyanate (FITC) was performed as described by Bernhardt et al. (1983). To monitor protein-protein interactions we employed the fluorescence energy transfer (FRET) in PM/CPM, CPM/FITC or CPM/heme donor/acceptor pairs (Davydov et al., 2000). O-Dealkylation of 7-ethoxy-resorufin (EROD) and 7-pentoxyresorufin (PROD) was measured at 30°C by direct fluorescent assay (Perrin et al., 1990). Analysis of the spectra were done using principal component analysis (PCA) technique (Davydov et al., 2000). All experiments were done in 0.1M Na-Hepes buffer (pH 7.4) containing 1mM DTE, 1 mM EDTA.

RESULTS AND DISCUSSION

Process of Subunit Exchange in Homo- and Hetero-Oligomers of CYP1A2 and CYP2B4. We monitored the process of subunit exchange in P450 oligomers in solution using FRET technique (Erijman and Weber, 1991) between cytochrome P450 molecules labeled with two different fluorescent maleimide probes. In these experiments we heve used either CPM/FITC or CPM/PM donor/acceptor pairs. In the absence of detergent the equilibrium of oligomerization was totally shifted towards oligomers and subunit exchange was very slow (*fig. 1a*). Introductdion of low concentration (0.025%.-0.05%) of Emulgen-913 into the system importantly facilitates the process of subunit exchange (*fig. 1b*). In these conditions the monomers and oligomers of P450 eisxst in the dynamic equilibrium although the constant of dissociation of oligomers remain to be rather low (K_d=0.2 μM). The solution of P450 1A2 and P450 2B4 hemoproteins, taken in micromolar concentrations in the presence of detergent (0.05% Emulgen-913), was chosen for further studies of the interactions of these hemoproteins.

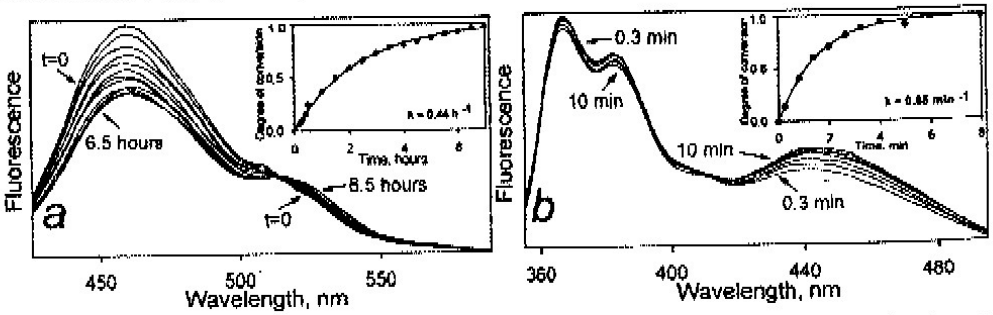

Fig. 1. Fluorescence energy transfer upon the process of subunit exchange in P450 oligomers. *(a)* Series of spectra (excitation at 385 nm) recorded in the intervals of 10 - 20 minutes after mixing of CYP2B4-CPM with CYP2B4-FITC (1 μM of each) in the absence of detergent Inset shows the kinetics of the process and the results of the fitting by the first reaction equation, which gives the rate constant of the decay of oligomers (k_{off}). *(b)* Series of spectra (excitation at 325 nm) recorded in 30 sec. intervals after mixing of CYP1A2-PM with CYP2B4-CPM (1 μM of each) in the presence of 0.05% Emulgen-913. Inset shows the kinetic curve of the process.

Mutual Effects of CYP1A2 and CYP2B4 on Their Interactions with NADPH-cytochrome P450 Reductase. Interactions of CPM-labeled CPR with CYP1A2 and CYP2B4 were monitored by a decrease in the intensity of fluorescence due to energy trabsfer from CPM to the heme chromophore (Davydov et al., 2000). Taken separately, CYP1A2 and CYP2B4 exhibited similar values of the dissociation constant (K_d) of their complexes with CPR (0.044 ± 0.02 μM and 0.037 ± 0.01 μM. correspondingly). However, when the reductase was titrated by the CYP1A2+CYP2B4 mixtures in the presence of 7-ethoxyresorufin (ER), a highly specific substrate of CYP1A2, the dependence of the apparent K_d of the P450-CPR complex on the molar ratio of the P450 species is given by an asymmetric bell-shaped curve (Fig. 3). The maximal value of K_d of 0.5 ± 0.2 μM is reached at the CYP1A2 : CYP2B4 molar ratio of 1:5 – 1:3. These results strongly supports a hypothesys that the

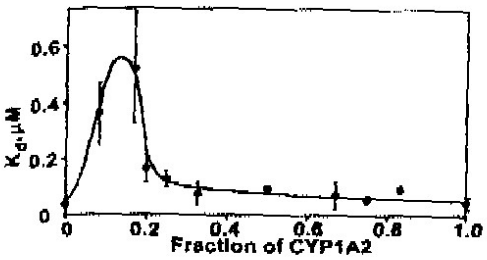

Fig. 2. Effect of the content of CYP1A2 in its mixture with CYP2B4 on the effective K_d of their complex with CPR in the presence of 7-etoxyresorufin (..mM). K_d was determined by titration of CPM-labeled CPR (0.02 μM) by 1A2, 2B4 or their mixture. Conditions: 0.1M Hepes pH 7.4, 0.05% Emulgen-913, 10μM 7-ethoxyresorufin. Each point represents an average of 4 - 5 repetitive experiments. The confidence intervals shown for each point are calculated for P≤0.05.

formation of the heterooligomers of CYP2B4 and CYP1A2 hemoproteins in the presence of ER partially hides the P450 2B4 enzyme, which is inactive in the oxidation of this substrate, from the interactions with the flavoprotein partner

Effect of the CYP1A2-CYP2B4 Interactions on Their Activity. To probe the reciprocal effect of these enzymes on their functional interactions with CPR we have studied the catalytic activities of the mixtures of these two hemoprtoteins taken at various ratios but at the constant total concentration (1 μM). CYP1A2 was highly active in the reaction of the oxidative deethylation of ER (EROD), while the activity of CYP2B4 in this reaction was negligibly low. If EROD activity of CYP1A2 is not affected by its interaction with CYP2B4, the apparent specific activity of CYP1A2+CYP2B4 mixture has to be proportional to the molar content of CYP1A2. However, the plot of the EROD activity versus CYP1A2 content importantly deviates from linearity and exhibits an apparent activation of the CYP1A2 enzyme in the mixture with CYP2B4 (*fig. 3a, curve 2*). Therefore, in the presence of CYP1A2 and ER, CYP2B4 appears to be at least partially excluded from the interaction with the flavoprotein.

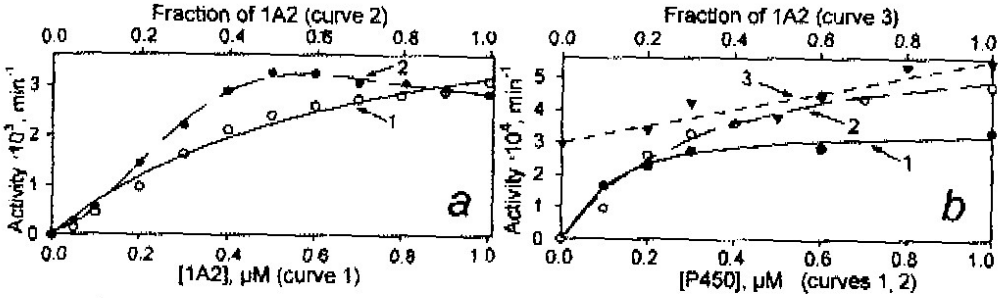

Fig. 3. Effect of the CYP1A2-CYP2B4 interactions on their association with CPR monitored by catalytic activity of P450. (a) Dealkylation of etoxyresorufin (CYP1A2-specific). Curve 1 shows the titration of 0.02 μM CPR by 1A2 fitted by the equation of binary association (K_d=0.3μM); curve 2 was obtained at the constant total concentration of the cytochromes (1 μM), but variable CYP1A2:CYP2B molar ratio, at 0.02μM CPR. *(b)* Dealkylation of pentoxyresorufin. Curves 1and 2 show the titration of 0.02 μM CPR by CYP1A2 (K_d=0.06 μM, curve 1) and CYP2B4 (K_d=0.25μM, curve 2); curve 3 was obtained at the constant total concentration of the cytochromes (1 μM), but variable CYP1A2:CYP2B molar ratio, at 0.02uM CPR. Other conditions were as those specified for *Fig. 2*.

At the same time, when ER was replaced by pentoxyresorufin (PR), which is metabolized by both enzymes, the plot of the activity versus the content of CYP1A2 was linear (*fig. 2b, curve 3*). Thus, in this case when both enzymes were capable to bind and methabolize the substrate, P450-P450 interactians has apparently no effect on their activity and interactions with the reductase.

These results appear to be in good agreement with the above data on the reciprocal effects of P450 1A2 and P450 2B4 on their interactions with reductase. Taken together, these data support our initial hypothesis on the substrate-modulated distribution of P450 isophorms between "open" and "closed" positions in the oligomer. The occupancy of the "open" subunit location is promoted by the substrate binding. In the presence of ER, which is a specific substrate for 1A2, occupancy of the "open" locations is preferred for the substrate-bound 1A2, whereas in the presence of PR both "closed" and "open" locations appear to be randomly distributed between 1A2 and 2B4 hemoproteins. This mechanism might by of high importance for the regulation of the degree of coupling of MMO and production of ROS in this system.

ACKNOWLEDGEMENTS

This research was supported in part by a Russian Foundation of Basic Research (RFBR) Grant 97-4-49132 to D.R.D. and INTAS Research Collaborative Grant 96-1343 to D.R.D. and G.H.B.H.

REFERENCES

Alston, K., Robinson, R.C., Park, S.S., Gelboin, H.V., and Friedman, F.K., 1991, Interactions among cytochromes P-450 in the endoplasmic reticulum. Detection of chemically cross-linked complexes with monoclonal antibodies, *J. Biol. Chem.* **266:**, 735-739.

Alterman, MA., and Dowgii A.I. , 1990, A simple and rapid method for the purification of cytochrome P-450 (form LM4), *Biomed. Chromatogr.* **4:** 221-222.

Bernhardt R., Ngoc Dao N.T., Stiel H., Schwarze W., Friedrich J., Janig G.R., and Ruckpaul K., 1983, Modification of cytochrome P-450 with fluorescein isothiocyanate, *Biochim Biophys Acta* , **745:** 2140-2148

Davydov, R.M., Khanina, O.Yu., Iagofarov, S., Uvarov ,V. Yu., and Archakov, A.I., 1986, Effect of lipids and substrates on the kinetics of binding of ferrocytochrome P-450 to CO. *Biokhimia* **51:** 125-129

Davydov, D.R., Knyshko, T.V., and Hui Bon Hoa, G., 1992, High pressure induced inactivation of ferrous cytochrome P-450 LM2 (IIB4) CO-complex: Evidence for the presence of two conformers in the oligomer, *Biochem. Biophys. Res. Commun.* **188:** 216 - 221.

Davydov, D.R., Deprez, E., Hui Bon Hoa, G., Knyushko, T.V., Kuznetsova, G.P., Koen, Y.M., and Archakov, A.I., 1995, High-Pressure-Induced Transitions in Microsomal Cytochrome P450 2B4 in Solution - Evidence for Conformational Inhomogeneity in the Oligomers, *Arch. Biochem. Biophys.* **320:** 330-344.

Davydov D.R., Kariakin A.A., Petushkova N.A., and Peterson J.A., 2000, Association of cytochromes P450 with their reductases: opposite sign of the electrostatic interactions in P450BM-3 as compared with the microsomal 2B4 system. *Biochemistry*, **39:** 6489-6497

Erijman L. and Weber G., 1993, Use of sensitized fluorescence for thestudy of the exchange of subunits in protein aggregates, *Photochemistry and Photobiology*, **57:** 411-415.

Imai Y, and Sato, R, 1974, A gel electrophoretically homogenious preparation of cytochrome P-450 from liver microsomes of phenobarbital pretreated rabbits, *Biochem. Biophys. Res. Commun*, **60:** 8-14

Kanaeva, I.P., Dedinskii, I.R., Skotselyas, E.D., Krainev A.G., Guleva, I.V., Sevryukova, I.F., Koen, Y.M., Kuznetsova, G.P., Bachmanova, G.I., and Archakov, A.I., 1992, Comparative study of monomeric reconstituted and membrane microsomal monooxygenase systems of the rabbit liver. 1. Properties of NADPH-cytochrome P450 reductase and cytochrome P450 LM2 (2B4) monomers, *Arch Biochem Biophys*, **298:** 395-402

Kariakin A.V. Davydov D.R., 1988, Kinetics of the electron transfer reactions in the monooxygenase system. *Vestnik Akad. Med. Nauk SSSR.* **1988**(1): 53-62.

Korzekwa K.R., Krishnamachary N., Shou M., Ogai A., Parise R.A., Rettie A.E., Gonzalez F.J., and Tracy T.S., 1998, Evaluation of atypical cytochrome P450 kinetics with two-substrate models: evidence that multiple substrates can simultaneously bind to cytochrome P450 active sites, *Biochemistry*, **37:** 4137-4147.

Perrin, R., Minn, A., Ghersi-Egea, J.F., Grassiot, M.C., and Siest, G., 1990, Distribution of cytochrome P450 activities towards alkoxyresorufin derivatives in rat brain regions, subcellular fractions and isolated cerebral microvessels, *Biochem Pharmacol*, **40:** 2145-2151.

Ravichandran, K.G., Boddupalli, S.S., Hasermann, C.A., Peterson, J.A., and Deisenhofer, J., 1993, Crystal structure of hemoprotein domain of P450BM-3, a prototype for microsomal P450's, *Science* **261:**731-736.

Schwarz, D., Pirrwitz, J., Meyer, H.W., Coon, M.J., and Ruckpaul K., 1990, Membrane topology of microsomal cytochrome P-450: saturation transfer EPR and freeze-fracture electron microscopy studies. *Biochem. Biophys. Res. Commun.* **171:** 175-181

Tsuprun, V.L., Myasoedova, K.N., Berndt, P., Sograf, O.N., Orlova, E.V., Chernyak, V.Ya., Archakov, A.I., and Skulachev, V.P., 1986, Quaternary structure of the liver microsomal cytochrome P-450, *FEBS Lett* **205:** 35-40.

Wendel, I., Behlke, J., and Janig, G.R. , 1983, Hydrodynamic studies on the association of cytochrome P-450, *Biomed. Biochim. Acta* **42:** 633-640.