Oxygen Binding to P-450$_{\text{cam}}$ Induces Conformational Changes of Putidaredoxin in the Ferrous P-450$_{\text{cam}}$–Reduced Putidaredoxin Complex

Hideo Shimada$^1$, Masashi Unno$^1$, Yoko Kimata$^1$, Ryu Makino$^2$, Futoshi Masuya$^3$, Takashi Obata$^3$, Hiroshi Hori$^3$, and Yuzuru Ishimura$^1$

Summary. P-450$_{\text{cam}}$ catalyzes the conversion of d-camphor to 5-exo-hydroxy-camphor at the expense of 1 mole each of NADH and oxygen. Two reducing equivalents from NADH are transferred to P-450$_{\text{cam}}$ via two redox-linked proteins, putidaredoxin reductase (PdR) and putidaredoxin (Pd). Pd serves as a one-electron shuttle between PdR and P-450$_{\text{cam}}$. Reduced Pd has been known to form a tight complex with ferric or ferrous P-450$_{\text{cam}}$. The former complex yields oxidized Pd and ferrous P-450$_{\text{cam}}$. Binding of oxygen to the latter complex affords the product formation, degrading into 5-exo-hydroxy-camphor, water, oxidized Pd, and ferric P-450$_{\text{cam}}$. In this study, we show evidence that in the ferrous P-450$_{\text{cam}}$–reduced Pd complex, binding of a ligand such as O$_2$, CO, and NO to P-450$_{\text{cam}}$ induces the conformational changes of reduced Pd at least at the redox center. The significance of this finding is discussed.

Key words. Cytochrome P-450—Iron-sulfur protein—Putidaredoxin—EPR—Oxygen

Introduction

Cytochrome P-450 (P-450) is a group of heme proteins that participates in the monoxygenation reactions of a wide variety of hydrophobic substances including steroids, fatty acids, and hydrocarbons [1]. More than 400 species of P-450 have been isolated from animals, plants, insects, and microorganisms and have been recognized to form a gene superfamily [2]. Among many P-450 species, P-450$_{\text{cam}}$ inducible in a soil bacterium Pseudomonas putida on growth on d-camphor has been the focus of P-450 research because of the ease of isolation of the enzyme in large quantities and its ability to catalyze the oxidation of an inactivated carbon [3,4]. P-450$_{\text{cam}}$ hydroxylates d-camphor at the 5-exo position with nearly 100% stereospecificity at the

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$^1$Department of Biochemistry, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160, Japan
$^2$Department of Biochemistry, Faculty of Science, Rikkyo University, Nishi-ikebukuro, Toshima-ku, Tokyo 171, Japan
$^3$Division of Biophysical Engineering, Department of Systems and Human Science, Graduate School of Engineering Science, Osaka University, Toyonaka, Osaka 560, Japan
expense of 1 mole each of NADH and molecular oxygen. Two electrons from NADH are transferred to P-450cam via an electron-transfer chain composed of putidaredoxin reductase (PdR), a flavine adenine dinucleotide- (FAD-) containing protein, and putidaredoxin (Pd), a 2Fe-2S iron-sulfur protein. Pd serves as a one-electron carrier from PdR to P-450cam. In the reaction, reduced Pd donates an electron to ferric camphor-bound P-450cam, yielding the ferrous form of the enzyme, which subsequently combines with molecular oxygen to form an oxy-ferrous intermediate. Subsequent reduction of the oxy-intermediate by reduced Pd affords oxidized Pd, ferric P-450cam, water, and 5-exo-hydroxycamphor.

At the step at which Pd donates an electron to ferric P-450cam, Pd can be replaced by a variety of reductants including spinach ferredoxin, bovine adrenodoxin, and methyl viologen. But at the step of reduction of the oxy-intermediate, such reductants as just shown cannot replace Pd. Based on these and other results, Gunsalus and his co-workers considered that Pd has a role more than that of an electron donor to P-450cam and proposed that Pd serves as an effector in the monooxygenation catalyzed by P-450cam [5]. We have infrared evidence supporting this proposal: Pd binding to P-450cam induces changes in the active site structure of P-450cam as seen by a shift in C-O stretch frequency of CO-ferrous P-450cam from 1940 to 1932 cm⁻¹ [6]. The effector action exerted by Pd, however, is poorly understood. Therefore, for elucidation of the mechanisms of the effector action and also of the monooxygenation reaction, studies on the Pd–P-450cam complex are required.

In this chapter, we show for the first time evidence that in the ferrous P-450cam–reduced Pd complex, oxygen binding to P-450cam induces the conformational changes of reduced Pd by employing electron paramagnetic resonance (EPR) spectroscopy. The current finding suggests a possible regulation of the structure and function of Pd by oxygen.

Materials and Methods

Enzyme Preparations

The wild-type P-450cam and its mutants were expressed in Escherichia coli strain JM109 and purified with the procedures described previously [7]. Purified preparations with the RZ value (A392/A280) greater than 1.5 were employed in this study. Pd and PdR were expressed also in Escherichia coli strain JM109 and were purified according to the methods described by Gunsalus and Wagner [8]. Pd and PdR were found to be homogeneous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Pd was stored in 0.1 M Tris-HCl, pH 8.0, containing 10 mM 2-mercaptoethanol. For the EPR spectral measurement of Pd described next, 2-mercaptoethanol was removed from Pd by using centricon 10 (Amicon, Beverly, MA, USA) before the measurement. The concentrations of P-450cam, PdR, and Pd were determined spectrophotometrically by using extinction coefficients [8].

EPR Spectroscopy

For spectroscopy, 150–200 μl of oxidized Pd or the mixture of oxidized Pd and ferric P-450cam in 50 mM potassium phosphate, pH 7.4, containing 50 mM KCl and 1 mM d-