Cytochrome P450BM-3: Mechanism of oxygen activation using site directed mutagenesis.

Hyeyeong Yeom
Korea Institute of Science and Technology, Cheongryang P.O.Box 131, Seoul

Introduction

Cytochrome P450 (P450) is a class of heme containing monooxygenases found in a numerous organisms, involved in a variety of oxidative metabolic reactions. P450 family is grouped into two classes depending on the electron transport system. Two protein system is observed in microsomal P450s, where the flavin containing reductase mediates electron transfer from NADPH to heme where the monooxygenation reaction occur. Bacterial system is consist of three proteins, redoxin, reductase and P450. P450BM-3 isolated from *Bacillus megaterium* is an unique enzyme among bacterial enzymes, having flavin containing reductase domain as a counterpart for electron transport (1). ω-n (n=1~3) hydroxylation of saturated and unsaturated fatty acid with various chain length and its amide analogues are the major function of P450BM-3. The heme domain protein of P450BM-3 is the first microsomal P450 whose crystal structure is solved (2). Detailed studies on P450BM-3 would provide valuable information on the nature of other microsomal P450s.

Throughout the whole family of P450s, there are two highly conserved region, one at the proximal site of the heme and one at the distal site of the heme. Proximal side of the enzyme possesses cysteine which is the origin of the strong absorption at 450 nm in a reduced carbon monoxide bound form. The distal site is believed as an oxygen binding pocket based on the crystal structure of ternary complex of carbon monoxide-camphor-P450cam (3). All the P450 enzyme possess acid-alcohol pair in I-helix of the protein with a few exception, implying that the acid-alcohol pair plays an important role in the mechanism of oxygen activation. Site directed mutagenesis on these amino acids showed that the presence of acid and threonine is important in the catalytic activity of the protein with varying degrees from protein to protein (4-8). Based on the results from various mutation studies, it is suggested that the acid-alcohol pair participate in the stabilization of oxygen bound intermediate and/or proton transfer pathway.

Here, the results from site directed mutation of threonine and glutamate in I-helix of P450BM-3 are presented. Mutation of threonine results in the uncoupling of the hydroxylation reaction without significant change in crystal structure. Unlike the threonine mutation showed similar effect regardless the substrate used, mutation of acid residue, glutamate, reduced catalytic activity of palimitate drastically, but has less effect on the activity toward laurate hydroxylation.

Experimental

Mutation of glutamate 267 to glutamine and threonine 268 to alanine was carried out by oligonucleotide directed mutagenesis using the "Muta-gene phagemid *in vitro* mutagenesis kit" from Bio-rad. Mutation was confirmed with a creation of silent mutation and DNA sequencing reaction using the sequenase 2.0 DNA sequencing kit from USB.

Intact proteins of wild type and mutant were expressed in DH5α without particular

induction system. Intact protein were purified using ammonium sulfate salt-cut, DE52 anion exchange column, S-200 gel filtration column and FPLC. SDS-PAGE was performed using the pharmacia phast gel system to confirm the protein size and purity.

Protein concentration was determined with the dithionite reduced CO difference spectrum ($\varepsilon 450 = 91{,}000\ M^{-1}cm^{-1}$) and the alkaline pyridine hemochromogen assay using 20% v/v pyridine and NaOH (9). Electronic absorption spectra were obtained with a Hitachi U-3300 spectrometer with a scan rate of 600nm/min. Substrate binding was measured by spectrometric titration of protein at 25°C. Binding of substrate caused the spin state change from low to high spin with Soret band shift from 418 to 392nm. Dissociation constants were determined from the reciprocal plot of ($\Delta A392+\Delta A418$) versus free substrate concentration.

The rate of NADPH oxidation was measured by monitoring the absorbance change at 340nm using an extinction coefficient of $6{,}220M^{-1}cm^{-1}$. The reaction was initiated with the addition of 150μM NADPH to the reaction mixture (0.1M KPi, pH 8.0, 200μM laurate (150μM myristate or palmitate)). Oxygen consumption rates were determined using a Clark type oxygen electrode (YSI membrane). The stoichiometric amount of hydrogen peroxide produced was measured with a horse radish peroxidase based assay. Oxidation of 2,2'-azino-di-[3-ethyl-benzothiazidine-6-sulfonic acid] was measured using the absorbance at 406nm. The amount of water produced was measured using a special cell which allowed simultaneous measurements of NADPH and oxygen. A Clark-type electrode was interfaced through a BAS27 electrometer to a data logging system composed of HP voltmeters and a personal computer running Lab Windows. For the product analysis, the reaction was carried out in the presence of excess NADPH and enzyme. Product and substrate were extracted using a C18-silica solid phase extraction column and methylated with etheral solution of diazomethane at room temperature overnight. Methylated product and substrate were dissolved with chloroform and analyzed using DB23 column. GC-EIMS was used to confirm the methylated fatty acid and its hydroxylated product.

Results and Discussion

Purification of the Glu267Gln and Thr268Ala mutant proteins were done using methods identical to that for wild type protein. The purified protein showed indistinguishable electronic absorption spectra from wild type in both substrate free and substrate bound form.

For kinetic study of Thr268A mutant, sodium laurate was chosen as a substrate. NADPH oxidation rate of the mutant (268 nmole/min/nmole of P450 used) is 28% of the wild type (941 nmole/min/nmole of P450 used). Similar decrease in the rate of O_2 consumption was observed upon mutation. While wild type protein showed no hydrogen peroxide formation, 67% of the reducing equivalents goes toward H_2O_2 formation in Thr268Ala mutant, giving about 16% yield of hydroxylated product. However, the distribution of product isomer is the same in both wild type and T268A mutant. In the other bacterial P450, P450cam, the mutation of corresponding threonine resulted in completed uncoupling of the reaction to hydrogen peroxide (4). Measurement of autoxidation rate in wild type and Thr252Ala mutant of P450cam suggests that the threonine residue plays a critical role in stabilization of oxygen bound intermediate, presumably through the formation of a hydrogen bond to the iron bound

oxygen. The role of threonine in eukaryotic P450s was suggested to be in the recognition of substrate rather than the stabilization of the intermediate. It is reported that P450d mutant Thr319Ala showed changes in activity depending on the substrate used and binding of asymmetrical ligand to mutant protein is different from wild type (7). Results from the present study on the role of threonine showed that the Thr to Ala mutant in P450BM-3 affects catalytic activity between two extremes of P450cam and P450d.

The presence of an acid residue in I-helix is critical for efficient hydroxylation in most P450s studied so far. In P450cam mutant Asp251Asn, the substrate on rate and autoxidation rate are not changed significantly upon mutation. It has been suggested that aspartate participate in proton transfer pathway through salt bridge (5). In P450BM-3 mutant Glu267Gln, Kd measurement showed that there is no change in substrate binding property with mutation in all three substrate used (laurate, myristate and palmitate. While the substrate binding properties of the mutant are close to wild type, the substrate dependent activity profile of the mutant is totally different from wild type. NADPH oxidation rates of Glu267Gln mutant are 147 (laurate), 39 (myristate) and 8.30 (palmitate) nmoles/min/nmoles of P450, which is 16, 1.6 and 0.3 % of the wild type activity. 35~47% of the reducing equivalents undergo oxidative pathway resulting in the formation of water. Transient spectrum during the reaction was observed in two slowest reactions, myristate and palmitate reaction with Glu267Gln mutant. The decay of spectral intermediate is highly dependent on the chain length. Although the exact nature of the intermediate is still under investigation, it is clear that the earlier steps prior to proton transfer and second electron transfer have little effect by the mutation. Participation of glutamate in efficient transfer of protons to the oxygenated intermediate is strongly suggested.

The mutation of acid-alcohol pair in I-helix of P450BM-3 reveals that the structure of the protein is insensitive to mutation while the mutation affects catalytic activity drastically. The presence of threonine residue is essential in complete coupling of hydroxylation reaction as observed in P450cam mutant although the P450BM-3 mutant is able to hydroxylate a part of substrate. The proton delivery system in P450BM-3 is assumed to different from that of P450cam as expected from the lack of salt bridge around acid residue in the crystal structure of P450BM-3. The water molecule present in the oxygen binding pocket might play a role in proton delivery to the oxygen bound intermediate. The active site of P450BM-3 can accommodate fatty acid with various chain length unlike P450cam, which showed tight substrate specificity. Variation in the effect of acid residue mutation of P450BM-3 with respect to the substrate used, implies reorganization of active site when different substrate is used.

Reference

1. Narhi, L. O. & Fulco, A. J. (1987) *J. Biol. Chem.* 262, 6683-6690
2. Ravichandran, K. G., Boddupalli, S. S., Haseman, C. A., Peterson, J. A. & Diesenhofer, J. (1993) *Science* 261, 731-736
3. Raag, R. & Poulos, T. L. (1989) *Biochemistry* 28, 7586-7592
4. Martinis, S. A., Atkins, W. M., Stayton, P. S. & Sligar, S. G. (1989) *J. Am. Chem. Soc.* 111, 9252-9253
5. Gerber, N. C. & Sligar, S. G. (1994) *J. Biol. Chem.* 269, 4260-4266

6. Imai, M. Shimada, H., Watanabe, Y., Matsushima-Hibiya, Y., Makino, R., Koga, H., Hirouchi, T. & Ishimura, Y. (1989) *Proc. Natl. Acad. Sci. USA* 86, 7823-7827
7.Ishigooga, M., Shimizu, T.,Hiroya, K. & hatano, M. (1992) *Biochemistry* 31, 1528-1531
8. Hiroya, K., Murakami, Y., Shimizu, T.,Hatano, M. & Ortiz de Montellano, P. R. (1994) *Arch. Biophys. Biochem.* 310, 397-401
9. Omura, T. & Sato, R. (1964) *J. Biol.Chem.* 239, 2370-2378