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Affinity labeling of rat cytochrome P450C24 (CYP24) and identification of Ser57 as an active site residue☆

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Abstract

25-hydroxyvitamin D_3- or 1α,25-dihydroxyvitamin D_3-24R-hydroxylase (cytochromeP450C24 or CYP24) has a dual role of removing 25-OH-D_3 from circulation and excess 1,25$(OH)_2D_3$ from kidney. As a result, CYP24 is an important multifunctional regulatory enzyme that maintains essential tissue-levels of Vitamin D hormone. As a part of our continuing interest in structure-function studies characterizing various binding proteins in the Vitamin D endocrine system, we targeted recombinant rat CYP24 with a radiolabeled 25-OH-D_3 affinity analog, and showed that the 25-OH-D_3-binding site was specifically labeled by this analog. An affinity labeled sample of CYP24 was subjected to MS/MS analysis, which identified Ser57 as the only amino acid residue in the entire length of the protein that was covalently modified by this analog. Site-directed mutagenesis was conducted to validate the role of Ser57 towards substrate-binding. S57A mutant displayed significantly lower binding capacity for 25-OH-D_3 and 1,25$(OH)_2D_3$. On the other hand, S57D mutant strongly enhanced binding for the substrates and conversion of 1,25$(OH)_2D_3$ to calcitroic acid. The affinity probe was anchored via the 3-hydroxyl group of 25-OH-D_3. Therefore, these results suggested that the 3-hydroxyl group (of 25-OH-D_3 and 1,25$(OH)_2D_3$) in the S57D mutant could be stabilized by hydrogen bonding or a salt bridge leading to enhanced substrate affinity and metabolism.

Keywords: Vitamin D cytochrome P450C24 or CYP24; Affinity labeling; Mutational analysis; Structure-function studies

1. Introduction

In the Vitamin D endocrine pathway, 25-hydroxyvitamin D_3- or 1α,25-dihydroxyvitamin D_3-24-hydroxylase (cytochrome P450C24, CYP24) converts 25-hydroxyvitamin D_3 (25-OH-D_3) to 24(R),25-dihydroxyvitamin D_3 (24,25$(OH)_2D_3$) and 1α,25-dihydroxyvitamin D_3(1,25$(OH)_2D_3$) to 1,24(R),25-trihydroxyvitamin D_3 (1,24,25$(OH)_3D_3$) prior to their ultimate catabolic degradation (Fig. 1). Therefore, CYP24 has a dual role of removing 25-OH-D_3 from circulation and excess 1,25$(OH)_2D_3$ from kidney. As a result, CYP24 is an important multifunctional regulatory enzyme in the Vitamin D pathway that maintains essential tissue levels of 1,25$(OH)_2D_3$.

1,25$(OH)_2D_3$ is a multi-functional hormone that regulates calcium homeostasis, immune function, cellular proliferation, and differentiation and apoptotic process for cell death. Recognizing the cell-regulatory properties of 1,25$(OH)_2D_3$, extensive efforts have been directed to develop 1,25$(OH)_2D_3$ and its synthetic analogs as therapeutic agents for many diseases including cancer. Biological actions of 1,25$(OH)_2D_3$ and its analogs are mediated via Vitamin D receptor (VDR), the nuclear receptor for 1,25$(OH)_2D_3$. Therefore, VDR has been an important target for developing 1,25$(OH)_2D_3$ analogs with therapeutic potential [1].

Bioavailability is an important consideration in the development of 1,25$(OH)_2D_3$-based drugs. Since CYP24 controls the catabolism of 1,25$(OH)_2D_3$ and majority of its synthetic analogs, bioavailability of these compounds is also regulated by CYP24. In this token, CYP24 is an important molecular target for developing 1,25$(OH)_2D_3$-based drugs. For example, analogs could be developed that would have reduced CYP24 activity and enhanced bioavailability. Additionally an inhibitor of CYP24 would also serve the same purpose.

However, realization of the above mentioned rationale has been seriously challenged by the lack of any structure-functional data of CYP24. CYP24 is a typical example of mitochondrial P450c enzymes containing multiple components including. NADPH ferrodoxin-reductase and an iron–sulfur protein required for the electron-transfer process. The unique constituent in this multi-component system

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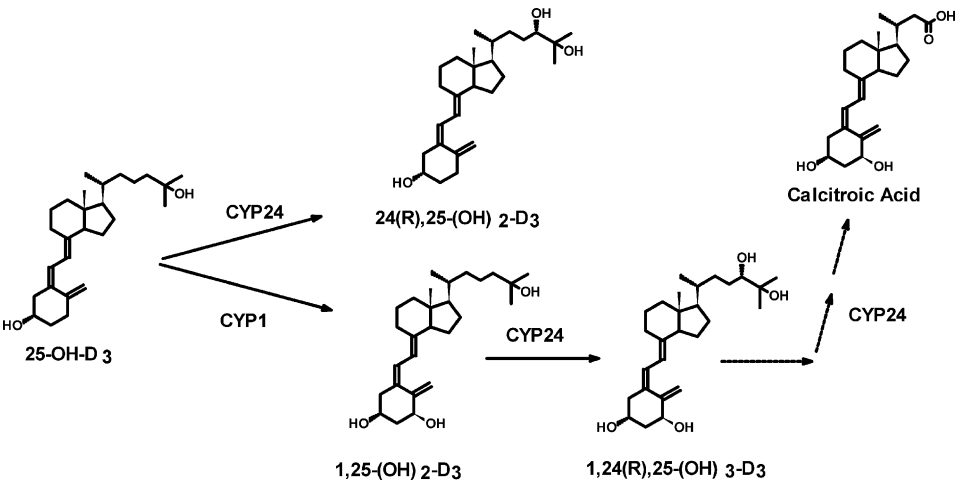

Fig. 1. Side chain modifications of Vitamin D metabolites by P450CYPs.

is the binding protein that binds the Vitamin D metabolites in a specific and spatially discrete way and presents them to the cytochrome for the oxidative transfer of molecular oxygen. In this respect, conformational fidelity of the Vitamin D ligand and orientation of the ligand inside the ligand-binding pocket of the binding protein component of CYP24 are crucial for the overall oxidative process. Therefore, delineation of the molecular structure of the binding pocket and identification of the key ligand-interacting amino acid residues are central to the structure-functional analysis of CYP24.

Affinity labeling is a biochemical technique in which a radioactively tagged ligand-mimic (affinity label) is covalently attached to the ligand-binding pocket, followed by the identification of the residue/residues that is/are labeled by the affinity label. In the Vitamin D endocrine pathway, this method was employed successfully to identify key amino acid residues inside the ligand binding pockets of Vitamin D-binding protein [2,3] and VDR [4] prior to the determination of the three-dimensional structures by X-ray crystallography [5–7].

In the present study, we employed 25-hydroxyvitamin D_3-3β–bromoacetate (25-OH-D_3-BE), a protein alkylating analog of 25-OH-D_3 to affinity label the ligand-binding pocket of recombinant rat CYP24 and identified a single residue that is alkylated by this compound by MS/MS technique. We mutated this residue to determine the impact of such changes in the biological activities of the mutated CYP24 versus the wild type to evaluate the fidelity of the labeling process. Results of these studies and their implication in delineating the three-dimensional structure of the ligand-binding pocket of CYP24 are discussed in this communication.

2. Materials and methods

$1,25(OH)_2D_3$ was a kind gift from Dr. Milan Uskokovic, Hoffmann La-Roche, Nutley, NJ. MS/MS Tandem mass spectrometric analysis (MS/MS analysis) was carried out at Tufts University Medical School (Boston, MA) core protein sequencing facility with Thermo Finnigan LCQ Deca MS/MS system consisting of a nanospray probe. Data was analyzed with Thermo Finnigan sequest browser search.

2.1. Synthesis

25-hydroxyvitamin D_3-3β-[2-^{14}C]bromoacetate (^{14}C-25-OH-D_3-BE, specific activity 10 mCi/mmol) was synthesized according to our previously published procedure [8]. Briefly, 25-OH-D_3 (Duphar, The Netherlands), [2-^{14}C]bromoacetic acid (Sigma sp. activity 10 mCi/mmol), dicyclohexylcarbodiimide and a catalytic amount of 4, 4′-*N*,*N*-dimethylamino-pyridine in anhydrous dichloromethane was stirred at 25 °C for 20 h followed by purification of the product by preparative thin layer chromatography on silica gel. Radiochemical purity of the desired compound was ascertained by HPLC-analysis of a sample of 25-OH-D_3-BE, spiked with a known amount of ^{14}C-25-OH-D_3-BE.

2.2. Expression of wild type and mutant rat P450C24 (CYP24)

A rat cytochrome P450C24 hydroxylase (CYP24) cDNA clone was used to prepare a functional *E*. coli expression construct with 5′-coding sequence [9]. Conditions were optimized for high expression of of the recombinant protein (40–50 mg/l). Protein expression was induced by 1.0 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) and 1.0 mM of δ-aminolevulinic acid (for the heme synthesis). Recombinant adrenodoxin and adrenodoxin reducatse were expressed and purified by established procedures. The crude enzyme was purified by adrenodoxin-sepharose affinity and hydroxylapetite chromatographies to a specific content of approximately 16 nm P450/mg of protein. The recombinant protein gave a typical P450 CO-reduced difference

spectra. Mutations in the wild type protein were confirmed by sequencing.

2.3. Substrate-binding and metabolic analysis of wild type and mutant CYP24s

Substrate-induced spectral changes were used to determine K_d values for various Vitamin D substrates for CYP24 [10]. Metabolic activity of wild type and mutant proteins was analyzed in enzyme reconstitution assays that consisted of NADPH, CYP24, ferredoxin and ferredoxin reductase. Reaction conditions were optimized for the concentration of each reactant and metabolic products were analyzed by HPLC. Special extraction conditions were used to recover both lipid- and aqueous-soluble metabolites through the use of acetic acid to charge-neutralize the polar and water soluble metabolite, calcitroic acid.

2.4. Affinity labeling of rat CYP24

A 36 μM solution of rat CYP24 in 0.4 M phosphate buffer containing 20% glycerol, pH 7.4. A 35 μl aliquot was diluted (1:10) with dH_2O, and 30 μl aliquots of the diluted sample were used for labeling study. Two samples of 30 μl aliquots of the protein samples were incubated at 4 °C with either ^{14}C-25-OH-D_3-3-BE alone or a mixture of ^{14}C-25-OH-D_3-3-BE and 10-fold molar excess of 25-OH-D_3 (steroids were dissolved in 2 μl of ethanol prior to the addition of protein samples) for 20 h followed by the addition of SDS-PAGE buffer. Samples were electrophoresed on a 10% SDS gel. At the end of the run the gel was stained with Coomasie Blue to visualize the protein band. Then the gel was dried and scanned for radioactivity (Bioscan Imaging Scanner system 200, Bioscan Inc., Washington, DC).

2.5. MS/MS analysis of CYP24-labeled with 25-OH-D_3-3-BE to identify amino acid residue/residues covalently modified by 25-OH-D_3-3-BE

A 20 μl aliquot (approximately 0.72 nmol) of the original solution of CYP24 (>95% purity, determined by SDS-PAGE/ Coomasie-staining, 36 μM solution in 0.4 M phosphate buffer containing 20% glycerol, pH 7.4) was diluted with 20 μl of dH_2O and incubated at 4 °C with 25-OH-D_3-3-BE (1 μg, 1.9 nmol) for 20 h followed by SDS-PAGE on a 10% SDS polyacrylamide gel. In a separate lane an equal amount of CYP24 was also electrophoresed. At the end of the electrophoresis, the gel was lightly stained with Coomasie Blue to visualize the protein band that was carefully cut out with razor blade. The gel-piece was washed twice with 50% aqueous CH_3CN and stored at −80 °C prior to MS/MS analysis. A similar size piece of the gel, which did not contain any protein was also saved for blank-analysis.

The gel-pieces (containing protein) were carboxymethylated with iodoacetamide by usual procedure and then digested with trypsin (1:100). The tryptic digest of each sample was injected into a HPLC (Thermo Finnigan Surveyor) containing a capillary C_{18} column (New Objective). HPLC was run in a 1% HCO_2H in H_2O to CH_3CN gradient, and the effluent was directly injected into a Thermo Finnigan LCQ Deca MS/MS system consisting of a nanospray probe.

The data was analyzed with Thermo Finnigan sequest Browser Search. Search was performed with modified weight (affinity ligand) of Ser, Cys, and Lys (amino acids with nucleophilic side chains). $X_{correletion}$ scores above 2.5 were considered as 'hits'.

3. Results and discussion

In the Vitamin D endocrine system 25-OH-D_3 is a common ligand for at least three binding proteins i.e. DBP, VDR and CYP24. We have previously shown that 25-OH-D_3-BE specifically labels the ligand binding domains of DBP [8] and VDR [11]. Therefore, in this investigation we chose 25-OH-D_3-BE as the potential affinity label for CYP24. As shown in Fig. 2, there was a radioactive band corresponding to the Coomasie Blue-stained band for CYP24 (lane 2). When incubation was carried out in the presence of approximately 10-fold excess of 25-OH-D_3 labeling was almost completely obliterated (lane 1). These results strongly suggested that ^{14}C-25-OH-D_3-3-BE covalently labeled the ligand-binding site of CYP24.

In the next step we affinity labeled purified CYP24 with 25-OH-D_3-BE, ran it on SDS-PAGE and isolated the band corresponding to CYP24. The protein was carboxymethy-

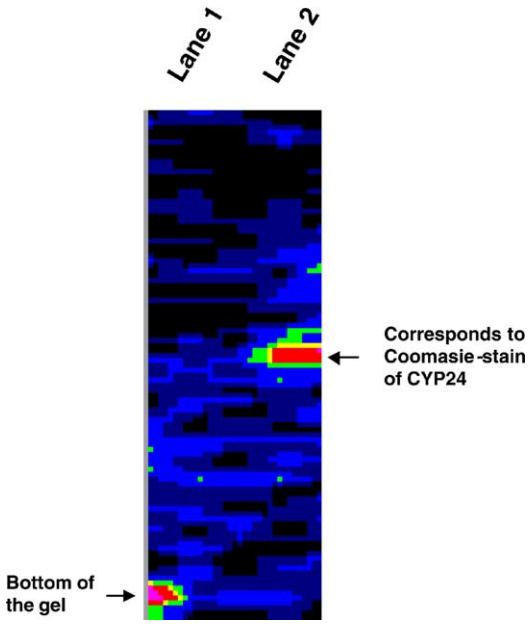

Fig. 2. Affinity labeling of rat CYP24 with ^{14}C-25-OH-D_3-BE. Lane 1 CYP24$^+$ ^{14}C-25-OH-D_3-BE$^+$ excess 25-OH-D_3. Lane 2 CYP24$^+$ ^{14}C-25-OH-D_3-BE.

lated and then digested with trypsin. The tryptic digest was subjected to HPLC and subsequent MS/MS analysis as described in Section 2. The data was analyzed with Thermo Finnigan sequest Browser Search, and the search was performed with modified weight of Ser, Cys and Lys, the amino acids with nucleophilic side chains that might form covalent bond with 25-OH-D_3-BE. Considering the $X_{correletion}$ scores above 2.5 as 'hits', we observed that the only amino acid residue that was modified by 25-OH-D_3-BE was Ser57. Since the 3-hydroxyl group of 25-OH-D_3 is chemically modified as a bromoacetate, this result strongly suggested that the 3-OH group of the ligand is juxtaposed with Ser57 in the ligand-binding pocket of CYP24.

Site-directed mutagenesis was used to validate the role of Ser57 in the binding of 25-OH-D_3. We constructed two mutants S57A and S57D, and compared substrate-binding (by spectral shift) and metabolic analysis of the mutants versus the wild type. With the S57A mutant the dissociation constants (K_d) for 25-OH-D_3 and 1,25$(OH)_2D_3$ were 391 and 127 nM, respectively compared with 324.5 and 61.5 nM for the same substrates with the wild type, showing significantly lower capacity for the binding of 25-OH-D_3 and 1,25$(OH)_2D_3$. Conversion of 1,25$(OH)_2D_3$ to calcitroic acid by the mutant was very similar to the wild type (Fig. 3). These results suggested that conversion of a hydroxymethyl group (in Ser57) to a methyl group (in S57A) did not change the environment in the binding pocket drastically to result in radical changes in biological activities of the mutant.

The S57D mutant, however, behaved very differently from the wild type. For example, dissociation constant for 25-OH-D_3 and 1,25$(OH)_2D_3$ for the mutants were 196 and 13.6 nM, respectively compared with 324.5 and 61.5 nM for the same substrates with the wild type, strongly suggesting higher binding capacity for the substrates. This observation was further supported by approximately three times higher side chain oxidation of 1,25$(OH)_2D_3$ to calcitroic by the mutant compared with the wild type enzyme (Fig. 3).

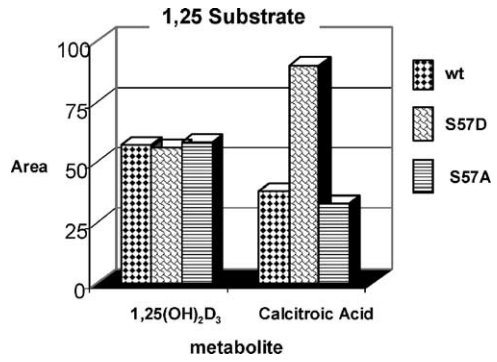

Fig. 3. Oxidation of 1,25$(OH)_2D_3$ to calcitroic acid by wild type CYP24 and Ser57 mutants.

Since the 3-hydroxyl group of 25-OH-D_3 is modified in the affinity label (25-OH-D_3-BE), the above mutation data strongly suggested changing Ser57–Asp57 drastically changed the binding environment. It could be argued that a direct hydrogen-bonding event took place between the 3-hydroxyl group (of 25-OH-D_3 and 1,25$(OH)_2D_3$) significantly increasing the substrate-binding capacity and catabolic capability of the mutant over the wild type. Alternatively, Asp57 could be pictured as forming a salt bridge with a juxtaposed basic amino acid that significantly changed the environment in the binding-pocket and resulted in enhanced substrate-binding and catabolism.

In conclusion, we have described, for the first time, structure functional analysis of CYP24, a key modulatory enzyme in the Vitamin D endocrine system, to identify Ser57 as an important contact point within the binding pocket of this protein. Identification of this residue will be crucial in developing a homology model of CYP24.

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