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Detection of 25-Hydroxyvitamin D_3 with an Enzyme modified Electrode

Harun F Ozbakir[1¶], David A Sambade[1¶], Sudipta Majumdar[1,2¶], Linda A Linday[3], Scott Banta[1] and Alan C West[1*]

[1]Department of Chemical Engineering, Columbia University, New York, USA

[2]Department of Chemistry, Indiana University of Pennsylvania, Indiana, Pennsylvania, USA

[3]Department of Pediatrics, Icahn School of Medicine at Mount Sinai, New York, USA

[¶]These authors contributed equally to this work

Abstract

Since testing for circulating vitamin D concentrations is relatively expensive and time consuming, rapid means of measurement are desired. As a step towards this goal, enzyme-modified electrodes responsive to 25-hydroxyvitamin D_3 (25(OH)D_3) have been developed. To make the enzyme, a synthetic gene encoding a human cytochrome P450 27B1 (CYP27B1) enzyme, which is a mitochondrial type heme-thiolate monooxygenase that converts 25(OH)D_3 into 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), was expressed and purified. The CYP27B1 enzyme was combined with NADPH-adrenodoxin reductase (ADR) and adrenodoxin (ADX) and activity was characterized using liquid chromatography mass spectrometry (LC-MS/MS). It was found that dihydroxyvitamin D_3 isomers were produced in addition to 1,25$(OH)_2D_3$. The enzyme was immobilized on glassy carbon electrodes using pH-adjusted Nafion® along with cobalt sepulchrate trichloride ($Co(sep)^{3+}$) as a redox mediator and electrode performance was characterized using cyclic and square wave voltammetry. The results demonstrate detection of 25(OH)D_3 in buffer within the physiological range (5-200 ng/ml).

Keywords: Vitamin D; Cytochrome P450; Enzyme modified electrode; Mediated electrochemistry

Introduction

The cytochromes P450 (CYP450) are a super family of heme-thiolate monooxygenases and can be found in organisms from various domains of life [1,2]. These enzymes are capable of metabolizing a diverse range of chemicals using different biotransformation reactions and are associated with biosynthesis of steroids, vitamins and lipids, xenobiotic and drug metabolism [1-8]. Thus, there has been immense biotechnological interest in these enzymes, and they have been utilized for applications in bioelectronic devices, biochips, bioreactors, and biosensor technologies [9-12].

The catalytic mechanism of the enzymatic CYP450 reaction involves several steps [13-15]. The two electrons necessary for the catalytic cycle are supplied by the electron donor NADPH and transferred via two electron-transfer proteins, NADPH-adrenodoxin reductase (ADR) and adrenodoxin (ADX) [7]. This pathway could potentially be utilized using electrochemical methods to detect $NADPH/NADP^+$ redox couple on an electrode (Figure 1A) [16-18]. As an alternative, non-native redox mediators can be used for electron delivery and electrochemical quantification of enzymatic activity (Figure 1B). Another alternative is direct electron transfer, where the enzyme can be deposited on the electrode by itself, can be attached to the electrode via gold nanoparticles [19], or can be immobilized to the electrode that is modified with polyelectrolyte multilayer films [20].

In vivo, the cytochrome P450 27B1 (CYP27B1) enzyme, which is a mitochondrial type CYP450, converts 25-hydroxyvitamin D (25(OH)D) into 1,25-dihydroxyvitamin D (1,25$(OH)_2$D). 25(OH)D is considered the best indicator of the body's vitamin D status [21]. Vitamin D is crucial for the human body due to its role in calcium and bone metabolism [21,22]. The best-known effect of vitamin D is the prevention of bone disease; vitamin D prevents rickets in growing children and osteomalacia (soft bones) in adults. Recent research has linked vitamin D to health benefits across the human lifespan. Recent data suggest vitamin D may reduce the risk of cancers, heart disease, autoimmune diseases, and type-2 diabetes [23]. In addition, low vitamin D levels have been linked to neuropsychiatric disorders, such as depression [24], Parkinson's disease [25], and Alzheimer's disease [26].

Vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) are the two common forms of this nutrient. Detection of vitamin D deficiency is generally accomplished using commercial assays for 25(OH)D. Radioimmunoassays (RIA), high pressure liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LC-MS/MS) are used for determining 25(OH)D_2 and 25(OH)D_3 values in serum [21]. These blood tests are performed in centralized laboratories; they are also expensive, and the results are delayed. In addition, two or three blood tests obtained over the course of several months may be necessary to indicate proper treatment. Another problem that should be addressed when analyzing the blood samples is the presence of 1,25$(OH)_2$D as the inhibitory effects of this product on level of 25(OH) D is not known [27-29].

Moreover, vitamin D supplementation has become a controversial topic. Many vitamin D experts disagree with the Institute of Medicine (IOM) recommendations regarding the amount of vitamin D for daily supplementation, as well as with the blood levels the IOM considers optimal [30]. Since measurement of 25(OH)D is relatively costly, blood level measurements are only recommended for people at known risk of vitamin D deficiency [23]; however many experts believe that

***Corresponding author:** Alan C West, Department of Chemical Engineering, Columbia University, New York, USA, Tel: 212-854-1754; E-mail: acw7@columbia.edu

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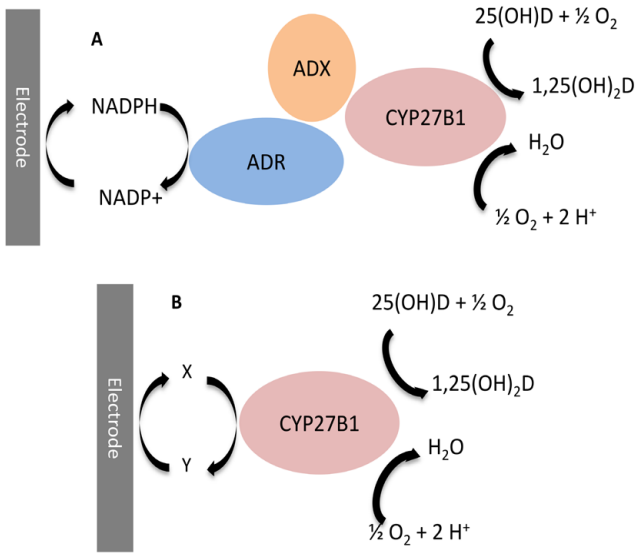

Figure 1: Electron transfer mechanisms in CYP450s. (A) Native mechanism with cofactor detection. (B) Mechanism with artificial redox mediators.

asymptomatic vitamin D deficiency is widespread and a larger patient population should be tested for their vitamin D levels. The availability of a quick and inexpensive 25(OH)D blood measurement may allow for optimization and individualization of vitamin D supplementation.

CYP27B1, which catalyzes hydroxylation at the 1α-position of 25(OH)D, has been cloned from several species including rats, mice, and humans with generally low expression levels and poor stability [31-33]. The human CYP27B1, a 55 kDa hemoprotein [27], is membrane associated, and has been cloned and expressed recombinantly in *E. coli* by several groups [28,34-38]. It has also been observed that recombinant CYP27B1 is active with other vitamin D isoforms (such as vitamin D_3 and 24, $25(OH)_2D_3$) when reconstituted in bacterial expression systems [34-36]. Various strategies have been used to optimize and increase the expression levels of CYP27B1 protein in *E. coli* including co-expression with molecular chaperones GroEL/ES [28,34]. The CYP27B1 enzyme cloned from the mouse kidney has been investigated, including at least one electrochemical study [39]. In the previous study, a surfactant material was used to solubilize and immobilize the enzyme, permitting electrochemical communication between the heme group of the enzyme and electrode. However this led to deleterious impact on catalytic activity. We have explored alternative approaches.

Cobalt sepulchrate trichloride ($Co(sep)^{3+}$), a non-native redox mediator, has been effectively utilized with other CYP450s [40-42]. Faulkner et al. reported that $Co(sep)^{3+}$ can be used in a CYP4A1 system with rates comparable to those obtained with NADPH [26]. Another research group constructed a biosensor by immobilizing CYP3A4 and $Co(sep)^{3+}$ on a Nafion® - modified electrode and reported relevant detection limits for the substrate of CYP3A4 [41]. Synthetic mediators like $Co(sep)^{3+}$ have multiple advantages such as facilitating quick, reversible electrochemistry, enhanced rates of reactions, and facile electrode design while maintaining flexibility in enzyme immobilization [43].

In the present work, we developed an enzymatic electrode for $25(OH)D_3$ detection in buffer, because testing for circulating $25(OH)D_3$ takes significant amount of time and is costly. As a first step, a synthetic gene encoding the human CYP27B1 was expressed in *E. coli*, purified, and its activity was identified using LC-MS/MS. The redox mediator $Co(sep)^{3+}$ was synthesized and utilized for electrochemical detection of CYP27B1 activity (Figure 1B). For this purpose, the mixture of purified CYP27B1, the redox mediator $Co(sep)^{3+}$ and Nafion® were immobilized on the glassy carbon electrode, and performance of the electrode was characterized in the physiological range of $25(OH)D_3$ using cyclic and square wave voltammetry.

Materials and Methods

Materials

NADPH, 3-((3-cholamidopropyl)dimethylamino)-1-propanesulfonate (CHAPS), lithium carbonate, sodium diethyldithiocarbamate trihydrate, hexane, dichloromethane, Nafion® - 117 solution, and $25(OH)D_3$ were obtained from Sigma Aldrich (St. Louis, MO, USA). Tris(ethylenediamine) cobalt(III) chloride trihydrate was obtained from Alfa Aesar. Anti-CYP27B1, anti-adrenodoxin and anti-adrenodoxin reductase primary antibodies and FITC-conjugated secondary antibodies were obtained from Abcam. A HisTrap™ HP column was obtained from GE Healthcare. Glassy carbon working electrode (model MF-2012) and Ag/AgCl reference electrode (model MF-2052) were obtained from BASi Inc.

Cloning, expression and purification of proteins

A gene encoding the human CYP27B1 gene with a C-terminal *myc*-tag and a 6 x *His*-tag was codon optimized for *E. coli* expression and chemically synthesized by DNA2.0 (DNA2.0, CA, USA). The N-terminal 32 amino acids of the 521 amino acid protein have been shown to constitute a pre-sequence, which targets the enzyme to the inner mitochondrial membrane and is cleaved to give the 489 amino acid mature protein [44]. Therefore, the first 31 amino acids were removed and Ser32 was mutated to Met32 by PCR to enable *E. coli* expression of the mature protein. The CYP27B1 gene was PCR-amplified using primers pr1 and pr2 (Table A in Supporting Information) producing 5'-*NdeI* and 3'-*EcoRI* restriction sites, respectively. The amplified PCR product was ligated into pre-digested pET20b vector, creating the plasmid pCYP.

Human ADX and ADR genes were PCR amplified from the pTC27A1 plasmid with C-terminal 6x *His*-tag using pr3 and pr4 for ADX, and pr5 and pr4 for ADR (Table A in Supporting Information) having 5'-*NdeI* and 3'-*EcoRI* restriction sites [45]. The amplified PCR products were ligated separately into pre-digested pMAL-c4E vector using the In-Fusion HD cloning kit (Clontech, USA). The plasmids containing ADX and ADR were named pAdx and pAdr, respectively. (Table B and C in Supporting Information)

All three plasmids pCYP, pAdx and pAdr were transformed into *E. coli* BL21 cells containing pGro7 plasmid which encodes chaperons GroEL/ES [34]. A single colony was used to inoculate 5 mL of TB broth containing 100 μg/mL ampicillin and 35 μg/mL chloramphenicol and incubated overnight at 37°C with shaking. The overnight cultures were added to 1 L TB broth containing the same antibiotics and incubated at 37°C shaking at 200 rpm until the cell density (OD_{600}) reached about 0.6. At this point, the transcriptions under the t7 (CYP27B1), tac (ADX and ADR), and araB (GroEL/ES) promoters were induced by IPTG and arabinose at a final concentration of 0.5 mM and 200 μg/mL, respectively. δ-Aminolevulinic acid (ALA) was also supplemented to a final concentration of 1.0 mM. The cultures were shaken at 28°C for 20 h and cells were harvested by centrifugation. The cell pellets were resuspended in 50 mL of buffer containing 100 mM Tris-HCl, 200 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, 0.1 mM PMSF, 1% CHAPS (w/v)

and 20% glycerol (v/v) (pH 7.4). The suspension was sonicated to lyse the cells, and cleared by centrifugation. The supernatant was applied to a 5 mL HisTrap™ HP column (GE Healthcare) and eluted with a linear gradient (50 mL) of 0 to 100 mM imidazole containing 100 mM Tris-HCl, 200 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, 0.1 mM PMSF, 1% CHAPS (w/v) and 20% glycerol (v/v) (pH 7.4). Fractions containing the purified enzymes were collected and dialyzed to remove the imidazole against 100 mM Tris-HCl, 200 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, 0.1% CHAPS (w/v) and 20% glycerol (v/v).

Western blot analysis

Solubilized CYP27B1, ADX and ADR prepared from recombinant *E. coli* cells were subjected to electrophoresis on 4-12% linear gradient polyacrylamide SDS gels and then transferred electrophoretically to nitrocellulose membrane using Semi-Dry Blotting unit (Fisher Biotech). CYP27B1, ADX and ADR membranes were probed with anti-CYP27B1, anti-adrenodoxin and anti-adrenodoxin reductase antibodies (Abcam, USA) and then followed by FITC conjugated secondary antibody (Abcam, USA). Antibody bound membranes were visualized by the Maestro *in vivo* imaging system.

Measurement of CYP27B1 activity and LC-MS/MS analysis

Activity measurements were carried out in a mixture consisting of 192 μM NADPH, 20 μM human ADX, 0.45 μM ADR, 5-10 μM 25(OH)D_3 and 100-200 μM CYP27B1, in a buffer containing 50 mM Tris-HCl, 0.1 mM EDTA, 0.1% CHAPS and 0.2% glycerol (pH 7.4). After preincubation at 37°C for 2 minutes, reactions were initiated with addition of NADPH. The reactions were stopped by addition of acetonitrile after 2-10 minutes [34]. After quick centrifugation, the clarified supernatants were extracted with ethyl acetate and the organic phase was transferred and dried under nitrogen flow. The organic phase was then resuspended in 4-phenyl-1,2,4-triazoline-3,5 dione (PTAD, 1 mg/ml in acetonitrile) and left for derivatization for one hour at room temperature. The same volume of DI water was added to quench the reaction. Samples were injected into the LC-MS/MS for quantification of metabolites. Reverse-phase UPLC with a Waters BEH C18 column was used for separation of the products. The gradient was initiated with 50% solvent A (water with 0.1% formic acid) and 50% solvent B (acetonitrile with 0.1% formic acid), then solvent B was increased to 65% over 2.5 minutes, with a flow rate of 0.3 mL/min.

Chemical synthesis of cobalt sepulchrate trichloride

The synthetic protocol was adapted from the literature [46]. In brief, NH_4OH (28% in DI H_2O, 50 mL) and CH_2O (37% w/w in H_2O, 50 mL) were added separately to a stirring suspension of Li_2CO_3 (2.303 g) and $[Co(en)_3]Cl_3.3H_2O$ (0.8989 g, 2.25 mmol) in DI H_2O (20 mL) at a rate of 0.83 mL/min utilizing peristaltic pumps. Upon complete addition of NH_4OH and, the reaction mixture was stirred for an additional 45 minutes, after which any unreacted Li_2CO_3 was filtered off and rinsed with aliquots of DI H_2O.

A solution of $Na[SCNEt_2].3H_2O$ (0.222 M in DI H_2O) was then added to the filtrate and the mixture was allowed to stir for 45 minutes. A dark orange-red solid was collected via vacuum filtration and was washed with 20:80 dichloromethane/hexane solutions until the filtrate was no longer green in color. The crystals were dried under vacuum and then suspended in acetonitrile. Concentrated HCl was added drop-wise to the suspension, resulting in the formation of bright-yellow crystals and the solution turning yellow-green. The mixture was then heated for 10 minutes, and the crystals were collected via vacuum filtration and rinsed with aliquots of acetonitrile in 50.1% overall yield. ^1H NMR (300 MHz wide bore, D_2O): δ(ppm) 4.0 (d, 12 H), 3.3 (dd, 12 H); UV/Vis (Molecular Devices SpectraMax M2 Spectrophotometer): λ 339.4 nm (ϵ 115.4 M^{-1} cm^{-1}), λ 469.7 nm (ϵ 108.9 M^{-1} cm^{-1}).

The ^1H NMR spectrum of $Co(sep)Cl_3$ dissolved in D_2O is in agreement with values published in the literature [47]. The UV-Vis spectrum exhibited a broad absorption band at 469.7 nm with ϵ = 108.9 M^{-1} cm^{-1} and a shoulder at 339.4 nm with ϵ = 115.4 M^{-1} cm^{-1}; these values are in close agreement with λ_{max} and ϵ_{max} values published in the literature [48].

Electrode preparation and electrochemical measurements

The working electrode was prepared by polishing the glassy carbon electrode (GCE) with aqueous slurry of 0.5 μm alumina for one minute using a Buehler rotary polisher. The working electrode was then sequentially rinsed with methanol and DI H_2O and sonicated in DI H_2O for five minutes to remove residual polishing material. This process was then repeated a second time where the working electrode was sonicated in absolute ethanol for five minutes. The GCE was then rinsed with DI H_2O and dried under a gentle nitrogen flow for approximately 5 minutes.

Nafion-117 stock solution was diluted to 1% w/v using 2-propanol, and it was pH adjusted with Tris-buffer, yielding a 1% solution (pH 7.4) of solution A. This was followed by the preparation of a 1 mM solution of $Co(sep)^{3+}$ in 0.1 M PBS (pH 7.4), yielding solution B. Purified CYP27B1 was used as solution C. The following electrode coating procedure was used: 5 μL of A was drop-cast onto the clean/dry GCE, followed sequentially by 5 μL of B and 5 μL of **C**. The electrode was then dried under a gentle flow of nitrogen for approximately 10 minutes.

Upon receiving 25(OH)D_3 from the manufacturer, it was dissolved in neat ethanol to make a 10 mM stock solution which was stored at -20°C. When needed for electrochemistry experiments, the stock solution was diluted with DI H_2O to a concentration of 0.49 μM; this solution was then serially diluted to obtain samples of other substrate concentrations in the physiological range. Water-diluted samples were not stored for longer than the duration of the experiment.

All electrochemical measurements were performed at room temperature (~25°C) using 0.1 M PBS (pH 7.4) as electrolyte. In addition to the GCE working electrode, a Ag/AgCl (3 M NaCl) reference electrode and a platinum wire counter electrode were used for all experiments. A salt-bridge-based electrochemical cell was created in order to enhance biosensor response. To construct the cell, a piece of Fisher-brand Q5 filter paper was saturated in electrolyte solution (1 mL) and placed into a petri dish. Electrodes were then placed in contact with the filter paper to allow for ionic current flow between them. A descriptive sketch of the cell can be found in Supporting Information (Figure A in Supporting Information).

Cyclic voltammetric (CV) analyses were performed on an EG&G PAR Potentiostat/Galvanostat model 273A under the control of CorrWare software; CorrView software was used for the visualization of results. All CV experiments were conducted at a scan rate of 100 mV/s and across a variable range of potentials. Square wave voltammetric (SWV) analyses were carried out on a μAutolab potentiostat at 15 Hz, 4.05 mV step height, and 25 mV amplitude on a potential range from 0-800 mV.

Results

The human CYP27B1 enzyme was expressed in *E. coli* with the assistance of chaperone protein GroEL/ES. The human CYP27B1

enzyme was partially purified using the appended polyhistidine-tag and immobilized metal affinity chromatography. Since CYP27B1 is a membrane-bound protein, CHAPS detergent was included for solubilization. No further purification was performed as the enzyme is known to be only marginally stable [28].

SDS/PAGE (Figure 2A) and Western blot analysis (Figure 2B) were used to assess the enzyme expression and purification. Since it is partially purified, SDS/PAGE analysis does not show a clear band, but Western blot confirms expression of CYP27B1 with a major band at 60 kDa. ADR and ADX, which are two extrinsic electron transfer proteins necessary for the catalytic cycle, were also expressed in *E. coli*, and purified with immobilized metal affinity chromatography. Purification of ADX and ADR were also assessed with SDS/PAGE and Western blot analysis (Figure 2), both confirming their purities.

The hydroxylation of 25(OH)D to $1,25(OH)_2D$ is catalyzed by CYP27B1 in the presence of NADPH as an electron donor, and ADX and ADR as electron-transfer proteins. In order to quantify the amount of $1,25(OH)_2D$ and other possible metabolites that were produced, LC-MS/MS was used. After preincubation, the reactions were initiated with the addition of NADPH. The reactions were stopped by addition of acetonitrile after 2-10 minutes and analyzed with LC-MS/MS. Figure 3 (panels A and B) shows the liquid chromatography profile of a sample where all the reactants in the enzymatic assay are present. After comparing with retention time of the standards, we observed $1,25(OH)_2D_3$ production, which is eluted at 1.33 minutes (Figure 3B), whereas for the same sample we also observed the formation of a $(OH)_2D_3$ isomer, which has a retention time similar but not identical to $24,25(OH)_2D_3$ (Figure 3A).

Experiments were performed with varying concentrations of $25(OH)D_3$ present in the assay and compared to controls without the addition of CYP27B1. The top two panels of Figure 4 show the results

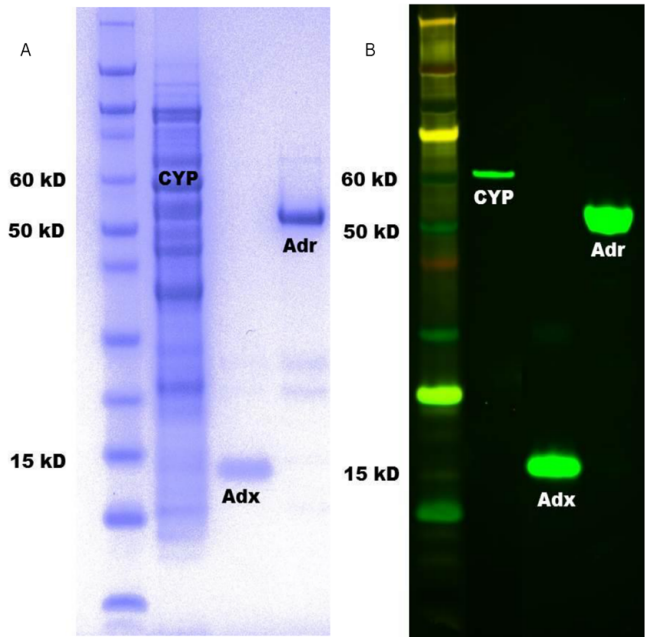

Figure 2: Analysis of purity of CYP27B1, ADX and ADR(A) SDS/PAGE of CYP27B1 (lane 2), ADX (lane 3), and ADR (lane 4) after nickel affinity chromatography. (B) Western blot of CYP27B1 (lane 2), ADX (lane 3), and ADR (lane 4) after nickel affinity chromatography. Lane 1 (leftmost one in both panels) is the protein standard.

for the production of $1,25(OH)_2D_3$ (A) and the $(OH)_2D_3$ isomer (B) as a function of initial $25(OH)D_3$ present in the assay. Concentrations of both $1,25(OH)_2D_3$ and $(OH)_2D_3$ isomer increased linearly as the initial concentration of the reactant in the assay was increased. The control experiments indicated the presence of a background signal which is likely some $1,25(OH)_2D_3$ and $(OH)_2D_3$ present in the $25(OH)D_3$ stock solution (Figure 4).

Kinetic experiments were also performed where the enzymatic reaction was stopped at different times in order to measure $1,25(OH)_2D_3$ concentration as a function of time. Figure 4C shows the amount of $1,25(OH)_2D_3$ produced for different reaction durations. Product formation starts with a value of 3.5 nM, showing a rapid production of $1,25(OH)_2D_3$, reaching 6.5 nM after 6 minutes. Lastly, Figure 4D shows the final concentration of $25(OH)D_3$ as a function of the initial concentration of the enzyme CYP27B1. We have observed that consumption of the substrate increased when the amount of CYP27B1 was increased in the assay, and this reaction is most relevant to the development of a sensor for Vitamin D detection. Experiments and controls summarized in Figure 4 suggest that recombinantly expressed CYP27B1 is successful in converting $25(OH)D_3$ into the products $1,25(OH)_2D_3$ and a $(OH)_2D_3$ isomer, although the yields for $1,25(OH)_2D_3$ are low.

Electrochemical experiments performed in bulk electrolyte systems did not yield reproducible results, likely due to the dissolution of materials off of the working electrode. To overcome these problems, a salt-bridge cell, wherein the electrodes were placed flush against a piece of filter paper saturated in electrolyte, was constructed. Control experiments utilizing well-characterized redox mediators were performed to ensure that effective charge transfer between electrodes was possible before applying this system to the development of a vitamin D sensor.

Cyclic voltammograms of the GCE/Nafion®/$Co(sep)^{3+}$/CYP27B1 system demonstrated two cathodic peaks, one indicative of the $Co^{3+/2+}$ transition at the more negative potential and the other occurring at the approximate redox potential of CYP27B1 [38]. Square wave voltammetry experiments with the same system were in agreement with this observation (Figure B in Supporting Information). Both peaks were responsive upon the addition of substrate; however, in the absence of $Co(sep)^{3+}$, the peak corresponding to the $Fe^{3+/2+}$ transition of the heme group was either not observed or poorly defined. Further control experiments with GCE/Nafion®/$Co(sep)^{3+}$ system that were conducted using SWV confirmed this conclusion (Figure C in Supporting Information). CVs of GCE modified only with Nafion® did not exhibit electrochemical activity as expected. Figure 5 summarizes the various behaviors observed in CV analyses and suggests that the electron transfer to the heme group of the enzyme was enhanced by the presence of $Co(sep)^{3+}$(Figure 5).

Discussion

Testing for circulating vitamin D concentrations is presently achieved using costly and prolonged techniques. In order to develop a rapid way of measuring it, an enzyme modified electrode sensitive to $25(OH)D_3$ has been improved. As a first step, the human CYP27B1 enzyme was made, and its activity was determined using LC-MS/MS. The purified CYP27B1 was immobilized on a glassy carbon electrode with the redox mediator $Co(sep)^{3+}$ and Nafion® membrane. The performance of this modified electrode was characterized electrochemically, using cyclic and square wave voltammetry.

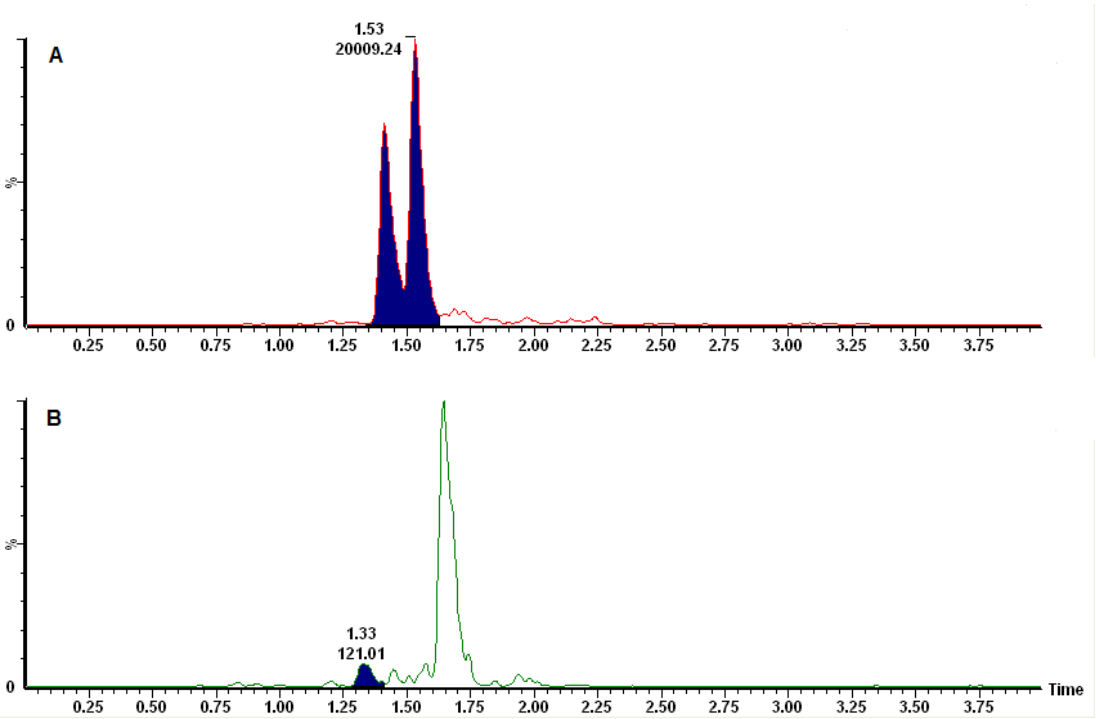

Figure 3: Characterization of products with liquid chromatography, All the reactants in the enzymatic assay are present. Filled area in the panel A shows $(OH)_2D_3$ isomer and filled area in the panel B shows $1,25(OH)_2D_3$ for the same sample. Abscissa shows the retention time in minutes whereas ordinate shows percent absorption in arbitrary units.

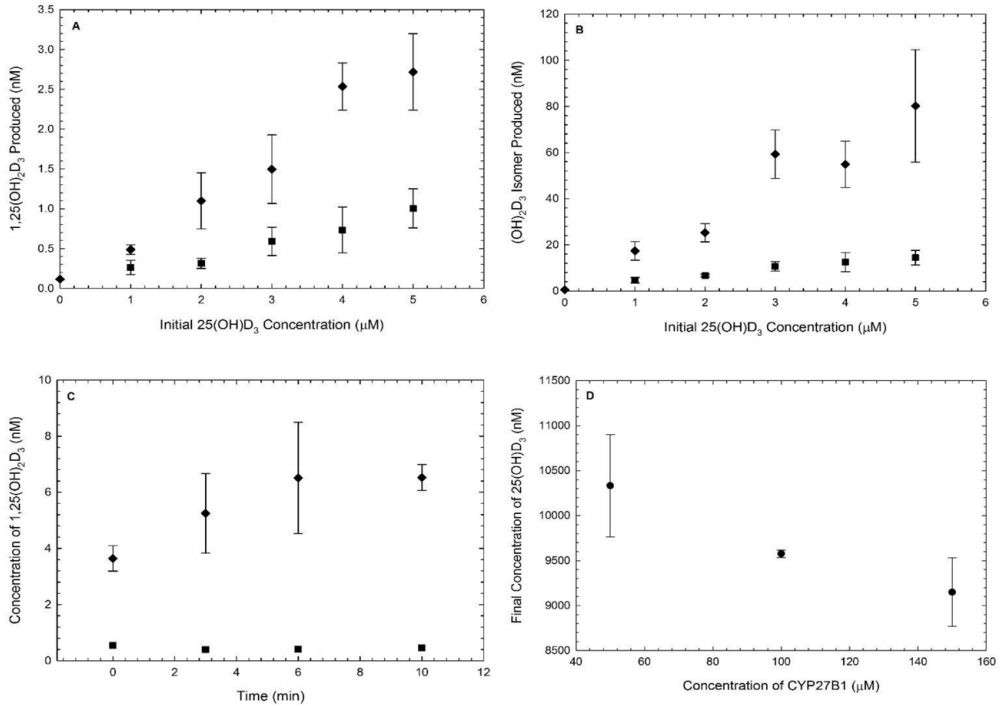

Figure 4: Results of the kinetic experiments, LC-MS/MS was used to quantify the concentration of products and other metabolites. The experiments were conducted at least in duplicate and error bars represent the standard error. (A) Concentration of $1,25(OH)_2D_3$ produced as a function of initial $25(OH)D_3$ concentration with the complete reaction mixture (♦) and without the addition of CYP27B1 (■). (B) Concentration of $(OH)_2D_3$ isomer produced as a function of initial $25(OH)D_3$ concentration with the complete reaction mixture (♦) and without the addition of CYP27B1 (■). (C) Concentration of $1,25(OH)_2D_3$ produced over time with the complete reaction mixture (♦) and without the addition of CYP27B1 (■). (D) Final concentration of $25(OH)D_3$ left in the reaction as a function of concentration of CYP27B1 in the assay.

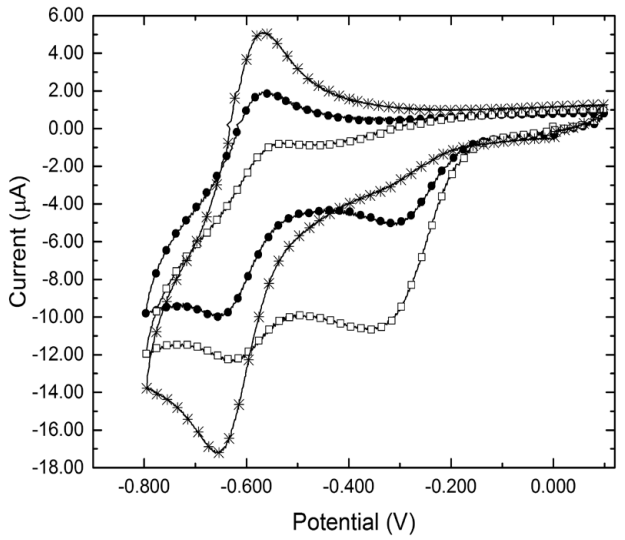

Figure 5: Representative cyclic voltammograms, Modified working electrode behavior of (*) GCE/Nafion®/Co(sep)$^{3+}$, (●) GCE/Nafion®/Co(sep)$^{3+}$/CYP27B1, and (□) GCE/Nafion®/Co(sep)$^{3+}$/CYP27B1 following the addition of 10 μL of 205.1 ng/mL substrate to the working electrode.

The CYP27B1 enzyme is able to catalyze the conversion of 25(OH)D_3 to 1,25(OH)$_2D_3$. A codon-optimized version of the human CYP27B1 gene was expressed in *E. coli* and partially purified. LC-MS/MS results show that under *in vitro* conditions, in addition to the expected 1,25(OH)$_2D_3$ production, the enzyme also catalyzes the formation of a (OH)$_2D_3$ isomer, similar to 24,25(OH)$_2D_3$. Possibly, when CYP27B1 is expressed in a bacterial expression system, some of the specificity of the reaction is lost. Uchida et al. [34] showed that fractions of overexpressed CYP27B1 are capable of 1α hydroxylation of both vitamin D_3 and 25(OH)D_3, the former not observed *in vivo*. Furthermore, Sawada et al. [35] and Sakaki et al. [36] demonstrated that human and mouse CYP27B1, respectively, overexpressed in a bacterial system metabolize 1α hydroxylation of 24,25(OH)$_2D_3$ as well as 25(OH)D_3. However, there is no specific report showing CYP27B1 catalyzing the formation of a (OH)$_2D_3$ isomer similar to 24,25(OH)$_2D_3$. Thus, an increased catalytic flexibility of this membrane-associated enzyme in an *in vitro* setting might explain the production of this isomer.

An important milestone for amperometric detection of 25(OH)D_3 is the immobilization of active CYP27B1 onto an electrode. A recent study reported the use of the CYP27B1 enzyme from mouse kidney and its immobilization onto an edge plane pyrolytic graphite electrode with a surfactant [39]. Although the heme group could electrochemically be detected, the surfactant used to solubilize and immobilize the enzyme was thought to have a deleterious impact on catalytic activity, and the system failed to quantify 25(OH)D_3 concentration electrochemically. Key differences in the present approach include use of Co(sep)$^{3+}$ as a redox mediator and of a Nafion® film for immobilization.

In cases where the redox center of an enzyme is buried in the structure and/or it is hard to detect the direct electron transfer to enzyme, an electrochemically active species (called a mediator) is used. This mediator first changes its redox state at the electrode and then shuttles the electrons to the redox site of the enzyme [49]. It is therefore possible to follow the response of the enzyme to its substrate by observing the changes in oxidation/reduction peak current of the mediator. Previous studies with various CYP450s showed that Co(sep)$^{3+}$ behaved as a mediator, in other words, detection of the corresponding substrates was possible by following the changes in the oxidation/reduction peak current of Co(sep)$^{3+}$ [40-42]. However, the presently constructed electrodes show electrochemical responses at the redox potential of Co(sep)$^{3+}$ as well as that of the heme group (Figure 5, Figures B and C in Supporting Information). In addition, a clear heme group peak was poorly defined in the absence of Co(sep)$^{3+}$ in film, suggesting that Co(sep)$^{3+}$ may enhance the electron transfer from the electrode to the heme group of CYP27B1. Amplification of the heme group signal with the addition of 25(OH)D_3 in the presence of Co(sep)$^{3+}$ suggests further that the mode of detection might be similar to direct electron transfer mechanisms. This mechanism is also analogous to the ones that were suggested by Abouzar et al. [19] and Poghossian et al. [20], where they use gold nanoparticles and polyelectrolyte multilayer to modify the sensor, respectively, and detect the changes in charged molecules directly.

The electrochemical detection of heme group in the presence of Co(sep)$^{3+}$ on the film suggests that Co(sep)$^{3+}$ may function as more than a mediator (i.e., as a 'promoter'). Despite the fact that it is not directly associated with the development of a new method for detection of 25(OH)D_3, it is important to understand the electron transfer process in this system and why Co(sep)$^{3+}$ might be acting differently than a typical redox mediator. It is possible that electrostatic interactions of the enzyme are altered due to the presence of Co(sep)$^{3+}$. The crystal structure of CYP27B1 is not available; however, previous simulation studies with another CYP450 crystal structure showed that substrate access channel of this enzyme has enough room to accommodate a molecule such as Co(sep)$^{3+}$ [50-52]. Therefore, electrostatic interactions of positively charged Co(sep)$^{3+}$ at the entrance of this channel might have effects on electron transfer rates to the redox center and on substrate binding [52]. Moreover, interaction of Co(sep)$^{3+}$ with the enzyme might alter the orientation of the latter so that heme group can be more accessible for electron transfer [53]. Another explanation for this 'more-than-a-mediator' behavior of Co(sep)$^{3+}$ might be due to cation-induced interfacial interactions. Armstrong et al. [54] demonstrated that use of multivalent cations such as Mg^{2+} and $Co(NH_3)_6^{3+}$ increased the electron exchange rate of a copper-containing protein and suggested that these cations may enhance the electron transfer.

The proof-of-concept developed in this study demonstrates that the detection of 25(OH)D_3 in buffer is possible, although behavior of the modified electrode across various 25(OH)D_3 concentrations has yet to be determined. The sensor is sensitive to 25(OH)D_3 in the physiological range (5-200 ng/ml) although optimization of the electrode assembly and mode of detection will be required in order to operate the sensor in the entire range. Furthermore, specificity of the modified electrode and 25(OH)D detection from blood-derived aliquots will be an important next step to determine the impact of interferents.

Conclusion

The human CYP27B1 enzyme has been expressed in *E. coli*, partially purified and its activity has been demonstrated using LC-MS/MS. A glassy carbon electrode modified with this enzyme, a non-native redox mediator Co(sep)$^{3+}$, and pH-adjusted Nafion® has been shown to respond to the circulating form of vitamin D, 25(OH)D_3. Using cyclic and square wave voltammetry, the GCE/Nafion®/Co(sep)$^{3+}$/CYP27B1 electrode has been shown to be successful in detecting 25(OH)D_3 in buffer within the physiological range (5-200 ng/ml).

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