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Purification and characterization of bovine steroid 21-hydroxylase (P450c21) efficiently expressed in *Escherichia coli* ☆

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Abstract

Steroid 21-hydroxylase, P450c21, is responsible for the conversion of progesterone and 17α-hydroxyprogesterone to their 21-hydroxylated derivatives. P450c21 has been poorly investigated because of difficulty in obtaining sufficient quantities of purified protein. To solve the problem, we have attempted to express the bovine P450c21 in *Escherichia coli* as a stable form. The N-terminal membrane anchor and basic regions of P450c21 were replaced by the basic region of CYP2C3. The engineered P450c21 was expressed at a level higher than 1.2 μmol/L culture (>60 mg/L) when coexpressed with molecular chaperones GroES/GroEL. Utilizing three steps of column chromatography, the protein was highly purified to the specific content 16.6 nmol/mg (91.2% purity). The purified protein is a monomer in the presence of 1% sodium cholate as determined by gel filtration analysis, suggesting that this membrane anchor-truncated form of P450c21 is more soluble than the native form. The purified enzyme showed typical substrate-binding difference spectra and 21-hydroxylase activities for both progesterone and 17α-hydroxyprogesterone. Truncation of the membrane anchor increases solubility of P450c21 facilitating expression of this protein in *E. coli* yielding sufficient quantities for both biochemical and biophysical studies.

Keywords: CYP21; *Escherichia coli* expression; Molecular chaperone; Steroid hormone; Congenital adrenal hyperplasia

Steroid 21-hydroxylase P450c21 encoded by the *CYP21* gene converts progesterone and 17α-hydroxyprogesterone to 11-deoxycorticosterone and 11-deoxycortisol, respectively, which are essential for the biosynthesis of aldosterone and cortisol. This enzyme is predominantly expressed in the adrenal cortex under the control of ACTH via the cAMP-dependent signaling pathway [1–3]. In humans, there are two CYP21 genes, a pseudogene CYP21A and a functional CYP21B, arranged in tandem on chromosome 6 [4,5]. This tandem arrangement of the CYP21B gene with the pseudogene and resultant gene crossover is proposed to be one cause of deficiency of this gene [5].

Deficiency of P450c21 found in ~1:10,000 live births is the major cause (90–95%) of congenital adrenal hyperplasia (CAH) [6], inherited as a monogenic autosomal recessive trait closely linked to the HLA major histocompatibility complex [7]. By defects of the CYP21 gene, synthesis of aldosterone and cortisol is impaired. This inefficient cortisol synthesis signals the hypothalamus to increase the level of corticotropin-releasing hormone that stimulates ACTH release in the pituitary. Due to ACTH overstimulation, the adrenal glands become hyperplastic. Rather than cortisol, the adrenals produce excess sex hormone precursors that do not require 21-hydroxylation for their synthesis. Once secreted, these hormones are further metabolized to active androgens, testosterone, and dihydrotestosterone. These hormonal conditions show three types of clinical symptoms, referred to as the salt-wasting, simple virilizing, and non-classical phenotypes.

Historically, steroid 21-hydroxylation is known as the first example of a P450-mediated monooxygenase reaction

☆ *Abbreviations:* HLA, human leukocyte antigen; CAH, congenital adrenal hyperplasia; DTT, dithiothreitol; ACTH, adrenocorticotropin hormone.

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as determined by light-reversible carbon monoxide inhibition [8]. Approximately 20 years after the discovery of this P450, P450c21 was purified from the bovine adrenal cortex [9]. The purified protein was stable in the presence of the detergent Emulgen 913 or in liposomes and formed a complex of 1:1 ratio with NADPH-dependent P450 reductase (CPR) although the complex was in an aggregated form [10]. Although stable in the presence of detergents or lipids, its hydrophobic nature and difficulties in obtaining quantities of the purified protein have hampered in depth study of P450c21. To fully understand this protein, sufficient quantities of the purified P450c21 must be available. However, P450c21 has not been successfully expressed as an active form in *Escherichia coli* [11]. We have developed an *E. coli* expression system for mammalian P450s [12–21] and have attempted to apply this system to P450c21 for obtaining significant quantities of the purified protein. Here, we report the first successful expression of P450c21 in *E. coli*, purification and characterization of this steroid hydroxylase.

Materials and methods

Construction of the CYP21 expression vector. The synthetic oligonucleotides used for PCR were: c3b21-N 5′-ACGCATatggctaaaaagacatcatct aaaggtaagCTCCCACCTCTGGTCCCCGGCTTCCTGC (the underline indicates the *Nde*I site), b21his-C 5′-ACGAAGCTTCAatggtgatggtgatg gtgCTGGGCACTGGCGCTCTCCCAGGC (the underline indicates the *Hin*dIII site).

To facilitate the expression, the membrane anchor and basic region of P450c21 were replaced with MAKKTSSKGK from CYP2C3 [22] as indicated by small letters in the c3b21-N primer. At C-terminus, the 6× histidine tag (small letters in the primer b21his-C) was added to facilitate the purification of the expressed protein. The PCR was carried out using P450c21pCMV [23] as template and the product was inserted at the *Eco*RV site in pBluescript. After sequence analysis, the *Nde*I/*Hin*dIII fragment was cut out and subcloned into the pCWori+ vector [24], yielding an expression plasmid c3B21pCW. The host *E. coli* DH5α was cotransformed with the c3B21pCW plasmid and pGro12, a molecular chaperone expression vector [25], and screened on LB plates supplemented with 0.1 mg/ml ampicillin and 0.04 mg/ml kanamycin.

Expression of CYP21 in E. coli. An overnight culture (1.6 ml) was diluted into 250 ml TB media supplemented with 0.1 mg/ml ampicillin and 0.04 mg/ml kanamycin. After incubation at 37 °C for 5 h and 40 min, 1 mM IPTG (for induction of P450c21 expression), 1 mM δ-aminolevulinic acid (a precursor of heme biosynthesis), and 4 mg/ml arabinose (for induction of GroES/GroEL expression) were added. The culture was incubated another 38 h at 27 °C, cells were harvested by centrifugation (2600 rpm for 15 min, GS-6 R, Beckman), suspended in 50 ml of a lysozyme buffer (250 mM sucrose, 50 mM Tris–HCl (pH 7.4), 0.5 mM EDTA, and 1 mg/ml lysozyme), and centrifuged again. Cell pellets were sonicated in 25 ml buffer A (50 mM potassium phosphate (pH 7.4), 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA, 500 mM sodium acetate, 1.5% sodium cholate, 1% Tween 20, and 0.1 mM PMSF). After centrifugation (95,000 rpm for 10 min, TL100-4, Beckman), supernatants were pooled and frozen in liquid nitrogen prior to purification.

Purification of CYP21. Supernatants (approximately 50 ml) were applied on a Ni–NTA agarose column (12 ml bed volume) equilibrated with buffer A. The column was washed with 100 ml buffer A supplemented with 20 mM imidazole followed with 20 ml buffer B (20 mM potassium phosphate (pH 7.4), 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA, 1.0% sodium cholate, 1% Tween 20, and 100 μM PMSF) and eluted with buffer C (100 mM imidazole acetate (pH 7.4), 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA, 1.0% sodium cholate, 1% Tween 20, and 0.1 mM PMSF). The eluate was diluted with one volume of solution D (20% glycerol, 0.1 mM DTT, 0.1 mM EDTA, 1.0% sodium cholate, and 0.5% Tween 20) and applied to DEAE-Sepharose (15 ml bed volume) equilibrated with buffer E (20 mM potassium phosphate (pH 7.4), 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA, 1.0% sodium cholate, and 0.5% Tween 20). The column was washed with 40 ml buffer E. Flow-through fractions were pooled and dialyzed against buffer F (50 mM phosphate buffer (pH 6.8), 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA, and 1.0% sodium cholate). The dialyzed protein solution was applied to SP-Sepharose (12 ml bed volume) equilibrated with buffer F. The column was washed with 40 ml buffer F plus 50 mM NaCl and P450c21 was eluted with 50–300 mM NaCl gradient in buffer F. The buffer was replaced by dialysis against buffer G (50 mM phosphate buffer (pH 7.4), 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA, and 1.0% sodium cholate) and the purified P450c21 was concentrated using a centrifugal device (Amicon Ultra-4) before being stored in liquid N_2.

Gel filtration. The purified P450c21 (500 mM × 50 μl) was applied on a Sephadex G200 column (10 × 300 mm). The protein was monitored by measuring the absorbance at 280 and 420 nm. Gel filtration was carried out at room temperature with the buffer containing 50 mM potassium phosphate (pH 7.4), 150 mM NaCl, 20% glycerol, 0.1 mM EDTA, and 0.1 mM DTT supplemented with 1% sodium cholate, 0.25% Cymal 5, or 0.056% Cymal 6.

Enzyme assay. The radioactive substrates, [1,2,6,7-^{3}H]17α-hydroxyprogesterone, [4-^{14}C]progesterone, and [1,2,6,7-^{3}H]progesterone, were purchased from American Radiolabeled Chemicals Inc. The 21-hydroxylation activity was reconstituted with recombinant rat CPR purified from *E. coli* [26,27]. The purified P450c21 (0.5–5 pmol) with 3-fold molar excess of reductase was mixed with radiolabeled substrates on ice and diluted with 0.5 ml of the pre-warmed reaction buffer (50 mM KPi (pH 7.4), 20% glycerol, 2 mM DTT, 0.1 mM EDTA, and 0.002% Cymal 5). After 30 s pre-incubation at 37 °C, the reaction was initiated by the addition of 5 μl of 100 mM NADPH. At each time point, the reaction mixture (100 μl) was transferred into 100 μl of 5 M guanidine thiocyanate and extracted twice with 1 ml toluene. The toluene extracts were combined, dried under air, and dissolved in 50 μl methanol. The reaction products were displayed on a thin-layer chromatographic plate (Kieselgel 60F_{254}, Merk) with ethyl acetate/dichloromethane 25:75 for 17α-hydroxyprogesterone and 20:80 for progesterone. The reaction was analyzed using a phosphorimager (Personal Molecular Imager FX, Bio-Rad) and/or a liquid scintillation counter (Wallac 1409, Pharmacia).

Results

Expression and purification of the P450c21

To express the bovine P450c21, the amino terminal membrane anchor and basic region of the protein were replaced with MAKKTSSKGK as described in Materials and methods. This modification alone was not successful for the expression, but P450c21 was efficiently expressed as an active form at the level of 1.2 μmol/L culture upon coexpression with molecular chaperones GroES/GroEL in *E. coli* DH5α (Fig. 1).

As shown in Fig. 2 and Table 1, the protein was highly purified by three steps of column chromatography. Bovine P450c21 having a C-terminal 4× His-tag did not bind to Ni–NTA agarose while that with 6× His-tag tightly bound. The column was washed with 20 mM imidazole without a detectable loss of P450, which efficiently removed most *E. coli* proteins. Therefore, the eluate from Ni–NTA agarose showed only minor contaminants on SDS–PAGE

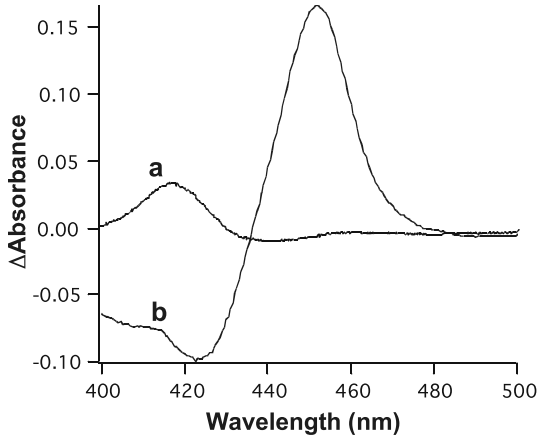

Fig. 1. Reduced CO-difference spectrum of P450c21. Transformed *E. coli* DH5α cells grown in the presence (b) and absence (a) of 4 mg/ml arabinose were solubilized. After ultracentrifugation, the supernatants were analyzed by measurement of the reduced CO-difference spectrum.

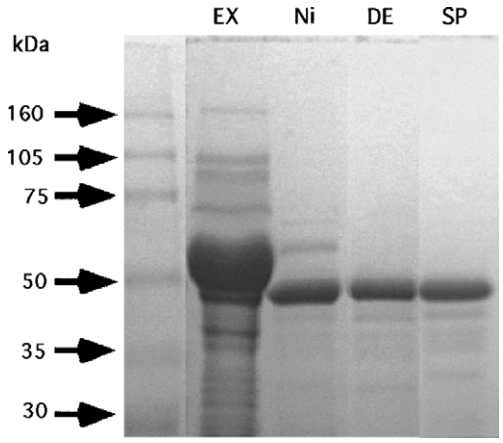

Fig. 2. SDS–PAGE of P450c21. Lane Ex, the *E. coli* extract before purification; Ni, the eluate from a Ni–NTA agarose column; DE, the pass-through fraction from a DEAE-Sepharose column; SP, the main fraction eluted from an SP-Sepharose column.

Table 1
Purification of P450c21 expressed in *E. coli*

	Proteins (mg)	P450 nmol (mg)	Spec. Cont. (nmol/mg)	Purity (%)	Recovery (%)
Extract	2630	644 (35.2)	0.28	1.33	100
Ni–NTA agarose	214	592 (32.4)	2.8	15.1	92
DEAE-Sepharose	56	544 (29.7)	9.7	53.1	84
SP-Sepharose	23	382 (20.9)	16.6	90.9	59

P450c21 expressed in *E. coli* (500 ml of culture media) was extracted and purified as described in Materials and methods. P450 was determined by the formation of reduced CO-complexes.

(Fig. 2, lane Ni) which were removed by subsequent DEAE-Sepharose and SP-Sepharose chromatography. P450c21 was estimated to be 91% in purity based on the specific content calculated from reduced CO-spectrum and protein assay (Table 1).

Spectral analysis of the purified protein

The reduced CO-difference spectrum of the purified P450c21 showed a typical P450 spectrum with a peak at 452 nm but no P420, an inactive form of P450, indicating that the purified P450c21 is free of inactive P420 and is stable under analytical conditions. To examine substrate binding, progesterone, 17-OH-progesterone, pregnenolone, and 17-OH-pregnenolone were added to the purified P450c21. As shown in Fig. 3A, 17-OH-progesterone produced a larger spectral change compared with progesterone although both substrates showed typical type I binding spectra, suggesting that 17-OH-progesterone binds P450c21 with a higher affinity than progesterone. Pregnenolone and 17-OH-pregnenolone did not produce spectral changes. As seen in Fig. 3B, the purified protein showed the Soret peak

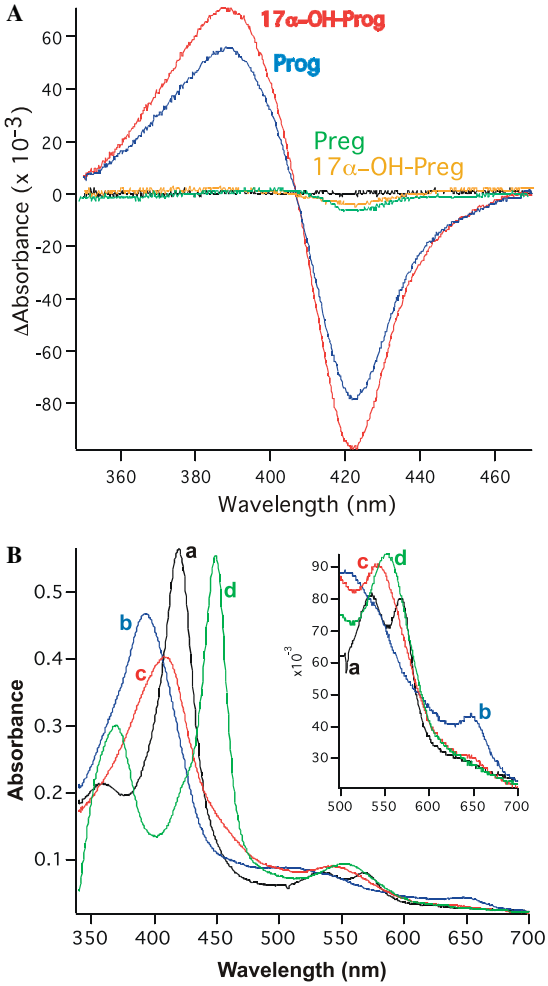

Fig. 3. Spectral analysis of the purified P450c21. (A) Substrate-binding difference spectra were determined with 100 μM each of 17-OH-progesterone (17-OH-Prog), progesterone (Prog), 17-OH-pregnenolone (17-OH-Preg), or pregnenolone (Preg). (B) A typical low spin spectrum (a) of the purified P450c21 was converted to a high spin form (b) by an addition of 17α-hydroxyprogesterone (0.5 mM). The P450 was reduced with sodium dithionite (c) and the reduced CO-complex (d) was formed. Spectra in the range 500–700 nm wavelength are enlarged in inset. Soret peaks of spectra (a), (b), (c), and (d) are seen at 420, 393, 408, and 449 nm, respectively.

of the substrate-free ferric form at 420 nm. The Soret peak was completely shifted to 393 nm by the addition of 17-OH-progesterone, indicating that the substrate converts the spin state of heme iron from 6-coordinate/low spin to 5-coordinate/high spin.

Oligomeric state of purified P450c21

To estimate the oligomeric state of the N-terminal membrane anchor-truncated P450c21, gel filtration was performed. P450c21 showed a single peak with a slight shoulder (Fig. 4A). The molecular size of the major peak was estimated to be 80.7 kDa in the presence of 1% cholate. Compared with the calculated size (53 kDa), purified P450c21 may be a monomer surrounded by a micelle of cholate. Although the intensity of the shoulder peak increased, P450 was eluted as a major peak having estimated molecular sizes of 124 and 167 kDa in the presence of 0.25% Cymal 5 (CMC = 0.12%) and 0.056% Cymal 6 (CMC = 0.028%), respectively. Because the elution patterns are similar to each other, the apparent increased size of the major peak with Cymal 5 and Cymal 6 may not be due to oligomerization but to difference in sizes of micelles formed by each detergent. Therefore, sodium cholate is a preferable detergent to stabilize the purified P450c21.

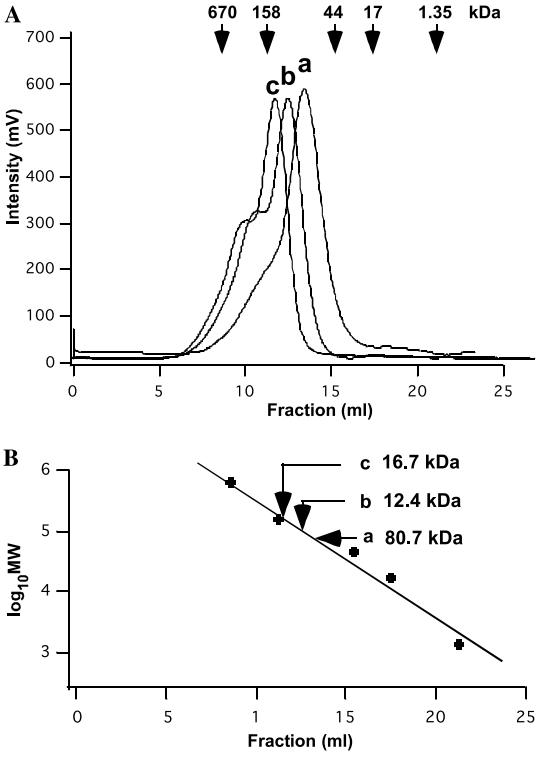

Fig. 4. Gel filtration analysis of the purified P450c21. (A) The gel filtration was performed in the presence of 1% cholate (a), 0.25% Cymal 5 (b), or 0.056% Cymal 6 (c). The concentrations of the detergents were adjusted to approximately 2-fold of their CMC. The proteins were monitored with absorbance at 280 and 420 nm. (B) The size of the major peak in each detergent (a, b, and c) was determined on a standard linear line of size markers. Thyroglobulin (670,000), bovine γ-globulin (158,000), chicken ovalbumin (44,000), equine myoglobin (17,000), and vitamin B12 (1350) were used as size markers.

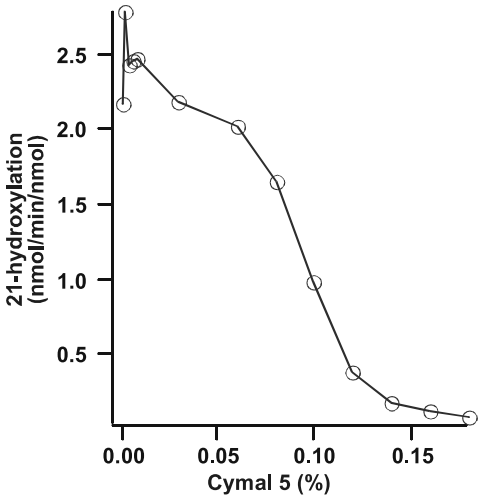

Fig. 5. Detergent dependence of 21-hydroxylation of progesterone using Cymal 5. The reaction (0.5 ml) was carried out using the purified P450c21 (1 pmol) and CPR (3 pmol) was incubated with ^{14}C-labeled progesterone at 37 °C for 4 min as described in Materials and methods. The concentration of Cymal 5 was varied in the range of 0.001–0.2%.

Enzymatic activities

To examine the 21-hydroxylation reaction, the detergent concentration in the reconstituted reaction system was optimized. In this study, Cymal 5 was used as a detergent for the enzymatic analysis. Kominami et al. reported that 21-hydroxylation of progesterone by P450c21 purified from the bovine adrenal cortex was maximized at 0.0034% of Emulgen 913 (CMC = 0.0035%). Therefore, we expected that an optimal concentration of Cymal 5 for the 21-hydroxylation reaction might be at the CMC (0.12%) of this detergent. However, the concentration of Cymal 5 around its CMC produced fairly low activities of 21-hydroxylation as seen in Fig. 5. The optimal condition was determined at 0.002% Cymal 5, which was used in the studies reported here. Although P450c21 has been purified from bovine adrenal cortex, kinetic parameters in the reconstitution system having a detergent have not been reported. The P450c21 expressed in *E. coli* efficiently catalyzed 21-hydroxylation of both 17-OH-progesterone and progesterone. V_{max} (nmol/min/nmol) and K_m (μM) obtained in this study were 10.8 ± 5.3 and 1.04 ± 0.53 for 17-OH-progesterone, and 12.9 ± 5.8 and 1.88 ± 0.85 for progesterone. We conclude that the N-terminal membrane anchor and the sequence of the basic region do not significantly affect either substrate-specificity or 21-hydroxylase activities.

Discussion

P450c21 has been efficiently expressed in *E. coli* for the first time. This success depends on N-terminal modification

and coexpression with molecular chaperones GroEL/GroES. Scott et al. [28] reported that a truncation of N-terminal membrane anchor increased the expression levels of P450s in the CYP2B subfamily. It was also true for several other P450s [29]. In addition to the truncation, replacement of the basic region of P450s with a corresponding sequence of an efficiently expressed P450 was also frequently carried out for the expression of mammalian P450s in *E. coli*. Previously, the basic regions of CYP17 and CYP2C11 have been used for successful expression of CYP19 (aromatase) in *E. coli* [15,16]. Also the basic region of CYP2E1 has been used for the expression of CYP2C8 and CYP2A6 [30]. In this study, we have used the modified sequence of the basic region MAKKTSSKGK from CYP2C3 [22] for the expression of CYP21 because the sequence was used for the expression of several soluble and monomeric forms of P450s [31]. With this modification alone, however, this P450 was not expressed in *E. coli* (Fig. 1). Only upon coexpression of molecular chaperones, GroES and GroEL which is also essential to the expression of mitochondrial vitamin D3 1α-hydroxylase CYP27B1 [20,32], could P450c21 be obtained. Therefore, the coexpression of molecular chaperones can be useful for the expression of active proteins in *E. coli*.

With the N-terminal modification, P450c21 has become more soluble than the native protein and bound neither ω-amino-octyl-Sepharose (hydrophobic affinity) nor hydroxyapatite that were used for the purification of this enzyme from tissues [9]. However, the modified P450c21 purified from *E. coli* still required a detergent for stability. After removal of the membrane anchor, several P450s have been shown to be oligomers in solution. CYP2C3 having the modification used in this study was shown to be a dimer in the absence of detergent but monomer in the presence of 0.5% cholate [22]. Scott et al. [28] reported that a modified form of CYP2B1 expressed in *E. coli* was mostly hexamer in the absence of detergent and was monomer with 0.25% cholate. The P450c21 expressed in this study is not as soluble as CYP2C3 or CYP2B1 and immediately precipitated when the purified sample was diluted with a solution having no detergent. However, this protein was determined to be monomeric in 1% cholate (Fig. 4). Based on results from size-exclusion chromatography, Kominami et al. [10,33] suggested that the native P450c21 forms an oligomer in micelles of Emulgen 913 or in liposomes. Compared with the truncated form of P450c21, the membrane anchor domain may be the major cause of this oligomerization.

In an in vitro reconstituted system using liposomes, P450c21 has been previously reported to catalyze 17-OH-progesterone better than progesterone because of approximately two times higher V_{max} but a similar K_d [34]. In our study, the modified P450c21 also preferred 17-OH-progesterone to progesterone with approximately 2-fold less K_m but similar V_{max} (Fig. 6). The inconsistency of the two studies may be due to differences in experimental systems. Because a very low concentration of detergent was used

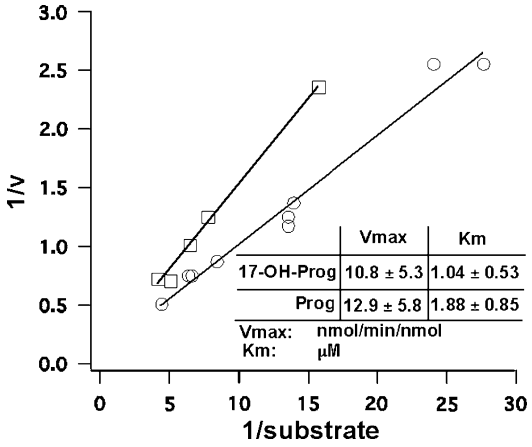

Fig. 6. 21-Hydroxylation of progesterone and 17-OH-progesterone by the purified P450c21. The activities of P450c21 were analyzed in the presence of 0.002% Cymal 5 using ^{3}H-labeled progesterone (squares) and 17-hydroxyprogesterone (circles) as described in Materials and methods. The V_{max} and K_m values were determined by using the least-squares method.

for our reconstitution system, most substrates may be in the solution, while most substrate molecules may be present in phospholipid bilayer in the liposome system. Therefore, the rate-determining steps of the hydroxylation reactions could be different between these reconstitution systems.

The highly purified P450c21 could be concentrated above 600 μM in the presence of 1% sodium cholate without any apparent loss of activities. Using this protein, we are currently investigating the structure/function relationship of P450c21 by resonance Raman spectrometry and crystallization.

Acknowledgments

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