Construction of a P450c27 fusion enzyme: a useful tool for analysis of vitamin D₃ 25-hydroxylase activity

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Liver mitochondrial P450c27, encoded by the CYP27 gene, can catalyse the 25-hydroxylation of vitamin D₃ and the 27-hydroxylation of sterols. To facilitate the study of this enzyme in cell culture systems, we engineered a fusion protein consisting of P450c27 coupled to its electron-transport accessory proteins, ferredoxin and ferredoxin reductase, and assessed its enzyme activity by measuring the C-25 and C-27 (side-chain) hydroxylation of 1 α -hydroxyvitamin D₃ (1 α -OH-D₃). When incubated with 1 α -OH-D₃, COS-1 cells transfected with a vector expressing the fusion protein produced 1 α ,25-(OH)₂D₃ and 1 α ,27-(OH)₂D₃ about four times more efficiently than did cells transfected with

three individual components of the fusion. When incubated with the natural substrate, vitamin D₃, the efficiency of hydroxylation was lower than that for 1 α -OH-D₃ but still 1.7-fold higher for the fusion protein than for its individual components. The fusion protein was also able to reproduce qualitatively and quantitatively the activity shown by P450c27 in liver cells *in situ*. The P450c27–ferredoxin reductase–ferredoxin fusion construct represents a valuable tool for establishing the substrate specificity of this liver cytochrome and for evaluating its potential for activating pro-drug analogues of vitamin D.

INTRODUCTION

Activation of vitamin D to its hormonal form requires sequential hydroxylations in the liver at C-25 and in the kidney at C-1. Whereas the 1-hydroxylation of 25-hydroxyvitamin D₃ (25-OH-D₃) is tightly regulated [1,2] to maintain 1 α ,25-(OH)₂D₃ concentration within a narrow range in the blood (10⁻⁹–10⁻¹⁰ M), the vitamin D₃ 25-hydroxylase appears to be less tightly regulated [3]. When compared with its natural substrate, vitamin D₃, 1 α -OH-D₃ is a much better substrate for the vitamin D₃ 25-hydroxylase [4]. This facilitates the administration of 1 α -hydroxylated vitamin D analogues as pro-drugs [5] to be activated through 25-hydroxylation in the liver. The vitamin D₃ 25-hydroxylase is a type-I (mitochondrial) cytochrome P-450 enzyme [6]. Cytochrome P450c27, encoded by the CYP27 gene, has been postulated to catalyse vitamin D₃ 25-hydroxylase and sterol 27-hydroxylase activities [7–9].

Type-I P450 enzymes are found in the mitochondria where they act as terminal oxidases, receiving electrons from NADPH through two intermediates, ferredoxin (Adx) and ferredoxin reductase (AdRed). The P450 component of the cytochrome P-450 system is intimately associated with the inner mitochondrial membrane, and Adx and AdRed are loosely associated with this membrane [10]. Hydroxylation proceeds when NADPH transfers a pair of electrons to the flavoprotein, AdRed. The electrons are then passed to Adx, an iron–sulphur protein, which then donates the electrons to the cytochrome P-450 which transfers a hydroxy group to its substrate. It has been suggested that the rate of hydroxylation by cytochromes P-450 may not be solely determined by access of the substrate to the P450, but also by the time needed for each of the pairwise associations: first AdRed with Adx, then Adx with the P450 [11]. This has been confirmed using the related type-I cytochrome P450, P450_{scc}, for which Harikrishna et al. [12] demonstrated increased enzymic efficiency

by constructing a fusion protein in which the cytochrome P-450 was linked to Adx and AdRed.

We constructed a fusion enzyme (F2c27) of the human P450c27 system. Transfection studies showed that the F2c27 fusion protein could generate the metabolites previously observed from P450c27 [13]. The efficiency of 1 α -OH-D₃ metabolism was greater in cells transfected with the F2c27 fusion construct than in cells co-transfected with cDNAs encoding all three components of the P450c27 system.

EXPERIMENTAL

Materials

The simian virus 40 (SV40)-transformed African Green Monkey kidney cell line COS-1 was purchased from the American Tissue Culture Collection. The vitamin D analogues 1 α -OH-D₃ and 1 α ,27-(OH)₂D₃ were provided by Dr. Martin Calverley (Leo Pharmaceuticals, Ballerup, Denmark). The plasmids expressing P450c27 (pSG5-CYP27) [13], Adx (pEAdx) [14], AdRed (pEAR) [15] and the P450_{scc} fusion protein termed F2 (pF2) [12] were synthesized previously.

Construction of pF2c27

The cDNA for human P450c27 was isolated as an *EcoRI* fragment purified from a 1% agarose gel using geneclon II (Bio101 Inc., La Jolla, CA, U.S.A.). This purified template (10 ng) was then used in a 100 μ l PCR using oligonucleotide (Oligo) 1 (5'-GGTACCATGGCTGCGCTGGGCTGC-3') corresponding to the sense direction of P450c27 and containing a *KpnI* site at its 5' six bases. Oligo 2 (5'-GACTAGTGCC-GTCGGTGCCTGTCTCTGCAGGAA-3') corresponds to the

Abbreviations used: D₃, vitamin D₃; OH or (OH)₂, hydroxy or dihydroxy, e.g. 1 α ,25-(OH)₂D₃ is 1 α ,25-dihydroxyvitamin D₃ (note that in the nomenclature used no distinction between C-26 and C-27 is implied); HIM, hexane/isopropyl alcohol/methanol; AdRed, adrenodoxin reductase (ferredoxin reductase); Adx, adrenodoxin (ferredoxin); Oligo, oligonucleotide.

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antisense direction of P450c27 at the 3' end of the coding region; it removes the stop codon from P450c27 cDNA and contains the *SpeI* linker region, which we have previously used to express P450 fusion proteins [12,16]. PCR amplification was then carried out with *Taq* DNA polymerase in a thermal cycler programmed for 20 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 2.5 min, followed by a final extension of 7 min at 72 °C. The resulting PCR product was directly ligated to the pCRII vector (Invitrogen, La Jolla, CA, U.S.A.) and then the fragment was sequenced through the internal *Bam*HI and *Sst*I sites to ensure that no PCR artifacts had been introduced. Subsequent manipulations are detailed in Figure 1. All ligation junctions were sequenced to ensure that no ligation artifacts occurred.

Transfection of COS-1 cells

COS-1 cells were transfected by the DEAE-dextran methods as modified by Levesque et al. [17]. Cells were incubated with DEAE-dextran (500 µg/ml) and (i) pF2c27, (ii) pSG5-CYP27, pEAdx and pEAR, (iii) pEAdx and pEAR or (iv) pF2 in PBS. The quantity of each plasmid used in transfection was determined to give equimolar amounts of each component of the P-450 system. The plasmid pRSVLuc was added to each transfection cocktail to correct for transfection efficiency. At 24 h after transfection, cells were washed twice with PBS, then the medium was replaced with Dulbecco's modified Eagle's medium + 0.1% BSA + 100 µM *N,N*-diphenylethylenediamine. Cells were then incubated for 48 h in the presence of 25 µM 1 α -OH-D₃, vitamin D₃ or vitamin D₂.

Analysis of P450c27 activity

The culture medium from cells incubated with 1 α -OH-D₃, vitamin D₃ or vitamin D₂ was extracted as previously described [18], and the total lipid extracts were separated by HPLC using a Zorbax-SIL (3 µm) column (0.62 cm × 8 cm) eluted with the solvent system hexane/isopropyl alcohol/methanol (HIM) (91:7:2, by vol.) at a flow rate of 1.3 ml/min for 1 α -OH-D₃ and its metabolites or HIM (94:5:1, by vol.) at a flow rate of 1 ml/min for vitamin D and its metabolites [19]. Metabolites were identified on the basis of their characteristic vitamin D chromophore ($\lambda_{\min}$ = 228 nm; $\lambda_{\max}$ = 265 nm; $\lambda_{\max}/\lambda_{\min}$ = 1.75) and by co-chromatography with synthetic standards. Metabolites were quantified by integration of UV peaks at 265 nm. Further characterization of 1 α -OH-D₃ metabolites was performed on HPLC using a Zorbax-CN (6 µm) column (0.46 cm × 25 cm) eluted with the solvent system HIM (91:7:2, by vol.) at a flow rate of 1 ml/min [20].

RESULTS

Construction of the fusion protein

We previously developed a strategy that readily allows the construction of mammalian expression vectors that code for a fusion protein comprising a mitochondrial cytochrome P-450 coupled to its electron-donor components, Adx and AdRed [12,16]. Of the several expression cassettes we have produced, the one designated pF2 produced the highest level of protein activity [12,16]. This fusion consisted of H₃N-P450-AdRed-Adx-COOH and allows rotational mobility of the Adx moiety and increases the interactions of Adx between both the AdRed and the P450 [12]. The construct contains small hydrophilic peptide linker sequences which are based on the hinge regions of naturally

occurring multidomain proteins [21]. The sequence of the peptide linker between the P450 and the AdRed sequences is Thr-Asp-Gly-Thr-Ser and that between the AdRed and Adx is Thr-Asp-Gly-Ala-Ser. To build a fusion of P450c27 we substituted human P450c27 cDNA sequences for P450scc. The mitochondrial leader signal of human P450c27 was retained but the 5'-untranslated region was removed and replaced by a *KpnI* site contained within Oligo 1 (5'-GGTACCATGGCTGCGCTGGCTGC-3'). Similarly, the translational stop codon and the 3'-untranslated region was removed and replaced with the sequences required to produce the P450-AdRed hydrophilic linker using Oligo 2 (5'-GACTAGTGCCGTCGGTGCACTGTCTCTGCAGGAA-3'). After PCR amplification, subcloning and sequencing, the P450c27 cDNA was cloned into pF2 (minus P450scc) to create pF2c27 (Figure 1).

Qualitative enzyme analysis of the P450c27 fusion protein

Analytical HPLC analysis of lipid extract from pF2c27-transfected COS cells incubated with 1 α -OH-D₃ gave the profile illustrated in Figure 2. The HPLC profile seen here is similar to that previously observed for COS cells transfected with a P450c27 cDNA expression vector [13], suggesting that these metabolites are probably 1 α ,24-(OH)₂D₃ (peak 2), 1 α ,25-(OH)₂D₃ (peak 3) and 1 α ,27-(OH)₂D₃ (peak 4). Co-chromatography of peak 3 and peak 4 with the synthetic standards 1 α ,25-(OH)₂D₃ and 1 α ,27-(OH)₂D₃ confirmed their identity (Table 1). Similarly, cells transfected with pF2c27 could generate 25-hydroxyvitamin D₃ from vitamin D₃ and 24-hydroxyvitamin D₂ from vitamin D₂ (results not shown). This confirms that the P450c27 fusion protein has the vitamin D₃ 25-hydroxylase activities previously attributed to the P450c27 system. The 1 α ,24-(OH)₂D₃ produced in subsequent transfection studies was not quantified because of difficulty in integrating the corresponding HPLC peak caused by a co-migrating contaminant peak.

Quantitative enzyme analysis of the P450c27 fusion protein

The hydroxylase activities of COS cells expressing the P450c27 fusion protein or the three individual components of the P450c27 system (P450c27, Adx and AdRed) were compared. The background 25-hydroxylase activity of the COS cells was examined by transfecting cells with control plasmids expressing either human Adx and AdRed or the previously characterized P450scc fusion protein F2 [12]. COS cells were transfected with equimolar amounts of each plasmid so that the P450c27 was present at concentrations that were previously determined to be optimal [13]. HPLC analysis of the transfected cells (Figure 3) demonstrated that COS cells transfected with the P450c27 fusion protein were able to generate greater amounts of 1 α ,25-(OH)₂D₃ and 1 α ,27-(OH)₂D₃ than the triple co-transfected cells, whereas control transfected cells produced levels of these two metabolites that were below the limit of detection for this system (25 pmol/24 h). When corrected for transfection efficiency, the cells expressing the P450c27 fusion protein were approximately 4 times more efficient at converting 1 α -OH-D₃ into its biologically active form than cells expressing the three individual components (see Table 2).

We also compared the ratio of the 25-hydroxylated metabolite with that of the 27-hydroxylated metabolite (see Table 2). Consistently, we observed that cells expressing the triple co-transfected system favoured production of 1 α ,27-(OH)₂D₃ while the cells transfected with the pF2c27 fusion construct favoured formation of 1 α ,25-(OH)₂D₃.

The ability of the P450c27 fusion protein to hydroxylate

Figure 1 Construction of pF2c27

The human P450c27 cDNA was PCR amplified using Oligos 1 and 2 as detailed in the Experimental section. The PCR product was then ligated into pCRII and sequenced through the internal *Bam*HI and *Sst*I sites. The P450c27 cDNA was then released from the pCRII vector as an *Eco*RI fragment and ligated into pUCSF [12], thus creating pSFc27. Subsequently, the internal coding sequence bound by the *Bam*HI and *Sst*I sites was replaced by the corresponding sequence isolated from the wild-type P450c27 cDNA to produce the plasmid pc27. This plasmid was then digested with *Kpn*I and *Spe*I and the cDNA insert was purified by electrophoresis. This was then ligated to similarly treated pF2 [12] lacking the P450scc component. This produced a plasmid (pF2Δ27) that lacks the 5' end of the human P450c27 cDNA because of the presence in the cDNA of an internal *Kpn*I site. Subsequently, the internal fragment of the human P450c27 cDNA flanked by the *Bam*HI and *Sst*I sites was removed and replaced with the corresponding fragment isolated from wild-type (unamplified) cDNA. Finally, the *Kpn*I fragment corresponding to the 5' end of the human CYP27 cDNA was ligated into the construct to produce pF2c27.

Figure 2 HPLC profile of lipid extract from COS cells expressing the CYP27 fusion protein

COS cells were transfected as described in the Experimental section. Total lipid extracts were separated on a Zorbax-SIL (3 μ m) column using the solvent system HIM (91:7:2, by vol.) at a flow rate of 1.3 ml/min. Metabolites of 1α -OH- D_3 were identified on the basis of their characteristic vitamin D chromophore and have been shaded. Int Std, internal standard.

Table 1 Chromatographic properties of metabolites generated in COS-1 cells transfected with the F2c27 fusion construct

The HPLC conditions for the two different chromatographic systems are given in the Experimental section.

Metabolite	Retention time (min)		Putative identity
	Zorbax-SIL	Zorbax-CN	
1α -OH- D_3 (Standard)	4.72	—	1α -OH- D_3
$1\alpha,25$ -(OH) $_2D_3$ (Standard)	12.08	13.91	$1\alpha,25$ -(OH) $_2D_3$
$1\alpha,27$ -(OH) $_2D_3$ (Standard)	13.56	15.28	$1\alpha,27$ -(OH) $_2D_3$
Peak 1	4.75	—	1α -OH- D_3
Peak 2	10.81	11.89	$1\alpha,24$ -(OH) $_2D_3$
Peak 3	12.12	13.82	$1\alpha,25$ -(OH) $_2D_3$
Peak 4	13.62	15.20	$1\alpha,27$ -(OH) $_2D_3$

natural vitamin D compounds in the form of vitamin D_3 and D_2 was also measured. As published previously [4,13], we found that both are hydroxylated much less efficiently than 1α -OH- D_3 . Vitamin D_3 is both 25- and 27-hydroxylated and the efficiency of this process is several-fold (~ 4.4) lower than for 1α -OH- D_3 . Furthermore, although the advantage seen for the fusion protein over the separate P450c27 components is still present when using vitamin D_3 as a substrate, it is not as pronounced as when 1α -OH- D_3 is the substrate (~ 1.7 -fold versus ~ 4 -fold). The efficiency of vitamin D_2 hydroxylation by both proteins is even lower. Indeed, vitamin D_2 is not 25-hydroxylated at all by the fusion protein or the triple co-transfected P450c27 but rather 24- and 27-hydroxylated. Again the fusion protein continues to perform 24-hydroxylation of vitamin D_2 more efficiently than its separate components.

DISCUSSION

We have engineered a functional P450c27 fusion protein that is a more efficient enzyme than the native reconstituted P450c27 system in transfected mammalian cells. The cDNAs encoding P450c27 and its electron-transfer proteins were coupled to form a single open reading frame which codes for a fusion enzyme.

Figure 3 Comparison of HPLC profiles from COS cells transfected with various mammalian expression vectors

COS cells were transfected as described in the Experimental section. Total lipid extracts were separated on a Zorbax-SIL (3 μ m) column using the solvent system HIM (91:7:2, by vol.) at a flow rate of 1.3 ml/min. The region of the chromatograms containing vitamin D metabolites (14–20 min) obtained from cells expressing the P450c27 fusion protein, the triple co-transfected P450 system or the control transfected system (Adx and AdRed) have been overlaid. Peaks generated in COS cells expressing the fusion hydroxylase (black) or with the triple co-transfected hydroxylase system (hatched), but not in the control transfected COS cells, have been shaded.

Table 2 Vitamin D_3 25-hydroxylase activity of COS-1 cells transfected with equimolar amounts of CYP27 DNA sequence

Cells were transfected with plasmid DNA such that equimolar amounts of each component of the P450 system were present. No empty plasmid was added to transfection cocktails to correct for equal amounts of DNA added to cells. Cells expressing the different CYP27 P450 systems were incubated with 1α -OH- D_3 for 48 h. Metabolites were extracted from the culture media, then isolated and quantified by HPLC. Values are expressed as the mean $\pm$ S.D. ($n = 3$). Values have been normalized for transfection efficiency. N.D. indicates metabolite not detected; N.Q. indicates that metabolite was detected but not quantified because of the presence of a contaminating peak.

	Metabolite production (pmol/24 h)			Ratio 25-OH/27-OH
	24-OH	25-OH	27-OH	
F2c27 – 1α -(OH) D_3	N.Q.	1639 ± 137	1007 ± 84	1.63 ± 0.01
P450c27 – 1α -(OH) D_3	N.Q.	391 ± 135	476 ± 123	0.82 ± 0.17
F2c27 – vitamin D_3	N.D.	375 ± 51	126 ± 15	2.96 ± 0.05
P450c27 – vitamin D_3	N.D.	218 ± 5	95 ± 7.5	2.32 ± 0.13
F2c27 – vitamin D_2	177 ± 6	N.D.	32 ± 2	$5.71 \pm 0.35^*$
P450c27 – vitamin D_2	116 ± 6	N.D.	50 ± 2	$2.31 \pm 0.01^*$

* Ratio of 24-OH/27-OH rather than 25-OH/27-OH since 25-OH-metabolite not detected.

Previous work using P450c21 (a type-II P450) expressed as a fusion protein with NADPH cytochrome *P*-450 reductase in yeast demonstrated that the enzyme activity was only retained when the P450 moiety was located at the N-terminus [22]. Using a type-I P450, P450sc, Harikrishna et al. [12] further showed that fusion enzymes with Adx located at the C-terminus are more stable than those containing AdRed at that position. Black et al. [16] showed that P450sc, a type-I P450, could also function when fused to P450 oxidoreductase, the type-II electron donor, but that this system was inferior to the fusion with Adx and AdRed, the type-I electron donor. Thus the P450c27 fusion enzyme was constructed so that the cDNA encoding the P450c27 precursor protein (including mitochondrial leader sequence) appeared at the N-terminus, linked to the cDNA encoding the

mature AdRed, which in turn was linked to the cDNA encoding the mature Adx. The strategy worked efficiently, as it did previously with P450_{scc}, indicating that it is probably generally applicable to other type-I P450 enzymes.

The P450c27 fusion protein is superior to the triple co-transfected P450c27 system for generating large quantities of 25-hydroxylated metabolites. Under all conditions tested, we observed greater quantities of 1 α ,25-(OH)₂D₃ produced by cells expressing the fusion protein when compared with triple co-transfected cells. When transfections were performed under conditions in which equimolar amounts of each component of the P450 system were present, the P450c27 fusion protein demonstrated enzyme activity four times higher than triple co-transfected cells. This increase in enzyme activity is similar to that previously demonstrated for the corresponding P450_{scc} fusion construct in which the apparent $V_{\max}$ for the conversion of 22-OH cholesterol to pregnenolone was increased 6-fold [12]. The efficiency of hydroxylation of vitamin D₃ and vitamin D₂ by the fusion protein is lower than for its artificial analogue 1 α -OH-D₃, an observation previously made for the separate cytochrome P-450 [4,13]. Furthermore the advantage of the fusion protein over the separate protein is not as marked with vitamin D₃ as substrate as with 1 α -OH-D₃ as substrate but is still 1.7-fold greater. Vitamin D₂ is a poor substrate for P450c27 irrespective of the environment in which it is placed. Thus, although the efficiencies of the P450 systems depend on the conditions used to assess their activities, it is evident that the P450c27 fusion protein is a more useful enzyme in that it provides maximal vitamin D 25-hydroxylase activity in transfected mammalian cells.

Consistent with previous studies [13], the metabolites 1 α ,24-(OH)₂D₃, 1 α ,25-(OH)₂D₃ and 1 α ,27-(OH)₂D₃ were generated in cells expressing either the P450c27 fusion protein or the triple co-transfected components of the P450c27 system. As 1 α ,24-(OH)₂D₃ represents only a minor product, we focused on the ratio of 25-hydroxylated to 27-hydroxylated metabolites. We found that the ratio of the 25-hydroxylated to 27-hydroxylated metabolites formed in these three different transfected cells systems varied. Previous studies in which human P450c27 was co-transfected into COS cells with bovine Adx (no AdRed was co-transfected) demonstrated that the 25-hydroxylated product [1 α ,25-(OH)₂D₃] was favoured when transfected cells were incubated with 1 α -OH-D₃ [13]. In this study, cells co-transfected with human P450c27, Adx and AdRed favoured the production of the 27-hydroxylated product whereas cells expressing the P450c27 fusion protein favoured 25-hydroxylation. Similar variations were observed in the ratio of 25- to 27-hydroxylated products using vitamin D₃ as substrate. We speculate that this altered ratio of 25-hydroxylated/27-hydroxylated products observed between these different transfection systems may be due to slight conformational modifications of the substrate-binding pocket of P450c27. Kinetic studies performed using the F2 P450_{scc} fusion enzyme suggested that the tertiary structure of the P450 moiety of that fusion protein was modified, leading to a 7.7-fold higher affinity (apparent K_m) of the substrate for its active site [12]. A similar modification of the substrate-binding pocket of P450c27 leading to preferential 25-hydroxylation of the substrate could be postulated for this fusion enzyme. In the case of the co-transfected cell systems, it is possible that the interaction of human P450c27 with either human or bovine Adx results in slightly different conformations of the P450 protein leading to altered preferential sites of hydroxylation.

The increased enzyme activity of the F2c27 fusion protein

provides a useful tool for metabolic studies of vitamin D analogues in transfected mammalian cell systems. The lack of regulation of the vitamin D₃ 25-hydroxylase suggests the potential to administer many vitamin D analogues as pro-drugs that become activated through 25-hydroxylation in the liver. *In vitro* studies in cell culture systems can be useful for determining which analogues may be administered as pro-drugs. Although hepatoma cell lines are very good models for studying and identifying the products of these enzymes, the large number of P450s in these cells do not allow easy identification of the specific P450 responsible for enzyme activity. We have used cells co-transfected with the three individual components of the P450c27 system expressed separately to demonstrate that the vitamin D pro-drugs MC1528 and MC1281 (Leo Pharmaceuticals) can be 25-hydroxylated to become the biologically active analogues MC1288 and MC1147 respectively [23]. The construction of a more efficient P450c27 fusion enzyme should allow us to detect activated metabolites generated from pro-drugs that might otherwise fall below our limits of detection. Similarly, generation of larger quantities of these activated pro-drugs will permit more rigorous analysis and identification of metabolites.

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