Engineering of a functional human NADH-dependent cytochrome P450 system

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A functional human NADH-dependent cytochrome P450 system has been developed by altering the cofactor preference of human NADPH cytochrome P450 reductase (CPR), the redox partner for P450s. This has been achieved by a single amino acid change of the conserved aromatic amino acid Trp-676, which covers the re-side of the FAD isoalloxazine ring in the nicotinamide-binding site. Of the mutations made, the substitution of Trp-676 with alanine (W676A) resulted in a functional NADH-dependent enzyme, which catalyzed the reduction of cytochrome *c* and ferricyanide as well as facilitated the metabolism of 7-ethoxyresorufin by CYP1A2. Kinetic analysis measuring cytochrome *c* activity revealed that the NADH-dependent k_{cat} of W676A is equivalent (90%) to the NADPH-dependent k_{cat} of the wild-type enzyme, with W676A having an approximately 1,000-fold higher specificity for NADH. The apparent K_M^{NADPH} and K_M^{NADH} values of W676A are 80- and 150-fold decreased, respectively. In accordance with structural data, which show a bipartite binding mode of NADPH, substitution of Trp-676 does not affect 2′-AMP binding as seen by the inhibition of both wild-type CPR and the W676A mutant. Furthermore, NADPH was a potent inhibitor of the W676A NADH-dependent cytochrome *c* reduction and CYP1A2 activity. Overall, the results show that Trp-676 of human CPR plays a major role in cofactor discrimination, and substitution of this conserved aromatic residue with alanine results in an efficient NADH-dependent cytochrome P450 system.

monooxygenases | NADPH-cytochrome P450 reductase | flavoproteins | nitric oxide synthase | ferredoxin reductase

NADPH cytochrome P450 reductase (CPR) (EC 1.6.2.4) is a microsomal dual flavin redox protein. Its main function is the transfer of electrons from NADPH via FAD and FMN cofactors to cytochrome P450 isoenzymes (1, 2), but it can also reduce foreign compounds such as anticancer drugs (3–5). CPR is the prototypical member (6, 7) of a growing number of mammalian dual flavin reductases belonging to the ferredoxin $NADP^+$ reductase (FNR) family (8, 9). Other members include nitric oxide synthases (10), methionine synthase reductase (11), and NR1 (12). These enzymes are multidomain proteins consisting of a FMN- and a FAD/NADPH-binding domain. The similarities between the individual dual flavin enzymes are further exemplified by their ability to reduce the same substrates (12–14). The high degree of sequence homology around the FMN- and FAD/NADPH-binding domains makes studies on one enzyme of direct relevance to other members of the family.

The mechanisms of electron flow and cofactor binding in CPR have been analyzed in several studies by chemical modification of amino acids or by site-directed mutagenesis (1, 2). Further insight has come from the x-ray structure of CPR (15) and related reductases (8, 16, 17). The binding site for NADPH in the published crystal structure of CPR is only partly resolved. The adenosine–ribose-binding region is well defined; however, the structure of the nicotinamide-binding region is unclear (15). Furthermore, it has to date not been possible to obtain a crystal structure of a wild-type member of the FNR family with NADPH bound.

Modeling studies have suggested that the conserved aromatic amino acid Trp-677 of rat CPR, which covers the re-side of the FAD isoalloxazine ring, is involved in NADPH binding. The proposed mechanism for binding involves the flipping of this aromatic residue to be replaced by the nicotinamide region of NADPH (15). On the basis of this model, we examined the effect of mutations to the C-terminal Trp-676 on catalytic activities of human CPR. The data obtained strengthen the critical role of this residue in nicotinamide binding. In addition, we report that cofactor specificity can be profoundly altered by alteration of this single residue while retaining a significant level of catalytic function.

Materials and Methods

Chemicals. Unless otherwise stated, chemicals and media were purchased from Sigma, restriction enzymes from Roche Diagnostic (Lewes, U.K.), and DNA-modifying enzymes from Promega.

Constructs. The cDNAs for human CPR and for human cytochrome P450 (CYP1A2) have been isolated as reported previously (18, 19). For expression in *Escherichia coli*, the bacterial *pelB* and *ompA* membrane leader sequences, respectively, have been fused to the cDNAs, and both modified enzymes were expressed off the single bacterial expression plasmid pB104 as previously described (20, 21). The plasmid pB104 was used as PCR template for mutagenesis of CPR by using a universal forward primer with a *Sma*I site (nucleotide 1774), and mismatching oligonucleotides (nucleotide 2005) with overhanging *Sfi*I sites as reverse primers.

To generate a soluble anchorless CPR (sCPR), exons 3 to 16 were further amplified by PCR by using 5′- and 3′-oligonucleotides with overhanging *Nde*I and *Xho*I restriction sites, respectively (18). This cDNA was ligated into the *Nde*I/*Xho*I sites of pET15b (Novagen) to generate the plasmid HPET-7. Mutants sW676A and sW676H were generated by PCR with HPET-7 as template by using the universal forward primer with a *Sma*I site (nucleotide 1774) and mismatching oligonucleotides (nucleotide 2014) with overhanging *Bam*HI sites as reverse primers.

The PCR fragments were separated on an agarose gel, isolated with a gel extraction kit (Qiagen, Chatsworth, CA) and cloned into the pGEM-T vector (Promega). They were then subcloned with *Sma*I/*Sfi*I or *Sma*I/*Bam*HI into either pB104 or HPET-7, respectively. The authenticity of all regions generated by PCR was confirmed by automated sequencing (Applied Biosystems).

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Abbreviations: CPR, cytochrome P450 reductase; sCPR, soluble anchorless CPR; FNR, $NADP^+$-ferredoxin reductase; EROD, 7-ethoxyresorufin-*O*-dealkylase.

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Expression of Recombinant Proteins in *E. coli* and Membrane Preparations. *E. coli* JM109 cells (Promega) were transformed with the respective expression vector, and bacterial membranes coexpressing CPR or CPR mutants and CYP1A2 were prepared as described (20, 21). Expression levels of CYP1A2 and CPR were determined by Western blot analysis by using polyclonal antibodies and the ECL detection kit (Amersham Pharmacia Biotech). The CYP1A2 concentration in bacterial membranes was determined spectrophotometrically (22).

Protein Purification. CPR and the mutants W676H and W676F were purified from *E. coli* membranes by using a combination of anion exchange and 2′,5′-ADP-Sepharose affinity chromatography. Bacterial membranes obtained from 1 liter of culture were solubilized in 40 ml of equilibration buffer (20 mM Tris-HCl, pH 7.7/1 mM EDTA/10% (vol/vol) glycerol/1 mM DTT/0.1 mM phenylmethylsulfonylfluoride) supplemented with 0.1% (vol/vol) Triton X-100 by shaking them for 30 min at 4°C. Insoluble fractions were precipitated by ultracentrifugation at 100,000 × *g* for 30 min at 4°C (Sorvall). The proteins were further purified essentially as described for the purification of NR-1 (12).

The soluble anchorless sCPR and the mutants sW676A and sW676H expressed in *E. coli* BLR (DE3, pLysS) cells (Novagen) were purified by Ni-agarose affinity chromatography, as described (18). The proteins were eluted from the Ni-agarose column at an imidazole concentration of 60 mM and dialyzed overnight at 4°C against 20 mM potassium phosphate buffer (pH 7.7), 20% (V/V) glycerol. To cleave the 6× His tag, purified proteins were applied to a Ni-agarose affinity column, and the column was equilibrated with 1× thrombin cleavage buffer (20 mM Tris-HCl, pH 8.4/150 mM NaCl/2.5 mM $CaCl_2$). Cleavage was performed on the column with one column volume of 0.06 units thrombin (Novagen) per milliliter in 1× thrombin cleavage buffer for 1 h at room temperature. The cleaved proteins were eluted and the column washed with one column volume of cleavage buffer.

Purified proteins were exchanged into 20 mM potassium phosphate, pH 7.7/20% (vol/vol) glycerol, by using a PD-10 gel filtration column (Amersham Pharmacia Biotech). Protein concentrations were determined by Bradford analysis (Bio-Rad), and the purity of the enzymes was greater than 90% as estimated by SDS/PAGE.

Flavin Determination and Spectral Analysis. The flavin content of the purified enzymes was determined by isocratic separation over a 25 cm Spherisorb ODS-2 5 μm column (Hewlett–Packard) by using a Hewlett–Packard 1050 HPLC with fluorescence detector (23). Absorption spectra of the purified CPR and mutant enzymes were obtained by using a Shimadzu MPS-2000 spectrophotometer (18).

Enzyme Assays. The CPR-mediated reduction of ferricyanide was measured in 50 mM potassium phosphate buffer, pH 7.7, and the reduction of cytochrome *c* in 300 mM potassium phosphate buffer, pH 7.7 (3, 18). Kinetic analyses were performed with a constant concentration of cytochrome *c* (50 μM) and varying concentrations of NADPH and NADH. Nonlinear regression analysis was used to derive K_M and k_{cat} values. CYP1A2-dependent 7-ethoxyresorufin-*O*-dealkylase (EROD) activity was measured in a 96-well plate fluorescence reader (Labsystems, Chicago) essentially as described (24). Membranes containing 5 pmol CYP1A2 were preincubated in 96-well plates in 100 mM potassium phosphate buffer, pH 7.7, containing 2 μM ethoxyresorufin for 5 min at 37°C in a total volume of 200 μl. The reaction was initiated by injection of 10 μl of 10 mM NADPH or NADH. The amount of resorufin was measured every 30 seconds at excitation and emission wavelengths of 530 and 585 nm, respectively, and values from the linear range were taken to calculate catalytic rates. To measure reconstituted benzyloxyresorufin-*O*-dealkylase activity, 5 pmol of purified rat CYP2B1 (25) was preincubated for 10 min with 5 pmol of purified CPR and 20 μg L-α-dilauroylphosphatidyl choline in a total volume of 25 μl in 100 mM potassium phosphate buffer, pH 7.7. Two micromolars 7-benzyloxyresorufin (175 μl) in 100 mM potassium phosphate, pH 7.7, was then added and the reaction started with 10 μl NADPH (10 mM).

Table 1. Effect of mutations at Trp-676 on cytochrome P450 reductase-catalyzed enzyme activities

Mutant	Activity, min^{-1}		
	Cytochrome *c**	Ferricyanide	P450 activity
E. coli membranes			
CPR	475.9 ± 194.6†	n.a.	5.33 ± 1.80‡
W676F	106.0 ± 21.9	n.a.	2.08 ± 0.32
W676H	49.8 ± 21.6	n.a.	1.94 ± 0.3
W676A	6.0 ± 2.3	n.a.	0.06 ± 0.02
W676S;S677W	6.0 ± 2.3	n.a.	0.11 ± 0.02
V675W;W676V	8.6 ± 7.5	n.a.	0.45 ± 0.09
Purified enzymes			
CPR	2,880 ± 210§	5,080 ± 820§	8.26 ± 1.23¶
W676F	470 ± 40	2,340 ± 240	5.54 ± 0.26
W676H	610 ± 90	2,760 ± 190	2.48 ± 0.19

n.a., not analyzed.

**E. coli* membranes, nmol/min/mg.

†Reactions were carried out with 50 μM cytochrome *c* with 10 μg *E. coli* membranes coexpressing CYP1A2 and CPR. Reactions were initiated with NADPH (100 μM). Means ± SD of at least three independent experiments are given.

‡7-Ethoxyresorufin-*O*-dealkylation activity was measured with 5 pmol CYP1A2 coexpressed with CPR in *E. coli* by using 7-ethoxyresorufin (2 μM). The reaction was initiated with NADPH (500 μM). Means ± SD of at least three independent experiments are given.

§Reduction of cytochrome *c* was measured with 5 pmol enzyme as described above. The reduction of ferricyanide was measured with 5 pmol enzyme with 500 μM ferricyanide in the presence of NADPH (100 μM). Values shown are mean ± SD of triplicates determined.

¶The reconstitution of CYP2B1-catalysed 7-benzyloxyresorufin-*O*-dealkylase activity was measured with 5 pmol of CYP2B1 and 5 pmol of CPR by using 2 μM substrate as described for the EROD activity. Values represent mean ± SD of triplicate measurements.

Results

Enzymatic Activities of Trp-676 Mutants in *E. coli* Membranes and Purified Enzymes. Membranes isolated from *E. coli* coexpressing CPR and CYP1A2 in a one-plasmid system were used initially to assess the effects of mutations to Trp-676. Levels of CYP1A2 expression ranged between 300 and 800 pmol/mg and wild-type cytochrome *c* reductase activity between 200 and 600 nmol/min/mg of *E. coli* membranes. Western blot analysis of *E. coli* membranes expressing mutant reductase enzymes revealed levels of expression of mutant enzymes and CYP1A2 to be very similar to the wild type (data not shown).

The effect of the mutations on cytochrome *c* reductase and EROD activity in bacterial membranes is shown in Table 1. EROD turnover in membranes expressing wild-type CPR was 5.33 ± 1.8 min^{-1}. All mutants exhibited a reduction in enzyme activity. The most profound effects were observed with the W676A mutant and the mutants where the residues W676S:S677W and V675W:W676V were interchanged. In these cases, cytochrome *c* and EROD activity was almost completely lost (<10%). Mutants with planar (W676H) or aromatic (W676F) substitutions retained EROD activities; however, these were reduced to 36 and 39% of wild-type CPR, respectively.

Table 2. Effects of mutations to Trp-676 of cytochrome P450 reductase on cofactor preference

	Activity, min^{-1}			
	NADPH		NADH	
Mutant	Cytochrome *c*	Ferricyanide	Cytochrome *c*	Ferricyanide
sCPR	1,890 ± 50	2,770 ± 130	10 ± 1	10 ± 6
sW676H	410 ± 30	1,430 ± 80	90 ± 10	160 ± 1
sW676A	20 ± 2	30 ± 10	490 ± 40	780 ± 110

The reduction of cytochrome c and ferricyanide were measured with 5 pmol enzyme as described in the legend to Table 1, with final cofactor concentrations of 100 μM. Values represent mean ± SD of triplicate determinations.

The mutant enzymes W676H and W676F were further characterized after purification from *E. coli* membranes by anion exchange and 2′,5′-ADP-Sepharose affinity chromatography. Analysis of flavin content in the wild-type and mutant enzymes revealed a normal 1:1 ratio of FMN to FAD, indicating no effects on flavin cofactor binding (data not shown). Compared with wild-type CPR, the purified mutants W676H and W676F retained 21 and 16% of cytochrome *c* activity, respectively (Table 1). They also showed reduced activity in a reconstituted system by using rat CYP2B1 as electron acceptor, with W676F exhibiting 67% and W676H exhibiting 30% of the CYP2B1 activity observed with wild-type CPR (Table 1). The effects of these mutations on enzyme activity were also measured by using potassium ferricyanide as electron acceptor, which is reduced directly from the FAD cofactor (26). With both W676H and W676F, a reduction in ferricyanide activity of approximately 50% was observed (Table 1).

Attempts to purify the catalytically inactive mutant W676A by using 2′,5′-ADP-Sepharose affinity chromatography were unsuccessful because of a failure of the mutant enzyme to bind to the affinity matrix. This, coupled with the reduction in ferricyanide activity of the W676H and W676F mutants, was a strong indication of alterations in NADPH-binding characteristics.

W676A Changes Cofactor Specificity. To investigate the biochemical and physicochemical properties of W676A and W676H, we constructed N-terminal His-tagged mutants to facilitate protein purification by Nickel agarose chromatography. The truncated enzymes (residues 61–677) lacked the N-terminal membrane anchor sequence essential for CYP coupling. However, all other enzyme characteristics, including cytochrome *c* reductase activity and a FMN:FAD ratio of approximately 1:1 (data not shown), were retained after cleavage of the His-tag.

The specific activities of sCPR and the mutants sW676A and sW676H are summarized in Table 2. In sW676H and sW676A, NADPH-dependent cytochrome *c* reduction was reduced by 78 and 99% and ferricyanide activity by 48 and 99%, respectively. These results are consistent with the data obtained with *E. coli* membranes (Table 1) and confirmed the dramatic loss of reductase activity associated with the W676A substitution.

In view of the probable alterations in NADPH binding, we also tested NADH as electron donor (Table 2). As anticipated, wild-type sCPR exhibited only marginal NADH-dependent activities toward cytochrome *c* and ferricyanide. However, with sW676A, a 50- and 78-fold increase in cytochrome *c* and ferricyanide activity, respectively, was observed. The sW676H mutant also used NADH as cofactor, but the change in activity was less marked. These results indicate that Trp-676 plays a critical role in determining cofactor specificity.

To investigate the change in cofactor specificity in more detail, kinetic analysis was carried out with cytochrome *c* as an electron acceptor (Table 3). The k_{cat} for sW676A measured with NADH as hydride donor was 3,748 min^{-1}, which was approximately 10% lower than the k_{cat} for sCPR (4,082 min^{-1}) measured with NADPH. The apparent K_M^{NADPH} and K_M^{NADH} values for sW676A were markedly changed, with an approximately 80- and 150-fold decrease compared with the wild-type enzyme, respectively. Comparison of k_{cat}/K_M revealed an approximately 1,000-fold preference for NADH of sW676A compared with sCPR (Table 3). sW676H also exhibited lower apparent K_M^{NADPH} and K_M^{NADH} values in comparison with wild-type CPR. sW676H had an NADPH-dependent k_{cat} of 453 min^{-1} and an NADH-dependent k_{cat} of 1,897 min^{-1}. The comparison of k_{cat}/K_M revealed an approximately 10-fold preference for NADH by sW676H compared with the wild-type enzymes (Table 3).

Spectral Analyses. The visible absorption spectra of oxidized sCPR, sW676H, and sW676A are shown in Fig. 1. The sW676H enzyme was similar to wild-type sCPR, with λ_{max} at 375 and 455 nm. However, sW676A produced a slight red shift in the 455 nm peak to 472 nm. The peak at 380 nm also became broader, with a shoulder at 395 nm (Fig. 1*C*).

The reduction of the mutant enzymes to the air-stable blue semiquinone with a 5-fold molar excess of NADPH under aerobic conditions produced spectral changes similar to wild-type CPR, i.e., a decrease in λ_{max} absorption and an increase in absorption at 585 nm (Fig. 1 *A–C*). Interestingly, the oxidized spectra of all three proteins could also be reduced with NADH (Fig. 1 *D–F*). The peak in sCPR at 365 nm (Fig. 1*D*) is probably because of reduced NADH. The formation and stability of the spectrum after cofactor addition under aerobic conditions was followed at 585 nm, which is indicative for the semiquinone (1, 3, 25). The semiquinone spectra of both sCPR and sW676A were stable for up to 16 h after NADPH reduction and for up to 2 h after NADH reduction (Fig. 1). In contrast, the sW676H semiquinone was unstable and reoxidized greater than 50% within one hour after the addition of either NADPH or NADH (Fig. 1).

Inhibition Studies. To gain further information on the changes in the NADPH-binding site, we investigated the effects of the competitive inhibitor 2′-AMP (27) on cytochrome *c* reduction by using NADPH and NADH as cofactors. 2′-AMP inhibited both

Table 3. Kinetic analysis of cytochrome P450 reductase, and the sW676A and sW6776H mutants

	NADPH			NADH			
Mutant	K_M, μM	k_{cat}, min^{-1}	k_{cat}/K_M(A), $\mu M^{-1}\cdot min^{-1}$	K_M, μM	k_{cat}, min^{-1}	k_{cat}/K_M(B), $\mu M^{-1}\cdot min^{-1}$	NADPH/NADH A/B
sCPR	15.6 ± 7.1	4,082 ± 574	261.7	47,590.0 ± 12,570.0	2,677 ± 191	0.06	4362
sW676H	1.2 ± 0.3	453 ± 27	377.5	2,673.0 ± 644.7	1,897 ± 100	0.7	539
sW676A	0.2 ± 0.06	10 ± 0.5	50	318.2 ± 66.9	3,748 ± 159	11.8	4

Cytochrome c reduction has been measured with 5 pmol enzyme, with the exception of K_M^{NADPH} for sW677A, where 25 pmol was used. Kinetic analysis was performed with 50 μM cytochrome *c* and various concentrations of NADPH and NADH, as described in the legend to Table 1.

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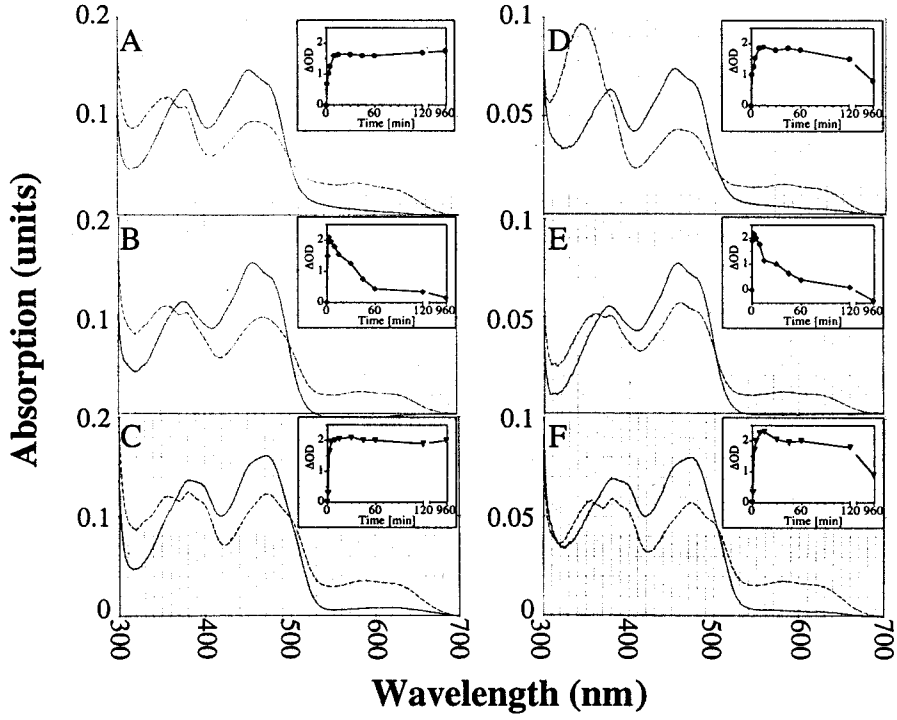

Fig. 1. Visible absorption spectra of cytochrome P450 reductase and mutants. Semiquinone spectra of sCPR (*A*, *D*), sW676H (*B*, *E*) and sW676A (*C*, *F*) were obtained after the addition of a 5-fold molar excess of NADPH (*A–C*) or NADH (*D–F*) under aerobic conditions, and recorded after equilibration has been reached. The reoxidation of the semiquinone (*Insets*) was followed at 585 nm for 16 h (solid lines: oxidized spectra; broken lines: reduced spectra).

wild-type and sW676A enzymes, with apparent IC_{50} values of 278 μM for the sCPR-mediated NADPH-dependent, and 453 μM for the sW676A-mediated NADH-dependent cytochrome *c* reduction (Fig. 2). These similar values indicate that 2′-AMP binding seems unaffected by the alanine replacement. However, NADPH was found to be a potent inhibitor of the sW676A-catalyzed NADH-dependent cytochrome *c* reduction (IC_{50} = 0.029 μM). In contrast, NADH had no effect on sCPR-catalyzed NADPH-dependent cytochrome *c* reduction (Fig. 2). These data correspond to the kinetic data, which showed a K_M of sW676A for NADPH in the nanomolar range, whereas the K_M of sCPR for NADH was in the millimolar range.

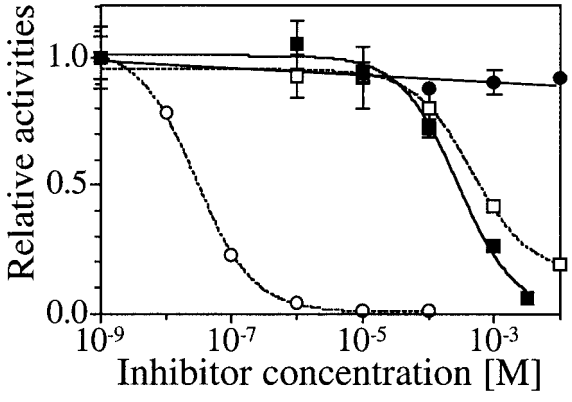

Fig. 2. Inhibition of NADPH- or NADH-dependent cytochrome P450 reductase activities by nucleotide analogues. Cytochrome *c* reduction was measured as described in the legend to Table 1 by using CPR with NADPH (15 μM, filled symbols) or W676A with NADH (300 μM, open symbols). The concentrations of NADPH and NADH corresponded to the respective apparent K_M values (Table 3). Inhibition studies were performed with 2′-AMP (1 μM–0 mM, squares), NADH (100 μM–10 mM, filled circle) and NADPH (10 nM–100 μM, open circle).

NADH as Cofactor for Cytochrome P450-Dependent Monooxygenase Activity. The above experiments were carried out with the soluble form of CPR, which does not couple with P450. To determine whether the change in cofactor specificity of the mutants could produce a functional NADH-dependent monooxygenase system, we measured EROD activities in *E. coli* membranes coexpressing CYP1A2 (Table 4). The NADH-dependent EROD activity of W676A was approximately 5-fold higher than in membranes coexpressing wild-type CPR or W676H (Table 4). This contrasts with the NADPH-dependent rates, which were 1% of the wild-type CPR (Table 1). It is notable that significant levels of NADH-dependent EROD activity are present in the wild-type CPR (Table 4), which are 10% of the activity with NADPH (Table 1). Under the conditions used, the NADH-dependent rate of the mutant W676A was approximately 50% of the wild-type enzyme with NADPH. Thus, the mutation of Trp-676 to alanine results in a functional NADH-dependent cytochrome P450 system.

The inhibitory effect of NADPH on NADH-mediated monooxygenase activity was also tested by using the CYP1A2 coexpression system. EROD activity was initiated with NADH (500 μM) and allowed to proceed for 5 min before the addition of NADPH (50 μM). The reactions were allowed to continue for an additional 5 min (Fig. 3). The addition of NADPH markedly

Table 4. CYP1A2-mediated monooxygenase activity using NADH as cofactor

Mutant	EROD*, min^{-1}
CPR	0.52 ± 0.02
W676H	0.45 ± 0.02
W676A	2.64 ± 0.05

*EROD activities were measured in *E. coli* membranes, as described in the legend to Table 1, with NADH (500 μM).

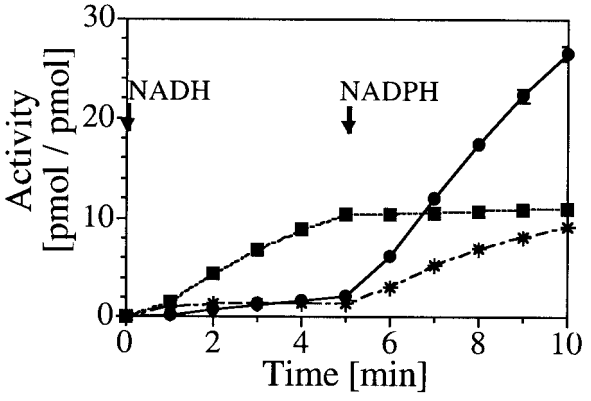

Fig. 3. Effect of NADPH on NADH-dependent 7-ethoxyresorufin metabolism. NADPH- and NADH-dependent EROD activities were measured with CYP1A2 (5 pmol) in *E. coli* membranes coexpressing either CPR or the mutants W676H and W676A, as described in the legend to Table 1. Reactions were started with NADH (500 μM). After 5 min, NADPH (50 μM) was added, and the reaction recorded for additional 5 min (CPR, circle; W676A, squares; W676H, asterix).

inhibited the rate of NADH-dependent EROD turnover in W676A from 2.64 ± 0.05 min^{-1} to 0.19 ± 0.2 min^{-1}. In contrast, enzyme activities of CPR and W677H were increased on NADPH addition to 5.37 ± 0.12 min^{-1} and 1.61 ± 0.06 min^{-1}, respectively. These values are essentially the same as the activities measured with NADPH alone (Table 1).

Discussion

The structural requirements for NADPH binding to cytochrome P450 reductase have been elusive, as the nicotinamide-binding site is unclear in the crystal structure (15). In this study, several mutations to Trp-676 of human CPR, which by homology with FNR seems to be involved in NADPH binding, have been made. Catalytic activities of CPR were significantly reduced by the replacement of Trp-676 with either an aromatic phenylalanine (W676F) or a planar charged histidine (W676H). The retention of activity for the W676H mutant was surprising, as there seems to be a general requirement in the FNR family for an aromatic residue covering the re-face of the FAD isoalloxazine ring. The W676H mutant could use both NADPH and NADH as cofactors with higher affinities than the wild-type enzyme. However, the catalytic activities toward cytochrome *c* reduction and EROD activity were about 5-fold higher with NADPH. The pH dependency of the NADPH-dependent cytochrome *c* reduction in W676H was not altered compared with wild-type CPR (data not shown), indicating that charge distribution around the isoalloxazine-binding site is not important in NADPH binding. These data indicate that Trp-676 plays predominantly a steric role in NADPH binding. Interestingly, the spectroscopic analysis showed that the semiquinone in W676H, unlike CPR, is unstable and rapidly reoxidized. The reason for this is unclear, but in view of the charged nature of the histidine residue, it may be related to differences in the redox potential.

Positional swapping of Trp-676 with the residues on either side significantly reduced both cytochrome *c* and P450 reductase activity, as did the substitution with Ala (W676A). The latter mutation was of most interest because it caused a change in cofactor preference for NADH with profound 50- and 78-fold increases in cytochrome *c* and ferricyanide reductase activities, respectively. Importantly, the k_{cat} of W676A by using NADH maintained approximately 90% of the turnover of the NADPH-activated wild-type CPR. Thus, the mutant enzyme is as efficient a catalyst as the wild-type enzyme at saturating concentrations of NADH.

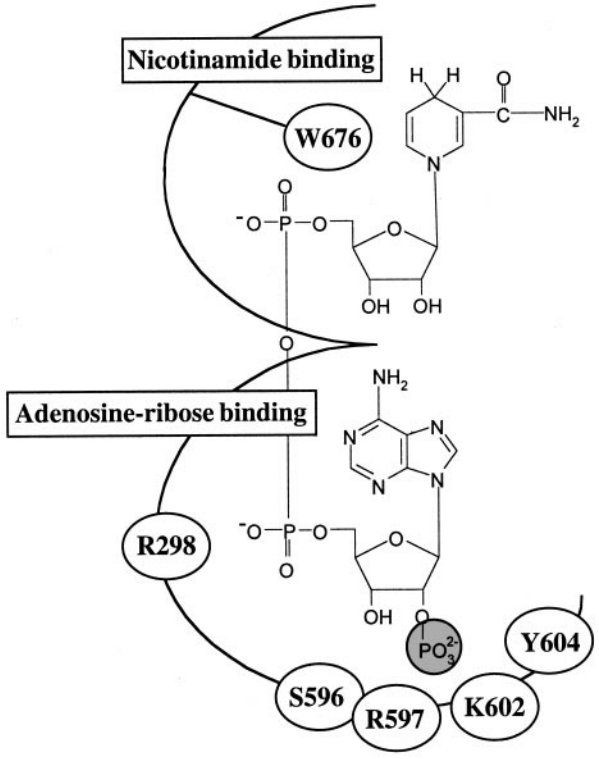

Fig. 4. Schematic model of the bipartite NADPH-binding mode. The model is based on our data and those of others (15, 28–30). The 2′-phosphate of NADPH is circled.

The contrasting size and electrostatic properties of the Trp-676 substitutions do not appear to cause significant changes to the overall structure of CPR as judged by flavin binding, mobility in SDS gels, or coupling with P450. However, the spectral analysis of W676A revealed a red shift in the visible spectrum, which could be caused by a change in the flavin electron environment possibly because of the loss of the shielding effect by the tryptophane. A similar spectral shift was observed in an analogous mutation (Y308S) of pea FNR, which has been attributed to the copurification of bound $NADP^+$ (28). However, inability of the Y308S spectrum to be perturbed on cofactor addition because of the presence of endogenous $NADP^+$ is in contrast to our results where NADPH- or NADH-dependent reduction of the W676A mutant was still observed. It is notable that the W676A mutant could not be purified by 2′,5′-ADP-Sepharose affinity chromatography, although 2′-AMP-binding seems not to be affected. This might be because of an effect on the 5′-phosphate-binding site, which is proximal to the mutation made.

The change in cofactor preference could be explained by a bipartite binding mode for NADPH, with both moieties, adenosine–ribose and nicotinamide, binding separately. The schematic model shown in Fig. 4 can be used to rationalize our findings and others (15, 28–30). The conserved aromatic residue Trp-676, which is placed in the nicotinamide-binding site and covers the isoalloxazine ring of the FAD cofactor, has been proposed to flip to facilitate nicotinamide binding and thus hydride transfer. With the loss of the side chain in the W676A mutant or the analogous Y308S mutant of pea FNR, this thermodynamically unfavored flipping is no longer required (28, 29). Hence, these enzymes have lower apparent K_M values for both nicotinamide cofactors because of the removal of this physical potentially rate-limiting step. In addition, the first defined structure of the nicotinamide-binding site of a member

of the FNR family with nicrotinamide bound could be resolved only in the Y308S mutant of pea FNR in which the substitution of Tyr-308 stabilized the enzyme/$NADP^+$ complex for x-ray crystallography (28). Several amino acids involved in binding of the adenosine-ribose moiety of NADPH have been proposed in CPR (Fig. 4), and specific charge interactions between these residues and the 2′-phosphate of NADPH appear responsible for discrimination against NADH (15, 30, 31). In accordance with the structural data, substitution of Trp-676 does not affect 2′-AMP binding as seen by the potent inhibition of both wild-type CPR and W676A by this compound. As nicotinamide binding is unfavorable in the wild-type enzyme, because of the side chain rearrangement of Trp-676, binding of the adenosine–ribose moiety through the specific charge interactions may be expected to be the first step in NADPH binding. In the W676A mutant, the side chain rearrangement of Trp-676 is not required, and both binding sites would be immediately accessible. Importantly, as the adenosine–ribose-binding site has not been altered in W676A, the electrostatic interaction does not seem to be sufficient to discriminate against NADH. In fact, both nicotinamide cofactors bind with higher affinities in the absence of Trp-676.

The W676A isoform is able to bind NADPH, and hydride transfer to FAD occurs; however, NADPH-dependent catalytic functions are impaired. Furthermore, NADPH is a strong inhibitor of the NADH-dependent enzyme activities of W676A. These observations could be explained by the high affinity of W676A for NADPH because of the specific 2′-phosphate binding. The release of the oxidized cofactor might then become a rate-limiting step for CPR function, as has been proposed for pea FNR (29).

Cytochrome P450s play a central role in the metabolism of drugs and xenobiotics as well as endogenous compounds such as steroid hormones. The specificity of P450s in regio- and stereospecific hydroxylation reactions also makes them important biocatalysts. Technological applications for P450s are therefore not only in preclinical drug development and toxicology (19, 32, 33) but also in areas ranging from gene therapy to the selective synthesis of pharmaceuticals and chemicals (34–36). However, a current limitation for the technical use of P450s is the requirement of relatively expensive reducing equivalents in form of NADPH. Several attempts have been made to develop cheaper electron sources for P450s (34, 37, 38). The ability of W676A to efficiently use NADH in the reduction of one-electron acceptors suggests it could be used as a cost-efficient alternative redox partner for P450 reactions. We have tested this concept through the coexpression of W676A with CYP1A2 in *E. coli*. The turnover of 7-ethoxyresorufin was 2.64 min^{-1} with NADH as cofactor by the W676A mutant, which compares favorably with the turnover of 5.33 min^{-1} by the wild-type enzyme by using NADPH. However, because NADPH is a strong inhibitor of W676A, the NADH-dependent monooxygenase system requires situations of low NADPH concentrations for optimal activity.

In conclusion, we have shown that the substitution of Trp-676 of human CPR with alanine results in an enzyme with an approximately 1,000-fold higher specificity for NADH than the wild-type enzyme. The experimental data establish an essential role of Trp-676 in NADPH cofactor binding and discrimination against NADH. The mutant enzyme is capable of providing electrons to cytochrome P450, indicating that substitution of Trp-676 with alanine results in a functional NADH-dependent monooxygenase system.

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