Role of Active Site Water Molecules and Substrate Hydroxyl Groups in Oxygen Activation by Cytochrome P450 158A2

A NEW MECHANISM OF PROTON TRANSFER

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Cytochrome P450 (P450 or CYP) enzymes are ubiquitous heme-based monooxygenases in nature and are involved in the biosynthesis of physiologically important compounds as well as in drug and other xenobiotic metabolism (1–3). Some microbial P450s are associated with polyketide biosynthetic gene clusters where they catalyze late-stage stereo- and regiospecific oxidations (4–7). For example, in Streptomyces coelicolor A3 (2) CYP158A2 is located in a three-gene operon, sco1206–sco1207, that also contains a type III polyketide synthase 1,3,6,8-tetra-hydroxynaphthalene synthase (8, 9) and a novel quinone-forming monooxygenase (momA) (10). This type III PKS catalyzes the sequential coupling of five molecules of malonyl-CoA to form 1,3,6,8-tetrahydroxynaphthalene, which is oxidized to flavilin by momA. CYP158A2 then catalyzes an unusual phenolic oxidative C-C coupling reaction, in which two or three molecules of flavilin are polymerized to biflavilin or trilavilin (7), red-brown pigments, which may afford physical protection to this soil bacterium, possibly against the deleterious effects of UV light radiation (11). During the general P450 catalytic cycle (1), dioxygen binding, protonation, and splitting of the oxygen–oxygen bond with generation of a peroxidyl iron complex (FeO03-) are critical steps for product formation. The most detailed understanding of the proton transfer systems in P450s is from the investigations with CYP101A1 (P450cam) (12–17) and CYP107A1 (P450eryF) (18–20). CYP101A1 contains a hydroxyl group at the position homologous to the distal threonine in the central I helix. This highly conserved threonine residue in a large number of the more than 5000 known P450s is proposed to be involved in stabilizing the oxy complex or hydroperoxy intermediate (17). In CYP101A1, it stabilizes the dioxygen complex by hydrogen bonding to the dioxygen itself (15) and a water molecule within hydrogen-bonding distance from the dioxygen (16). Proton transfer is thought to originate from the water molecule (15, 16). However, CYP101A1 does not contain this conserved threonine but instead contains an Ala245 (18). The recent crystal structure of the ferrous dioxygen complex of CYP101A1 suggests that the 5-hydroxyl group of the substrate 6-deoxyerythronolide B can directly donate a hydrogen bond to the iron-linked dioxygen for the proton transfer (19).

We reported the crystal structure of CYP158A2 containing two substrate molecules of flavilin in the active site (7). This crystal structure complex suggested that the flavilin 2-OH might be responsible for anchoring substrate to the enzyme and the 5-OH and/or 7-OH may stabilize catalytically important water molecules. Three water molecules (WAT505, WAT600, and WAT640) in the vicinity of the two flavilin molecules may have both a structural as well as a functional role in oxygen binding and/or proton transfer for oxygen activation. Interestingly, in CYP158A2 the residue position of the conserved threonine in the I-helix is also occupied by Ala245 as observed in the CYP107A1 (18). In CYP107A1 the postulated crucial catalytic water molecule (WAT519) present in the ferric enzyme is absent in the ferrous dioxygen-bound CYP158A2 complex (2, 5). Catalytic activity toward 2-hydroxy-1,4-naphthoquinone was 70-fold less than with flavilin.

From the x-ray crystal structure of CYP158A2 (Zhao, B., Guengerich, F. P., Bellamine, A., Lamb, D. C., Izumikawa, M., Lei, L., Podust, L. M., Sundaramoorthy, M., Reddy, L. M., Kelly, S. L., Kalaitzis, J. A., Stec, D., Voehler, M., Falck, J. R., Moore, B. S., Shimada, T., and Waterman, M. R. (2005) J. Biol. Chem. 280, 11599–11607), one of 18 cytochrome P450 (CYP) genes in the actinomyecete Streptomyces coelicolor, ordered active site water molecules (WAT505, WAT600, and WAT640), and hydroxyl groups of the substrate flavilin were proposed to participate in proton transfer and oxygen cleavage in this monooxygenase. To probe their roles in catalysis, we have studied the crystal structures of a substrate analogue (2-hydroxy-1,4-naphthoquinone) complex with ferric CYP158A2 (2.15 Å) and the flavilin ferrous dioxygen-bound CYP158A2 complex (1.8 Å). Catalytic activity toward 2-hydroxy-1,4-naphthoquinone was ~70-fold less than with flavilin. In the ferrous dioxygen-bound flavilin complex, the three water molecules in the ferric flavilin complex still occupy the same positions and form hydrogen bonds to the distal dioxygen atom. These findings suggest that CYP158A2 utilizes substrate hydroxyl groups to stabilize active site water and further assist in the iron-linked dioxygen activation. A continuous hydrogen-bonded water network connecting the active site to the protein surface (bulk solvent) not present in the other two ferrous dioxygen-bound P450 structures (CYP101A1/P450cam and CYP107A1/P450eryF) is proposed to participate in the proton-delivery cascade, leading to dioxygen bond scission. This ferrous-dioxygen structure suggests two classes of P450s based on the pathway of proton transfer, one using the highly conserved threonine in the I-helix (CYP101A1) and the other requiring hydroxyl groups of the substrate molecules either directly transferring protons (CYP107A1) or stabilizing a water pathway for proton transfer (CYP158A2).

The on-line version of this article (available at http://www.jbc.org) contains supplementary material.

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The abbreviations used are: P450 or CYP, cytochrome P450 monooxygenase; 2-OH NQ, 2-hydroxy-1,4-naphthoquinone; HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond coherence; KSI, kinetic solvent isotope effect.

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EXPERIMENTAL PROCEDURES

Protein Purification and Assays—CYP158A2 and Escherichia coli flavodoxin and flavodoxin reductase were expressed and purified as reported earlier (7, 21). 2-OH NQ was purchased from Sigma. Binding and catalytic activity assays and NMR spectral analysis with 2-OH NQ were carried out as described previously with flaviolin (7). Absorbance spectra were recorded using a double-beam Shimadzu UV-2401PC spectrophotometer or a modified Cary 14/OLIS instrument (OLIS, Bogart, GA). CYP158A2 (5 μM) in 20 mM Tris- HCl was divided between two tandem cuvettes. After thermal equilibration at 25 °C a baseline was established between 350 and 450 nm, and sequential additions (2 μl) of 0.1 M NQ (150 nmol). The reaction was started by the addition of NADPH to the sample cuvette to give a final ligand concentration in the range of 30–180 μM. An equal volume of methanol was added to the reference cuvette, and the difference spectrum was recorded after each titration. For activity assays, CYP158A2 (3 nmol), flavodoxin (20 nmol), and flavodoxin reductase (10 nmol) were reconstituted in 400 μl of methanol and 20 μl of concentrated 2-OH NQ (15 μM) dissolved in methanol were added to the sample cuvette to give a final ligand concentration in the range of 30–180 μM. An equal volume of methanol was added to the reference cuvette, and the difference spectrum was recorded after each titration.

Kinetic Solvent Isotope Assay—Buffer solutions were prepared by lyophilizing Tris-HCl buffers and then dissolveing the dry residue in D2O or H2O. Reaction mixtures contained CYP158A2 (1 nmol), flavodoxin (20 nmol), and flavodoxin reductase (10 nmol) and flavavin (250 nmol) in a total volume of 400 μl of freshly prepared various H2O/D2O mixtures of 20 mM Tris-HCl buffer (pH 7.5) containing 10% (v/v) glycerol. The turnover rate constants for flavavin oxidation, and the rates of formation of each product were analyzed using a nonlinear regression analysis described by Vidakovic et al. (24). The pD of the buffer was determined using the equation pH = pD + 0.4 (25, 26). The pH effect control incubations with 20 mM Tris-HCl buffer (pH 7.1) in H2O alone were carried out as above.

Crystallization—Crystals of CYP158A2 were grown by the hanging drop vapor diffusion method as described previously (7), using 20–28% (w/v) of polyethylene glycol 3350, 0.1 M bis-Tris (pH 6.5) as the mother liquor, and 1 mM flavavin. At 20 °C, the ferric flavavin-bound crystals appeared within a few days; for 2-OH NQ crystals, the substrate flavavin was replaced in the protein solution by 2 mM 2-OH NQ.

Preparation of the Ferrous Dioxygen-bound Complex—The procedure for preparation of the ferrous dioxygen-bound complex was described by Nagano et al. (19). CYP158A2 ferric flavavin-bound crystals were transferred into cryobuffer (50 mM bis-Tris (pH 6.5), 1 M flavavin, 25% polyethylene glycol 3350, and 20% glycerol, v/v), washed for 5 min, and then reduced with 1–5 mM sodium dithionite under anaerobic conditions (N2) in a glove box (Fisher, Vernon Hills, IL). The crystals were kept in the dithionite solution for another 5 min. After reduction, crystals were washed thoroughly by transfer sequentially into three fresh drops in deoxygenated cryobuffer for 10 min in the glove box. The reduced crystals were transferred to the oxygen saturated cryobuffer (atmospheric conditions) at −5 to −10 °C for 10 min. The crystals were cooled to liquid nitrogen temperature for x-ray analysis.

Data Collection and Structure Determination—All diffraction data were collected at 100 K at the Southeast Regional Collaborative Access Team (SER-CAT) 22-BM beamline at the Advanced Photon Source, Argonne National Laboratory, Argonne, IL. The x-ray data were processed and scaled with the HKL package programs HKL2000 (27). The ferric 2-OH NQ-bound crystals belong to the orthorhombic space group P212121 with unit cell parameters a = 4}

strate flavavin analogue lacking 5- and 7-hydroxyl groups (2-hydroxy-1,4-naphthoquinone (2-OH NQ)) and solved the ferric crystal structure of a CYP158A2-2-OH NQ complex at 2.15 Å. We measured the kinetics of formation and decay of the CYP158A2 ferrous dioxygen complex in the presence of flavavin as well as 2-OH NQ and subsequently obtained the structure of the ferrous dioxygen-bound complex with flavavin at 1.8 Å. The crystal structures of the different CYP158A2 complexes, combined with biochemical data, indicate that substrate hydroxyl groups stabilize a well ordered water chain from the active site to the protein surface providing a novel path for proton transfer and dioxygen scission.

Spectral and Kinetic Analyses of CYP158A2 Ferrous Dioxygen Complex—CYP158A2 (6.7–9.5 μM) was mixed with 50 mM potassium phosphate buffer (pH 7.4), 100 mM Tris-HCl (pH 7.7), 10 mM EDTA, 1.0 μM 5-deazaflavin, and either 25 μM flavavin or 375 μM 2-OH NQ, in a final volume of 7.0 ml in an all-glass tonometer, fitted with a sidearm attached to a cuvette for optical measurements. The contents were deaerated by >12 cycles of mild vacuum/argon using a manifold attached to a gas train, with prepurified argon passed through two Oxy-Trap devices attached in tandem (Alltech, Deerfield, IL).

Spectra of the ferric complexes were recorded in the cuvette, and the P450 (in the body of the tonometer) was reduced with sequential 2-min exposures (4–5) to a visible lamp until reduction was complete, as judged by the spectral changes (Cary 14-OLIS). When reduction was complete, the contents of the tonometer were introduced into one of the drive syringes of an OLIS RSM-1000 stopped-flow spectrophotometer, which had been scrubbed with Na2S2O4 (overnight), subsequently with anaerobic buffer containing a reduced safranine T/methyl viologen mixture, and finally with anaerobic 50 mM potassium phosphate buffer (22, 23).

The other syringe contained an aerated solution of the same substrate in 50 mM potassium phosphate buffer (pH 7.4). Equal amounts of the contents of the two syringes were mixed (23 °C), and the reaction was observed in the rapid monochromator-scanning mode (either 62 or 1000 scans s−1, depending on the reaction time). The absorbance spectra were used to select individual wavelengths for kinetic analysis, i.e. fitting to pseudo-first order kinetic plots for the formation and decay of the FeO2− complex.

Kinetic Solvent Isotope Assay—Buffer solutions were prepared by lyophilizing Tris-HCl buffers and then dissolving the dry residue in D2O or H2O. Reaction mixtures contained CYP158A2 (1 nmol), flavodoxin (20 nmol), and flavodoxin reductase (10 nmol) and flavavin (260 nmol) in a total volume of 400 μl of freshly prepared various H2O/D2O mixtures of 20 mM Tris-HCl buffer (pH 7.5) containing 10% (v/v) glycerol. The turnover rate constants for flavavin oxidation, and the rates of formation of each product were analyzed using a nonlinear regression analysis described by Vidakovic et al. (24). The pD of the buffer was determined using the equation pH = pD + 0.4 (25, 26). The pH effect control incubations with 20 mM Tris-HCl buffer (pH 7.1) in H2O alone were carried out as above.

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55.8 Å, \( b = 69.24 \) Å, and \( c = 103.74 \) Å. The structure was solved by molecular replacement using the program CNS1.1 (28) and the CYP158A2 flavin complex structure (Protein Data Bank code 1T93) as a search model. The initial model was built in O (29), and refinement was performed using CNS1.1 (28), and CNS parameter and topology files were generated by PRODRG (30). For the ferrous dioxygen-bound complex of CYP158A2 with flavin, crystals also belong to the orthorhombic space group \( P2_12_12_1 \) with unit cell parameters \( a = 55.0 \) Å, \( b = 70.94 \) Å, and \( c = 102.92 \) Å. The ferric flavin-bound complex (Protein Data Bank code 1T93) as an initial model was subjected to rigid body refinement followed by B-factor refinement and energy minimization using CNS1.1 (28). After this stage, the position and orientation of dioxygen was guided by both \( 2F_o - F_c \) and \( F_o - F_c \) electron density maps. The dioxygen ligand was fit to the electron density using O (29). The Fe–O bond was

### Table One: Data Collection and Refinement Statistics

|                        | Ferric 2-OH NQ-bound complex | Flavin ferrous dioxygen-bound complex |
|------------------------|-------------------------------|--------------------------------------|
| **Data collection statistics** |                               |                                      |
| Space group            | \( P2_12_12_1 \)               | \( P2_12_12_1 \)                      |
| Wavelength (Å)         | 1.035                         | 0.95                                 |
| Total observations     | 131,906                       | 194,245                              |
| Unique reflections     | 21,594                        | 37,024                               |
| Completeness (%)       | 96.1 (94)                     | 97.1 (89)                            |
| \( I/\sigma(I) \)       | 21.3 (1.9)                    | 18.3 (2.6)                           |
| \( R_{merge} \) (%)    | 10.0 (72)                     | 8.5 (35)                             |
| **Refinement statistics** |                               |                                      |
| Resolution range (Å)   | 10–2.15                       | 15–1.8                               |
| No. of reflections used in refinement | 20,339                        | 31,890                               |
| No. of water molecules | 185                           | 258                                  |
| Protein atoms          | 3,133                         | 3,104                                |
| Heme atoms             | 43                            | 43                                   |
| Ligand atoms           | 26\(^\text{a}\)               | 30\(^\text{a}/2\)\(^\text{a}\)        |
| \( R_{work}/R_{free} \) (%) | 22.6/28.1                    | 23.2/23.9                            |
| Root mean square deviation in bond lengths (Å) | 0.006                         | 0.007                                |
| Root mean square deviation in bond angles (°) | 1.4                           | 1.2                                  |

\(^{a}\) Values for the highest resolution shell in parentheses.

\(^{b}\) Two 2-OH NQ molecules.

\(^{c}\) Two flavin molecules.

\(^{d}\) Dioxygen atoms.

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**FIGURE 1. 2-OH NQ binding to CYP158A2.** CYP158A2 was present at 5 \( \mu \)M; see “Experimental Procedures” for details. Additions included 30–180 \( \mu \)M of 2-OH NQ. The \( K_d \) value was estimated to be 43 \( \mu \)M. The structures of 2-OH NQ and flavin are shown.

**FIGURE 2. Oxidation of 2-OH NQ by CYP158A2.** Oxidation reactions were carried out as described under “Experimental Procedures.” A, product profile obtained following the flavodoxin and flavodoxin reductase supported reaction using CYP158A2 (3 nmol) and 2-OH NQ (375 \( \mu \)M) at 37 °C for 2 h. S, substrate (2-OH NQ); P, dimeric product, (structure shown). B, negative control incubation, as in A but without CYP158A2.
restrained to be 1.9 Å using a force constant of 4.0 kcal/mol/Å², whereas the Fe–O–O bond angle was restrained to 130° using a force constant of 3.5 kcal/mol/Å². Final refinement statistics are given in TABLE ONE. The coordinates and associated structure factors have been deposited with the Protein Data Bank (accession codes ferric 2-OH NQ complex, 2D0E, and ferrous dioxygen-bound complex, 2D09).

RESULTS

2-OH NQ Binding and Activity—To identify the roles of substrate flavilin hydroxyl groups and catalytic water molecules in the ferric flavilin complex active site, 2-OH NQ was examined as a substrate analogue. 2-OH NQ produced a partial shift in the Soret band from 418 to 388 nm upon binding, indicating that 2-OH NQ can substitute for flavilin in the active site (Fig. 1). The binding affinity constant \( K_d \) was estimated to be \( 43 \mu M \), 6-fold higher than that found with flavilin \( (K_d = 7.3 \mu M) \) (7). Analysis of the reaction of CYP158A2 with 2-OH NQ by electrospray mass spectrometry indicated a single dimerization product with mass 346 (Fig. 2). The turnover number for this activity was estimated to be 0.02 min \(^{-1}\). The activity is 70 times less than with flavilin (1.4 min \(^{-1}\)) as substrate. These results suggest a role for the 5- and 7-hydroxyl groups of flavilin in CYP158A2 catalysis.

The chemical structure of the dimeric product was analyzed by multiple NMR approaches, as previously described for flavilin dimerization (7), including one-dimensional \(^1H\) and two-dimensional \(^1H^13C\) HMQC and \(^1H^13C\) HMBC experiments. The dimer was clearly a symmetric structure as judged by its \(^1H\) NMR spectra, which showed two doublets at \( \delta 8.08 \) (d, \( J = 7.5 \text{ Hz}, 2H, H-8/8 \)) and \( 8.04 \) (d, \( J = 7.5 \text{ Hz}, 2H, H-5/5 \)) and two triplets at \( \delta 7.84 \) (t, \( J = 14.8 \text{ Hz}, 2H, H-6/6 \)) and \( 7.78 \) (t, \( J = 14.8 \text{ Hz}, 2H, H-7/7 \)). This structure was assigned as 3,3'-bi-2-hydroxy-1,4-naphthoquinone (Fig. 2), which was further verified by analysis of the \(^1H^13C\) HMQC and \(^1H^13C\) HMBC spectra (see supplemental data).

Kinetics of Formation and Decomposition of the Ferrous-Dioxygen Complex—The ferric form of CYP158A2 has absorbance maxima at 415, 527, and 567 nm, even in the presence of a saturating concentration of flavilin (Fig. 3). Second-derivative spectroscopy (31, 32) indicated \( 16\% \) high spin iron (\( \lambda_{\text{max}} 390 \text{ nm} \)). Reduction of CYP158A2 with

FIGURE 3. UV-visible spectra of ferric and ferrous CYP158A2 in the presence of flavilin. The solution contained 9.5 \( \mu M \) CYP158A2, 25 \( \mu M \) flavilin, 50 mM potassium phosphate buffer (pH 7.4), 100 mM Tris-HCl, 10 mM EDTA, 1.0 \( \mu M \) 5-deazaflavin. Spectra were recorded of the ferric enzyme (Fe\(^{3+}\), — —) and, after anaerobiosis and photochemical reduction, of the ferrous complex (Fe\(^{2+}\), - - -). (Spectra were recorded intermediately during the photochemical reduction process.) A 5-fold expansion of the scale in the \( \alpha,\beta \)-region is shown.

FIGURE 4. Reaction of ferrous CYP158A2 with oxygen. The ferrous CYP158A2-flavilin complex (Fig. 3) was introduced into an anaerobic syringe of an OLS RSM-1000 spectrophotometer, and the contents were mixed with air-saturated buffer. A, initial spectral changes, collected every 10 ms during the reaction. The directions of the changes are shown with the arrows. B, kinetic course of the first part of the reaction (part A). The changes in \( A_{418} \) and \( A_{440} \) were fit to first order plots (\( k = 0.043 \pm 0.001 \) and \( 0.040 \pm 0.02 \text{ s}^{-1} \), respectively). C, later changes in the absorbance spectra. The first trace recorded was at 0.32 s. Traces are shown every 6.4 s, with the directions indicated with arrows. D, kinetic course of the second part of the reaction (part C). The changes in \( A_{360} \) and \( A_{300} \) were fit to first order plots (\( k = 0.043 \pm 0.001 \) and \( 0.040 \pm 0.02 \text{ s}^{-1} \), respectively). Residuals traces are shown above the kinetic traces in parts B and D.
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The electron density map was calculated using $aK$-weighted $2F_o - F_i$ coefficients and is contoured at 1.0σ. The proximal 2-OH NQ and distal 2-OH NQ molecules in the orange stick models are denoted "N1" and "N2", respectively. Note that there is no electron density for water molecules in the active site. Potential hydrogen bonds and the distances between the correspondent carbon atoms to iron atom are shown as dotted green lines.

When anaerobically reduced CYP158A2 (in the presence of 25 μM flaviolin) was mixed with air (~100 μM O2 final concentration), a rapid change in the spectrum to the putative FeO2$^{2+}$ complex was observed (Fig. 4A), at a rate of ~120 s$^{-1}$ (at 23 °C) (Fig. 4B). The spectrum converted to the original ferric form (Fig. 4C), at a rate of ~0.042 s$^{-1}$ (Fig. 4D). The kinetic experiments were repeated with 375 μM 2-OH NQ replacing flaviolin. The spectral changes (not presented) were similar to those observed with flaviolin, although the higher concentrations required for saturation had the effect of obscuring some of the visible spectra. The rate of formation of the FeO2$^{2+}$ complex was ~15 s$^{-1}$, and the rate of decay was ~0.09 s$^{-1}$ indicating that the FeO2$^{2+}$ complex was formed more slowly and decayed more rapidly with 2-OH NQ than flaviolin.

Crystal Structure of the Ferric CYP158A2/2-OH NQ complex—The overall features of the 2-OH NQ complex structure (2.15 Å) (Fig. 5, produced using program Setor (34)) exhibit a closed conformation as found in the flaviolin complex structure (7). Overlaying the structures of the complexes obtained with flaviolin and 2-OH NQ show a root mean square deviation of 0.61 Å between all Cα atoms of the two structures, which indicates that some differences occur in the overall enzyme structures because of the binding of different ligands into the active site. The largest differences in the two structures are found in the region of F/G helices. The complete F/G helices in the 2-OH NQ complex swing away from the structural core ~5° with respect to that in flaviolin complex. Consequently, the FG loop is repositioned slightly away from the active site compared with the flaviolin-bound crystal form.

As in the flaviolin complex (7), two molecules of 2-OH NQ are present in the active site pocket of its complex with the protein (Fig. 6). The positions of the two molecules of 2-OH NQ are clearly recognizable based on their electron densities, although the distal 2-OH NQ showed some disorder. The average crystallographic temperature factors of these two ligand molecules, 47.2 and 69.5 Å$^2$, respectively, are significantly higher than those of the two flaviolin molecules previously reported in the flaviolin-protein complex, 16.2 and 16.5 Å$^2$, respectively (7). The temperature factor differences between these two different ligands are consistent with the results of the ligand binding experiments, which show that an affinity of 2-OH NQ for CYP158A2 is ~6-fold less than for flaviolin. The location of the proximal 2-OH NQ places the carbon atoms (C-5, C-6, C-7, and C-8) near the heme iron between 3.7 and 5.4 Å (Fig. 6), closer than the corresponding carbon atoms in the flaviolin complex, 4.6–5.3 Å (7). The reason that 2-OH NQ molecules are closer to the heme (with respect to flaviolins) is that the absence of the 5- and 7-hydroxyl moieties provide more room in the active site so that they slide toward the F/G region to accommodate themselves in the relatively large CYP158A2 active site. The movements of 2-OH NQ may push the F/G helices slightly away from the structural core, which could be one of the reasons for the overall structural and enzymatic differ-

Na$_2$S$_2$O$_4$ or photochemically (with visible light in the presence of 5-deazaflavin (33), anaerobically) yielded a spectrum with $\lambda_{max}$ at 407 and 539 nm (Fig. 3).
FIGURE 7. Stereo view of the contentious hydrogen bonding network in the CYP158A2 complexes. A, the ferric flavolin complex is in cyan; B, the ferrous dioxygen-bound complex is in yellow. C, the ferric 2-OH NQ complex is in orange. The flavolin (orange) and 2-OH NQ (green) atoms are rendered as stick figures. The flavolin molecules are denoted F1 and F2 and the 2-OH NQ molecules as N1 and N2. Water molecules are displayed as red spheres. Dotted green lines show the presumed proton transfer pathway.

FIGURE 8. Superimposed active site structures of 2-OH NQ- and flavolin-bound CYP158A2. The orange and green stick models show the ferric flavolin complex and ferric 2-OH NQ complex structures, respectively. 2-OH NQ molecules are much more parallel to each other than the two flavolin molecules.
ences between these two complexes. In addition, there is no electron density for water molecules in the active site of the 2-OH NQ complex. The 2-OH NQ complex is missing the three well ordered active site water molecules (WAT505, WAT600, and WAT640) forming hydrogen bonds with the 5- and 7-hydroxyls in the flaviolin complex. The guanidium group of Arg288 forms both hydrogen bonds with the 2-OH in the proximal 2-OH NQ molecule and the C4-carbonyl in the distal 2-OH NQ and His287 also provides another new hydrogen bond to the carbonyl C4 in the proximal 2-OH NQ molecule to help stabilize the two molecules of 2-OH NQ in the active site instead of hydrogen bond to water molecule in ferric flaviolin complex. An interesting result from the 2-OH NQ and flaviolin complex structures is that the water chain identified in the flaviolin complex is still present (Fig. 7A). A hydrogen bonded network consisting of WAT505–WAT545–WAT548–WAT652–WAT630 extends to the carbonyl oxygen of Leu179 and the amide nitrogen of Leu287 located on the distal surface (Fig. 7C). It is of interest that one additional water (WAT630) is present in the network in the ferric CYP158A2 complex because the water chain is disturbed and does not reach the substrate in the 2-OH NQ complex. Another feature in the 2-OH NQ complex is that the overall geometry of the two 2-OH NQ molecules is more parallel than in the case of the two flaviolin molecules (Fig. 8). In the 2-OH NQ complex, the two 2-OH NQ molecules are positioned almost parallel to each other; whereas in the flaviolin complex, the two flaviolin molecules are present at an angle of ~30° because WAT508 and WAT600 interact with both flavioins via hydrogen bonding. Apparently water molecules also play an important role in establishing the geometry of the substrate in the active site.

Crystal Structure of the Ferrous Dioxygen-bound CYP158A2-Flaviolin Complex—The electron density maps for the ferrous CYP158A2-flaviolin-O2 complex clearly exhibit dioxygen coordinated to the heme iron sandwiched between the proximal flaviolin and heme (Fig. 9). The restrained Fe–O bond length is refined to 1.9 Å and the Fe–O–O angle is 123.5°, both in the normal range compared with other ferrous P450 dioxygen-bound complexes (16, 17, 19). The average crystallographic temperature factors of these two oxygen atoms are 48.5 Å², similar to CYP101A1 (16) but much higher than the CYP101A1 and CYP107A1 of Nagano et al. (17, 19), which suggests that partial occupation by the dioxygen ligand occurs in this ferrous CYP158A2 complex.
Proton Transfer and Oxygen Activation in CYP158A2

All active site water molecules observed in the ferric flaviolin complex are exactly reproducible in the ferrous dioxygen-bound complex, according to electron density maps. These well ordered waters provide direct hydrogen bonds with the two flaviolin molecules as described in the case of ferric complex (7). No new water molecules are detected, as in the case of CYP101A1 (16, 17), nor are existing water molecules in the ferric form pushed away from the active site, as in CYP107A1 (19). An unexpected result from this structure is that WAT529 and WAT664 not only hydrogen bond with the substrate flaviolin but also provide hydrogen bonding connections with the distal dioxygen atom. The WAT529 is in a position to form a relatively stronger hydrogen bond with the distal oxygen atom (at a distance of 2.96 Å) than WAT664 (3.26 Å). These hydrogen bonded interactions cause the dioxygen ligand to move toward the distal I helix cleft between Gly241 and Ala245. There are no significant structural changes observed between the ferrous-dioxygen and ferric complex forms in the active site for the two molecules of flaviolin and surrounding residues and water molecules upon dioxygen binding. A superposition of the ferrous dioxygen bound form with the ferric complexes is shown in Fig. 10. Only slight structural changes are observed for the distal flaviolin, and the distance of the proximal flaviolin to the heme does not change significantly. From the crystallographic analysis of the ferric complex, the water molecules WAT505, WAT600, and WAT640 are at distances of 4.3, 4.8, and 5.3 Å from the heme iron atom and are able to provide room for dioxygen binding. This situation is in contrast to the case of CYP107A1 where the water molecule (WAT519) is only 3.6 Å away from the heme iron atom, and this water is displaced in the ferrous dioxygen-bound complex (19). Thus, the dioxygen ligand in the ferrous CYP158A2 dioxygen-bound complex will not conflict with water molecules WAT528, WAT529, and WAT664, with the result that three waters occupy almost exactly the same positions as WAT505, WAT600, and WAT640, respectively, in the ferric form.

Kinetic Solvent Isotope Effects (KSIE)—The overall rate-limiting steps in P450 catalyzed reactions vary depending on the specific enzyme and the substrate (35). Proton transfer is one of the rate-limiting steps in the CYP101A1 catalytic cycle (24). The results above suggest that hydrogen-bound water molecules may participate in proton transfer relay for oxygen activation in CYP158A2. To explore this possibility, KSIE experiments were carried out and are summarized in Fig. 11. The effect of deuterium on the substrate flaviolin oxidation kinetics in CYP158A2 was investigated in mixed isotopic waters. Obviously, the rates of flaviolin consumption and the rates of each product formation were decreased at increasing content of deuterium oxide in the solvent. The overall catalytic activities in various D2O/H2O mixtures are decreased at increasing content of deuterium oxide in the solvent. The pH effect control (pH 7.1) results show that the rate of substrate consumption is 1.2 min⁻¹, which confirms that the KSIE results described above are because of the effect of deuterium solvent rather than a pH issue.

DISCUSSION

Proton transfer and dioxygen activation in P450s have been studied using ferrous dioxygen-bound complexes in CYP101A1 (16, 17) and CYP107A1 (19). In CYP101A1, the highly conserved Thr252 is important for dioxygen activation. Although no ordered waters were observed in the structure of the active site of ferric CYP101A1, Shlichting et al. (16) first reported that two new catalytic waters did interact with Thr252 in the ferrous form for proton transfer. Recently, Nagano and Poulos (17) further clarified the role of Thr252 as a hydrogen bond acceptor stabilizing the hydroperoxy intermediate. Thus, in CYP101A1 the protons are directly transferred to dioxygen via water molecules that appear only in the active site of the ferrous dioxygen complex. CYP107A1 is different from CYP101A1 because there are no direct hydrogen-bonded waters with dioxygen atoms. The predicted catalytic water molecule (WAT519) present in the ferric-substrate active site is displaced upon binding of dioxygen to the ferrous iron in the substrate complex. Instead, a substrate 5-hydroxyl group interacts with dioxygen, which suggests that the direct proton donor to dioxygen is the substrate hydroxyl group and not water (19). To address the proton transfer system and to evaluate the roles of substrate flavin hydroxyl groups and active site water molecules in CYP158A2, we utilized the flavin analogue 2-OH NQ, devoid of the two extra hydroxyls and found that a loss of catalytic activity with this substrate analog 2-OH NQ is associated with the loss of active site waters in the 2-OH NQ com-

FIGURE 11. Kinetic solvent isotope effects. The rates of flaviolin consumption and the rates of each product formation in the presence of the various D2O/H2O mixtures were determined as described under “Experimental Procedures.” The fraction of deuterium (x) is plotted against the ratio of activity measured in the D2O/H2O mixture (kx) to the rate measured in H2O (kH). The symbols used are ( ) flaviolin, ( ) 3,8-biflaviolin, ( ) 3,3-biflaviolin, ( ) undefined biflaviolin, and ( ) undefined triflaviolin (7).

FIGURE 12. Hydrogen bond networks for CYP158A2. Potential hydrogen bonds are dotted lines. The labeled distances are in Angstroms. The flaviolin molecules are denoted F1 and F2.
Proton Transfer and Oxygen Activation in CYP158A2

The active site waters are able to directly form hydrogen bonds with the distal dioxygen atom in the ferrous dioxygen-bound complex. These results allow an understanding of the role of both active site waters and hydroxyl groups of flavin in catalysis, providing insights into the mechanism of proton transfer and oxygen activation in CYP158A2.

The reaction of the ferrous CYP158A2-flaviolin complex with O₂ produced two sets of spectral changes (Fig. 4). The effect of varying the O₂ concentration on the rates was not done, but we propose that the rapid initial changes (Fig. 4A) represent the formation of an FeO₂(‘substrate’) complex, and the succeeding changes (Fig. 4C) represent the decay of the complex to regenerate ferreic CYP158A2. The estimated rate of the first reaction was ~120 s⁻¹ and the decay was 0.04 s⁻¹ in the presence of flavinol at 23 °C. This complex appears to be less stable than the ferrous dioxygen complexes reported for bacterial CYP101A1 (36, 37) and CYP108A1 (38) but has a stability similar to rabbit CYP1A2 (22, 39), human CYP2A6 (23), and the heme domain of CYP102A1 (36); it is more stable than the complexes of rabbit CYP2B4 (40) and bacterial CYP19A1 (41). The complex was formed less rapidly (~15 s⁻¹) and decayed somewhat faster (k = 0.09 s⁻¹) when flavinol was replaced with 2-OH NQ. These kinetic data may indicate that the active site water molecules can stabilize dioxygen binding.

No continuous hydrogen bonded connection between the dioxygen atoms and the protein surface has been observed in the ferrous dioxygen-bound crystal structures of CYP101A1 (16, 17) and CYP107A1 (19). However, in both the ferric and ferrous-oxo complexes of CYP158A2 we find a continuous hydrogen-bonded network to the bulk solvent that is postulated to be the direct proton relay pathway to dioxygen in CYP158A2. In the ferric state, a well ordered water chain consisting of WAT528–WAT544–WAT568–WAT503–WAT640–WAT505 (average temperature factor 21.7 Å²) forms an extensive network of hydrogen bonds and extends to the carbonyl oxygen of Leu179 and the amide nitrogens of Leu393 and Ileu394 on the distal surface (Fig. 7A). In the ferrous dioxygen-bound complex, a very similar well ordered hydrogen-bonded water chain consisting of WAT604–WAT521–WAT516–WAT506–WAT528–WAT529 (average temperature factor 20.6 Å²) was observed extending to the distal surface of the protein (Fig. 7B). In both cases this network joins the three water molecules in the active site described above. A similar water hydrogen bond network topology also exists in the 2-OH NQ complex, but the water network terminates in the vicinity of the two molecules of 2-OH NQ because of the absence of the three water molecules in the active site (Fig. 7C). Also, the results of KSIE experiments strongly support that proton transfer is through water molecules leading to dioxygen bond scission and also contributes to the rate-limiting action in the catalytic cycle of CYP158A2.

The ferric 2-OH NQ complex structure suggests that no activity should be detected because the 2-OH NQ, missing the 5- and 7-hydroxyl groups, creates a more hydrophobic environment around the benzene ring motif and disturbs the continuous water chain in the active site, disrupting the proton delivery pathway. However, a trace of activity was observed with 2-OH NQ as substrate. A possible explanation for this residual activity may be active site waters present in the 2-OH NQ ferrous dioxygen-bound form, as found in the case of ferrous CYP101A1 (16, 17). These active site waters might not be stable enough to transfer protons efficiently (although they may transiently exist in the ferrous form) because the two 2-OH NQ molecules are incapable of hydrogen bond donation to active site water molecules and cannot hold them for dioxygen activation. This explanation is consistent with the dramatically decreased activity of 2-OH NQ as a substrate, and it also accounts for the absence of dioxygen electron density in the 2-OH NQ crystal form produced in attempts to obtain a structure of 2-OH NQ ferrous dioxygen-bound complex. Further, we observed only one dimeric product from 2-OH NQ but four major products with flavinol as the substrate (7). Thus, the active site water molecules may influence the positions of the intermediate flavinol molecules during catalysis in addition to transferring protons to the dioxygen in CYP158A2.

In the ferrous dioxygen-bound complex, the catalytic water molecules are still present in the active site, and WAT528, WAT529, and WAT664 (Fig. 10) correspond to WAT505, WAT600, and WAT640 in the ferric flavinol complex. Thus CYP158A2 probably utilizes the hydroxyl groups of the substrate to stabilize the catalytic water molecules to make hydrogen bonds with the distal dioxygen molecule atom. Also, the catalytic waters hydrogen bonding to the distal dioxygen molecule will participate in a proton relay system to the iron-linked oxygen molecule. The 5- and 7-hydroxyl groups act as important catalytic groups in the acid-catalyzed dioxygen bond cleavage reaction, and CYP158A2 appears to achieve enzyme specificity by precise positioning of proton donor/acceptor groups of the substrate in the active site.

In summary, we propose that the WAT529 and WAT664 may be the direct proton donors to the iron-linked distal oxygen atom in CYP158A2 and that the substrate 5- and 7-hydroxyl groups assist in this process by helping to hold and orient these active site water molecules (Fig. 12). This continuous hydrogen bonded network, which connects iron-linked dioxygen in the active site to the protein surface, is proposed to serve as the proton transfer pathway in CYP158A2. Unlike CYPs 101A1 and 107A1, in CYP158A2 this hydrogen bonded network is unchanged between the ferric and ferrous dioxygen forms.

It is interesting to note, when comparing the three ferrous dioxygen-bound complexes now known for P450s, that each appears to have its own pathway for proton transfer for dioxygen activation. Because CYP101A1 is the only such structure having the highly conserved threonine in the I helix, it may be possible that when other such ferrous-dioxygen structures are determined that this residue will dictate a common proton path among all such P450s. However, comparison of CYP107A1 with CYP158A2 suggests that certain CYPs not having this conserved threonine use a very specific proton path involving substrate or active site water molecules or both. P450s may be classified into two types of enzymes based on the mechanism of oxygen activation. One is a self-assisted enzyme (e.g. CYP101A1) containing the highly conserved threonine in the I-helix, which stabilizes the oxy complex or hydroperoxy intermediate. The other is a substrate-assisted enzyme (e.g. CYP107A1 and CYP158A2) that does not contain the highly conserved threonine and requires a specific substrate containing proton donor/acceptor groups. In the latter class, two different proton transfer paths are predicted, one from a substrate hydroxyl group (CYP107A1) and the other from a water path from the surface to the liganded dioxygen, which is stabilized by substrate hydroxyl groups.

3 The three water molecules in the ferric CYP158A2-flavin active site complex are numbered WAT528, WAT505, WAT600, whereas in the ferrous CYP158A2-flavin-dioxygen complex they are numbered WAT528, WAT529, and WAT664. Both sets occupy the same positions, and we believe that they are probably the same water molecules. There are two reasons why the numbers are different; the total number of water molecules in the flavin ferric complex is 242, whereas in the flavin ferric dioxygen complex it is 258, and although there is no change in positions of waters in the proposed proton pathway between the two structures, some other water molecules are in different positions, which alters numbering of all water molecules.
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