Role of Active Site Residues and Solvent in Proton Transfer and the Modulation of Flavin Reduction Potential in Bacterial Morphinone Reductase*

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The reactions of several active site mutant forms of bacterial morphinone reductase (MR) with NADH and 2-cyclohexen-1-one as substrates have been studied by stopped-flow and steady-state kinetic methods and redox potentiometry. The enzymes were designed to (i) probe a role for potential proton donors (Tyr-72 and Tyr-356) in the oxidative half-reaction of MR; (ii) assess the function of a highly conserved tryptophan residue (Trp-106) in catalysis; (iii) investigate the role of Thr-32 in modulating the FMN reduction potential and catalysis. The Y72F and Y356F enzymes retained activity in both steady-state and stopped-flow kinetic studies, indicating they do not serve as key proton donors in the oxidative reaction of MR. Taken together with our recently published data (Messiha, H. L., Munro, A. W., Bruce, N. C., Barsukov, I., and Scrutton, N. S. (2005) J. Biol. Chem. 280, 4627–4631) that rule out roles for flavin reduction in MR by nicotinamide coenzyme indicates that hydride transfer from FMNH2 to 2-cyclohexen-1-one and codeinone (17). Mutagenesis, kinetic, and NMR studies have indicated that residues His-186 and Asn-189, which are conserved as His-191 and Asn-194 in OYE1, are important in ligand binding, and that His-186 is not the key proton donor required for the reduction of 2-cyclohexen-1-one (24). The turnover mechanism for MR is a ping-pong reaction comprising two half-reactions. The mechanism of flavin reduction and oxidation in MR (Fig. 1A) has been studied by stopped-flow and steady-state kinetic methods (25, 26). The temperature dependence of the primary kinetic isotope effect for flavin reduction in MR by nicotinamide coenzyme indicates that quantum mechanical tunneling plays a major role in hydride transfer (26). The oxidative half-reaction (Fig. 1, A and B) is fully rate-limiting in steady-state turnover with the substrate 2-cyclohexen-1-one and NADH at saturating concentrations and hydride transfer from FMNH2 to 2-cyclohexen-1-one occurs by tunneling (26). A large solvent isotope effect accompanies the oxidative half-reaction, and double isotope effects indicate that hydride transfer from the flavin N5 atom to 2-cyclohexen-1-one, and the protonation of 2-cyclohexen-1-one by an unknown donor, are coupled (26).

Morphinone reductase (MR) catalyzes the NADH-dependent saturation of the carbon–carbon bond of both morphinone and codeinone. The enzyme is dimeric, contains a single FMN per subunit, and belongs to the Old Yellow Enzyme family of flavoproteins (1, 2). The family includes the isoforms of OYE (3), estrogen-binding protein (EBP) from Candida albicans (4), pentaerythritol tetranitrate (PETN) reductase from Enterobacter cloacae (5), glycerol trinitrate reductase from Agrobacterium radiobacter (6), the xenobiotic reductases of Pseudomonas species (7), and 12-oxophytodienoic acid reductase from tomato (8) and Arabidopsis thaliana (9). More complex members include the bile acid-inducible flavoenzymes Bai H and Bai C from Eubacterium species (10), the bacterial FeS flavoenzymes tri- and dimethylamine dehydrogenases (11, 12), the histamine dehydrogenase from Nocardioides simplex (13), and the NADH oxidase of Thermoanaerobium brockii (14). Crystallographic structures are available for a number of these enzymes, including OYE1 (15), PETN reductase (16), MR (17), 12-oxophytodienoic acid reductase (18, 19), and trimethylamine dehydrogenase (20, 21).

The structure of MR has been determined at 2.2-Å resolution (17) and reveals a dimeric enzyme comprising two 8-fold β/α barrel domains, each bound to FMN. The active sites of MR, OYE1, and PETN reductase are highly conserved, and each enzyme catalyzes the reduction of the non-physiological substrate 2-cyclohexen-1-one (22). This reflects the general reactivity of each member protein with α/β unsaturated carbonyl compounds. The active site acid identified in OYE1 (Tyr-196) (23), and conserved in PETN reductase (Tyr-186) (16), is replaced by Cys-191 in MR, but Cys-191 does not act as a crucial acid in the mechanism of reduction of the olefinic bond found in 2-cyclohexen-1-one and codeinone (17). Mutagenesis, kinetic, and NMR studies have indicated that residues His-186 and Asn-189, which are conserved as His-191 and Asn-194 in OYE1, are important in ligand binding, and that His-186 is not the key proton donor required for the reduction of 2-cyclohexen-1-one (24). The turnover mechanism for MR is a ping-pong reaction comprising two half-reactions. The mechanism of flavin reduction and oxidation in MR (Fig. 1A) has been studied by stopped-flow and steady-state kinetic methods (25, 26). The temperature dependence of the primary kinetic isotope effect for flavin reduction in MR by nicotinamide coenzyme indicates that quantum mechanical tunneling plays a major role in hydride transfer (26). The oxidative half-reaction (Fig. 1, A and B) is fully rate-limiting in steady-state turnover with the substrate 2-cyclohexen-1-one and NADH at saturating concentrations and hydride transfer from FMNH2 to 2-cyclohexen-1-one occurs by tunneling (26). A large solvent isotope effect accompanies the oxidative half-reaction, and double isotope effects indicate that hydride transfer from the flavin N5 atom to 2-cyclohexen-1-one, and the protonation of 2-cyclohexen-1-one by an unknown donor, are coupled (26).

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Herein we report the isolation and properties of a number of mutant forms of MR with the intention of identifying the key proton donor required for the reduction of 2-cyclohexen-1-one and related compounds. Based on the crystal structure of MR, we have targeted all possible side-chain proton donors and demonstrated retention of activity for each mutant form. Based on the studies reported herein and our previously published mutagenesis studies in which we targeted residues Cys-191 and His-186 (24), we infer that solvent is the source of the proton donor required for the reduction of 2-cyclohexen-1-one.

A

Reductive half-reaction

\[ E_{\text{red}} + \text{NADH} \rightarrow E_{\text{red}}\text{NADH} \rightarrow E_{\text{red}}\text{NADH}^{+} \]

Oxidative half-reaction

\[ E_{\text{ox}} + 2\text{-cyclohexen-1-one} \rightarrow E_{\text{ox}}\text{2-cyclohexen-1-one} \]

B

![Diagram of the reaction mechanism](Image)

**FIG. 1.** A, minimal kinetic schemes for the reductive and oxidative half-reactions of MR. B, the oxidative half-reaction of MR showing the coupled nature of the proton transfer from the unidentified acid and hydride transfer from FMN2 to 2-cyclohexen-1-one.

**EXPERIMENTAL PROCEDURES**

**Materials—**All materials were of analytical grade. Mimetic Yellow 2 affinity chromatography resin was from Affinity Chromatography Ltd. Q-Sepharose resin was from Amersham Biosciences.

**Protein Purification and Mutagenesis—**Wild-type MR was purified from a recombinant strain of *Escherichia coli* expressing the enzyme from the cloned *morB* gene as described previously (27) but also incorporating a final chromatographic step using Q-Sepharose also as described previously (17). The Y72F, the Y356F, the W106F, and the T32A mutant MR enzymes were isolated using the QuikChange mutagenesis protocol (Stratagene) and the following oligonucleotides: 5′-TGC CCC ACC GCC GGC TTG GTC TAC AGC CGG GGG ATC-3′ and 5′-GAT CCC CGG CTT GTA GAC AAA GCC GCG GGT GGG GCA-3′ (Y72F), 5′-CCC GAC CCC ACC TCC TTC TGT GGC GCC GCG GAG GTC GGC-3′ and 5′-GCC GAC CTC GCC GCC GAA GAA GGT GCT GGG GTC GGG-3′ (Y356F), 5′-CGC ATC GCC CTG CAG CTG TTT CAC GTC GGT GTC TCC-3′ and 5′-GGA ACG GCC GAC GTG AAA CAG CTG CAG GGC GAT GCG CAT GAT CAC-3′ (T32A). Plasmid pMORB3 (1) was used as template for the mutagenesis reactions. The mutant genes were completely sequenced to ensure that spurious changes had not arisen during the mutagenesis reaction. Expression and purification of the mutant MR enzymes was as described for wild-type enzyme (17, 27) with the exception of W106F and T32A mutant enzymes, which did not bind to the Mimetic Yellow 2 resin. These enzymes were purified using Q-Sepharose resin as described for wild-type procedures (17), but with an additional column washing step involving the passage of 2 liters of buffer B (2 mM 2-mercaptoethanol in 30 mM Tris, pH 8.0) through the column followed by 2 liters of 230 mM NaCl in buffer B. The mutant enzymes were eluted from the column using the procedures adopted for the wild-type enzyme (17).

**Redox Potentiometry—**Redox titrations were performed at 25 ± 2 °C in 50 mM potassium phosphate buffer, pH 7.0, in a Belle Technology glove box under anaerobic conditions. Enzyme solutions (60–80 μM in 10 mM potassium phosphate buffer, pH 7.0, in a Belle Technology glove box) were electrochemically titrated according to the method of Dutton (28) using sodium dithionite as reductant and potassium ferricyanide as oxidant. Mediators were added to facilitate electrical communication between the enzyme and the electrode prior to titration. Typically, 5 μM 2-hydroxy-1,4-napthaquinone, 2 μM phenazine methosulfate, 1 μM methyl viologen, and 1 μM benzyl viologen were included in the titration to mediate in the range between +100 and −480 mV. The electrochemical potential of the enzyme solutions was measured using a Hanna pH 211 meter coupled to a platinum/calomel electrode (Thermolab Ltd.) that had been previously calibrated using the Fe²⁺/Fe³⁺ EDTA couple as a standard (+108 mV). The calomel electrode was corrected by +244 mV relative to the standard hydrogen electrode. The electrode was allowed to stabilize between each addition of reductant or oxidant and spectra (300–700 nm) were recorded using a Cary 50 UV-visible scanning spectrophotometer (Varian) via a fiber optic cable immersed in the enzyme solution and connected to the external spectrophotometer. The spectra of at least 30–40 points across a whole range of redox potentials during the process of reduction and oxidation were recorded. Titrations were performed over a 5- to 7-h period. Equilibration was achieved throughout the titrations, and no hysteretic behavior was observed. Corrections were made for small amounts of protein evaporation during titrations as described previously (26). Plots of the absorbance against redox potential were fitted to Equation 1, which is derived by extension of the Nernst equation and the Beer-Lambert law using Equation 1, as described previously (30),

\[ A_{\text{obs}} = \left( a + b \times E_{\text{red}} \right) / 1 + 10^{(E_{\text{mid}} - E_{\text{red}})/2.3} \]  

(Eq. 1)

where \( A_{\text{obs}} \) is the absorbance value at the peak for the oxidized flavin in wild-type MR and the mutants analyzed) at the electrode potential \( E \), and \( a, b \) are the absorbance values of the fully oxidized and reduced enzyme at this wavelength, respectively. \( E_{\text{mid}} \) is the midpoint potential for the concerted two-electron reduction of the MR flavin. Data manipulation and analysis were performed using GraFit software package version 5.0. All redox potentials are given relative to the standard hydrogen electrode.

**Kinetic Studies—**All kinetic studies were performed under strict anaerobic conditions (<5 ppm of O₂) within a glove-box environment (Belle Technology) to prevent oxidase activity of MR. Steady-state assays were performed using a Jasco V530 spectrophotometer at 25 °C. Reaction buffer was 50 mM potassium phosphate, pH 7.0, which was made anaerobic by bubbling with humidified Pureshield argon at 5 p.s.i. for 2 h prior to introduction into the glove box. NAHD solutions were made from pre-weighed powder and anaerobic buffer inside the glove box, and the concentration was determined spectrophotometrically (ε₄₅₀ = 6220 M⁻¹ cm⁻¹). Solutions of 2-cyclohexen-1-one were made by dilution of a stock (10 mM) into anaerobic buffer. The initial velocity in steady-state assays was determined from absorbance changes at 340 nm in a reaction cell volume of 1 ml; the desired concentration of...
substrate and cofactor solutions was achieved by making microliter additions to the cell. Least-squares fitting procedures to the standard Michaelis-Menten equation or to the simple substrate inhibition equation (31) were performed using GraFit software package version 5.0.

Stopped-flow kinetic experiments were performed under anaerobic conditions using an Applied Photophysics SX.17MV stopped-flow instrument, and transients were analyzed using non-linear least-squares regression using Spectrakinetics software (Applied Photophysics). Averages of five to seven individual transients were analyzed. Enzyme was contained in 50 mM potassium phosphate buffer, pH 7.0. In the reductive half-reaction, enzyme (20 nM) was mixed with NADH at different concentrations (at least >5-fold the enzyme concentration to ensure pseudo first order conditions). Flavin reduction was monitored at 462 nm; formation and decay of the oxidized enzyme-NADH charge-transfer species was monitored at 552 nm (25). In studies of the oxidative half-reaction, MR enzyme was titrated with sodium dithionite to produce the two-electron-reduced form of MR. Reduced MR (20 nM) was then mixed with 2-cyclohexen-1-one at different concentrations, and flavin oxidation was monitored at 462 nm. Equations used to analyze the reductive half-reaction and the oxidative half-reaction have been described elsewhere (25).

Computational Chemistry—To aid in interpreting the experimental results, computational studies were performed on wild-type and T32A MR. These calculations were based on model systems. The positions of the flavin and Thr-32 were defined from the crystal structure of oxidized MR (PDB accession code 1gwj (17)), and the FMN ribityl moiety was truncated to methane, forming lumiflavin, as in our previous studies (20). To eliminate strain present in the isoxazoline ring from the crystal structure, the oxidized lumiflavin was replaced by a copy optimized, using Gaussian 03 (www.gaussian.com/citation.htm), at the B3LYP/3–21G(d) level of theory. To obtain the LUMO energy of the lumiflavin in wild-type and T32A MR, QM/MM calculations were performed using ONIOM (34) within Gaussian 03. The amino acid (Thr or Ala, respectively) was treated molecular mechanically, using the UFF force field (35), with charges derived using the charge equilibration (QEQ) scheme (36). The electronic embedding scheme available in Gaussian 03 was used to account for electrostatic interactions in the QM/MM system. The lumiflavin was treated quantum mechanically using various levels of theory: HF/3–21G(d), B3LYP/3–21G(d), B3LYP6–31G(d), B3LYP6–31+G(d), B3LYP6–311+G(d), B3LYP6–311+G(d,p), and MP26–311++G(d,p). The corresponding two-electron reduction potential obtained was similar to our previous studies (20), using an empirical fit (at the appropriate level of theory) to experimentally measured reduction potentials of a range of flavin derivatives (37).

RESULTS

Active Site Structure and Implications for Catalysis—Our previous studies of MR (17) established a very close structural relationship with OYE1 (15) and PETN reductase (16). Overlay of the structures of the active sites of these enzymes indicates a high degree of sequence and structural conservation; in particular, residue Trp-106 (MR) occupies a similar position in each enzyme (Fig. 2A) and is conserved throughout the OYE family of enzymes (Fig. 2D). The corresponding residue in PETN reductase plays a major role in directing reactivity against nitroaromatic substrates along nitro reduction and hydride transfer pathways, the latter generating a Meisenheimer-hydride complex of the nitroaromatic substrate. High resolution structural studies indicate that in PETN reductase the tryptophan residue adopts multiple conformational states influenced by ligand (nitroaromatic) binding (38). The Meisenheimer-hydride pathway for nitroaromatic degradation is restricted to PETN reductase (22, 39), prompting the question as to the role of the conserved tryptophan residue in the remaining members of the OYE family. Also conserved throughout the family are residues known to be involved in ligand binding through interaction with the carbonyl moiety of the oxidizing substrate (e.g. 2-cyclohexen-1-one, selected steroids, and codeinone) and the reducing coenzyme (NADH or NADPH). In MR these residues are His-186 and Asn-189, but in PETN reductase the residue corresponding to Asn-189 (MR) is conservatively replaced by histidine (His-184). Our work (16,
24) and that of others (15, 40) has demonstrated that these are key determinants in ligand binding, but that the conserved histidine residue is not involved in proton donation to substrate. Thr-32 (MR) is also conserved throughout the family (Fig. 2C), and is known to modulate the potential of the FMN in OYE1 (41). A striking difference between MR and OYE1 and PETN reductase is the absence of a tyrosine residue corresponding to Tyr-196 (OYE1) and Tyr-186 (PETN reductase). In OYE1, Tyr-196 is the proton donor required for the reduction of two cyclic enone substrates (23), and structural data suggest a similar role in PETN reductase (16). In MR, however, the corresponding residue (Cys-191) is known not to donate a proton to oxidizing substrates (17), thereby prompting the question as to the source of the proton during reduction of two cyclic enone substrates. The structure of MR indicates two further residues (Tyr-72 and Tyr-356) that could potentially supply a proton in the oxidative half-reaction, and for this reason we have mutated each residue to phenylalanine and investigated the kinetics of substrate reduction by the Y72F and Y356F enzymes. We have also characterized (i) the T32A enzyme to probe effects on flavin potential and enzyme catalysis and (ii) the W106F enzyme to investigate any unforeseen role for the highly conserved tryptophan residue in the OYE family of enzymes.

**Spectral and Redox Properties of the Mutant MR Enzymes**—The mutant enzymes were purified in high yield and to homogeneity using procedures described under “Experimental Procedures.” Although the mutations are located close to the FMN isoalloxazine ring, only small perturbations were observed in the electronic absorption spectrum of the FMN (Fig. 3, A and B). Mutagenesis did not lead to loss of FMN during purification, and each mutant enzyme was fully associated with FMN following purification. With the exception of the T32A enzyme (Fig. 3C), potentiometric analysis revealed no major perturbations in the flavin redox potential representing conversion of oxidized FMN to hydroquinone FMN. The reduction potentials for the concerted two-electron reduction of the FMN are given in the legend to Fig. 3. As with wild-type MR (29), there was no evidence for the formation of a flavin semiquinone species during reduction or oxidation of the flavin. Potentiometric analysis indicated that the two-electron reduction potential of the T32A enzyme is −50 mV more negative than wild-type (−290 mV versus −242 mV for wild-type).

Computational chemistry studies were performed to gain further insight into the impact of the T32A mutation on the electronic properties of the flavin. This yields calculated differences in the two-electron reduction potential between wild-type and T32A ranging from −48.9 to −47.2 mV depending on the level of theory used, supporting the hypothesis that removal of the hydrogen bond between Thr-32 hydroxyl and flavin O4 reduces the two-electron reduction potential of the flavin by −50 mV. To address the general role of this hydrogen bond, equivalent calculations were also performed on wild-type and T32A OYE. These again suggested that removal of the hydrogen bond between Thr hydroxyl and flavin O4 reduces the two-electron reduction potential of the flavin by −50 mV, yielding calculated differences between wild-type and T32A ranging from −49.0 to −47.3 mV depending on the level of theory used, consistent with the lowering of the two-electron reduction potential observed experimentally for OYE (41). Additionally, the mutation T32A increases the energy of the LUMO by −0.2 eV (ranging from 0.18 to 0.21 eV depending on the level of theory used), consistent with the removal of the hydroxyl leading to a compromised rate of flavin reduction (see Table III below).

**Steady-state Kinetic Properties of the Wild-type and Mutant Enzymes**—The steady-state kinetic parameters for wild-type and mutant enzymes with 2-cyclohexen-1-one as substrate are presented in Table I. Plots of initial velocity versus 2-cyclohexen-1-one concentration at fixed NADH concentration were hyperbolic except for the T32A and Y356F enzymes, which showed marked inhibition at high 2-cyclohexen-1-one concentrations (Figs. 4, A and B). One possible explanation of this is that, at high substrate concentrations, a 2-cyclohexen-1-one molecule inhibits the catalytic site by binding close to the si-face of the flavin in T32A and Y356F mutant enzyme, occupying volume that is occluded by Thr-32 and Tyr-356 side chains in the wild-type, Y72F, and W106F mutant enzymes. The turnover values for the Y72F and Y356F enzymes are
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TABLE I

| Enzyme | $k_{cat}$ | $K_m$ 2-cyclohexen-1-one | $K_m$ NADH |
|-------|---------|------------------------|------------|
| Wild-type | 0.80 ± 0.01 | 4.2 ± 0.2 | 8.9 ± 0.6 |
| Y72F | 0.81 ± 0.01 | 3.3 ± 0.2 | 8.8 ± 0.9 |
| Y356F | 0.49 ± 0.03 | 2.9 ± 0.3 | 61.2 ± 3.2 |
| W106F | 0.148 ± 0.004 | 15.1 ± 0.9 | 78.9 ± 6.3 |
| T32A | 0.28 ± 0.02 | 1.2 ± 0.1 | 48.9 ± 2.3 |

$^a$ Values were determined at 150 μM NADH.

TABLE II

| Enzyme | $k_{cat}$ | $K_m$ codeinone |
|-------|---------|----------------|
| Wild-type | 16.32 ± 0.2 | 225.4 ± 11.3 |
| Y72F | 9.1 ± 0.2 | 429.7 ± 42.7 |
| Y356F | 2.4 ± 0.003 | 15.7 ± 0.8 |
| W106F | 0.9 ± 0.009 | 837.3 ± 28 |
| T32A | 0.2 ± 0.003 | 13.4 ± 0.9 |

$^a$ Values were determined at 150 μM NADH.

Steady-state kinetic parameters for wild-type and mutant MR enzymes with NADH as reducing coenzyme and 2-cyclohexen-1-one as oxidizing substrate.

The reaction conditions for all assays were: 0.05–0.5 μM enzyme in 50 mM potassium phosphate buffer, pH 7.0, at 25 °C under anaerobic conditions.

Steady-state kinetic parameters for wild-type and mutant MR enzymes with NADH as reducing coenzyme and codeinone as oxidizing substrate.

Steady-state kinetic parameters were obtained with the T32A enzyme giving rise to a ~80-fold reduction in turnover number. A key observation is that mutants Y72F and Y356F both retain high levels of activity, consistent with Tyr-72 and Tyr-356 not being the source of the proton required for the oxidative half-reaction. There is some variation in the Michaelis constants measured for the mutant and wild-type enzymes, but in the absence of high resolution structural data for the enzyme-codeinone complex we are unable to rationalize at the molecular level the effects of these mutations on substrate binding.

Stopped-flow Studies of the Wild-type and Mutant Enzymes—Stopped-flow kinetic studies were performed to provide further insight into the effects of mutation on catalysis. A kinetic scheme (Fig. 1A) for the reductive half-reaction of wild-type and mutant enzymes has been published in our previous work, where further details pertinent to data analysis and the calculation of rate constants can be obtained (17, 25). Reductive half-reaction studies were performed by mixing oxidized enzyme with coenzyme under anaerobic conditions and the observed rate constant for flavin reduction, and $E_{ox}/NADH$ charge-transfer formation and decay were calculated by appropriate fitting of reaction transients measured at the flavin peak absorption (462 nm) and at longer wavelength (552 nm), respectively. Kinetic constants for flavin reduction are presented in Table III. Like wild-type enzyme, the rate of flavin reduction is independent of NADH concentration in the first order regime for the Y72F enzyme. The Y356F and W106F enzymes, however, showed a hyperbolic dependence of the flavin reduction rate on NADH concentration from which limiting rates of flavin reduction were calculated (Table III). The elevated $K_p$ values for these mutant enzymes are consistent with the elevated $K_m$ values for NADH seen in steady-state turnover (Table I). Reaction traces for the T32A enzyme were biphasic (Fig. 5A), and observed rate constants for each kinetic phase were found to be independent of NADH concentration in the pseudo first order regime (Fig. 5A). Mutation of Thr-32 to alanine effects a major reduction (~5-fold for the fast phase) in the rate of hydride transfer from NADH to FMN. The rate of the fast phase is similar to the turnover value measured in steady-state assays (Table I), suggesting that, unlike with wild-type enzyme (17, 25), flavin reduction is rate-limiting in steady-state turnover with 2-cyclohexen-1-one. The rate constant for the slower phase, the origin of which is uncertain, is less than the steady-state turnover value, indicating it is not relevant to steady-state catalysis.

In wild-type MR, evidence for the formation and decay of an oxidized enzyme-NADH charge-transfer intermediate is obtained from reaction traces measured at 552 nm (25). The decay phase (loss of absorption at 552 nm) is concomitant with hydride transfer to FMN, and consequently has identical kinetics to the rate of FMN bleaching measured at 462 nm. Qualitatively, similar reaction traces were observed for the

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**FIG. 5.** A, kinetic traces observed at 454 nm for the reaction of the T32A mutant MR (main panel) and at 462 nm for the reaction of wild-type MR (inset) with NADH. In the main panel, the solid line is the fit to a double exponential equation; the dashed gray line is the fit to a single exponential equation. Reaction conditions: enzyme (20 μM) with 2-cyclohexen-1-one; NADH (100–500 μM) in 50 mM potassium phosphate buffer, pH 7.0, at 10 °C.

**TABLE III**

Kinetic parameters obtained from stopped-flow studies of the reductive half-reactions of wild-type and mutant forms of MR with NADH as reducing substrate by measuring absorption changes at 452 nm that correspond to reduction of the flavin cofactor

| Enzyme  | kₚₑᵈ | Kᵣ for enzyme-NADH complex |
|---------|------|-----------------------------|
| Wild-type | 22 ± 2 | s⁻¹ |
| Y72F    | 11 ± 0.05 | 421 ± 10 μM |
| Y356F   | 18 ± 0.1 | 234 ± 12 μM |
| W106F   | 4.7 ± 0.06 | 0.594 ± 0.006 s⁻¹ |
| T32A*   | 0.47 ± 0.04 | |

*The kinetic traces obtained for the T32A enzyme were biphasic. The rate constants for both phases are independent of NADH concentration in the pseudo first order regime. Rate constants for wild-type and Y72F enzymes were also independent of NADH concentration in the pseudo first order regime, whereas a hyperbolic dependence of rate constant on NADH concentration was seen for the W106F and Y356F enzymes. Reaction conditions: enzymes (20 μM); NADH (100–500 μM) in 50 mM potassium phosphate buffer, pH 7.0, at 10 °C.*

**TABLE IV**

Kinetic parameters obtained for the formation and decay of enzyme-NADH charge-transfer complexes in the reductive half-reaction of wild-type and mutant MR enzymes from analysis of kinetic traces at 552 nm

| Enzyme  | kₚₑᵈ | kᵣ⁻¹ | Kᵣ for enzyme-NADH complex |
|---------|------|------|-----------------------------|
| Wild-type | 12.3 ± 0.4 × 10⁵ | 7 | s⁻¹ |
| Y72F    | 19.3 ± 0.5 × 10⁵ | 7 | 6 |
| Y356F   | 0.06 | ND | |
| W106F   | 4.4 ± 0.06 × 10² | 95 | |
| T32A*   | ND | ND | |

*ND, not determinable from stopped-flow analysis.

Kinetic constants were determined from absorption transients at 552 nm following the mixing of NADH with wild-type and mutant MR enzymes. Kinetic transients at 552 nm show a characteristic increase followed by a decrease in absorption, and observed rate constants for the formation and decay of the E-NADH charge-transfer complex were determined by fitting to a standard double exponential function (25). The observed rate constant, kᵣ, for formation of the charge-transfer complex was too fast to be measured by the stopped-flow method for the Y356F and T32A enzymes, but a decrease in absorption at 552 nm (charge-transfer decay) was seen for these enzymes, indicating that the charge-transfer complex had formed in the dead time of the stopped-flow instrument. For the other enzymes, values of kᵣ (the second order rate constant for formation of the E-NADH charge-transfer species), kᵣ⁻¹ (the value of the ordinate intercept, minus the limiting rate of flavin reduction), and Kᵣ (the dissociation constant of the E-NADH complex) were calculated from linear plots of kₑᵈ vs. NADH concentration. For a more detailed discussion of the kinetic scheme used to model the data see Ref 17. Reaction conditions: enzymes (20 μM), except for Y72F (10 μM); NADH (100–1200 μM) in 50 mM potassium phosphate buffer, pH 7.0, at 10 °C.

Y72F and W106F enzymes, from which the second order rate constant, kᵣ, for formation of the E-NADH charge-transfer species and the first order rate constant, kᵣ⁻¹ (i.e. the value of the ordinate intercept minus the limiting rate of flavin reduction) from plots of observed rate versus NADH concentration (17) were calculated (Table IV). These plots also enabled calculation of the dissociation constant of the E-NADH complex (Table IV).

Analysis of this type indicated that the dissociation constant for W106F enzyme (230 μM; Table IV) is identical to that measured through analysis of reaction transients at 462 nm (234 μM; Table III), which is also consistent with the increased Kᵣ value for NADH observed in steady-state turnover (Table I). Similar analysis indicated that the dissociation constants for the wild-type and Y72F enzymes were much lower in value (6 and 2 μM, respectively), which is consistent with the independ-
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Table V

| Enzyme | Fast phase | Slow phase |
|--------|------------|------------|
|        | $k_{on}$  | $K_d$      | $k_{low}$ | $K_d$     |
| Wild-type | 0.9 ± 0.02 | 5.7 ± 0.5  | 0.02 ± 0.004 | 6.6 ± 1.3 |
| Y72F    | 1.1 ± 0.02 | 5.8 ± 0.4  | 0.064 ± 0.004 | 4 ± 0.8   |
| Y365F   | 0.5 ± 0.01 | 2.6 ± 0.2  | 0.037 ± 0.002 | 6.8 ± 1.1 |
| W106F   | 0.22 ± 0.01 | 30 ± 2.0  | None       | None      |
| T32A    | 2.54 ± 0.05 | 10.4 ± 0.6 | 0.148 ± 0.008 | None      |

DISCUSSION

Determination of the crystal structure of MR (17) has facilitated interrogation of the reaction mechanism using mutagenesis and kinetic methods, and enabled detailed structural and mechanistic comparisons with the reaction mechanisms of highly related enzymes such as OYE1 and PETN reductase.

Our work described in this report has demonstrated that the conserved tryptophan residue in the active site of OYE family members is not essential for the reduction of $a/b$ unsaturated carbonyl compounds, although in PETN reductase it plays a major role in pathway selection for the degradation of nitroaromatic compounds presumably by influencing the geometry of attack of the hydride ion on the aromatic nucleus of these substrates (38). OYE and MR are known to reduce nitroaromatic substrates, but reduction of these compounds does not proceed through the Meisenheimer reduction pathway (22). One can only infer that other residues in the active site of OYE1 and MR, in addition to Trp-106, influence the geometry of attack of the hydride anion, and that this is not optimal for Meisenheimer formation in OYE1 and MR.

A key aspect of the mechanism of reduction of "generic" substrates such as 2-cyclohexen-1-one is the need to protonate the substrate in addition to catalyzing hydride transfer from the flavin N5 position to substrate (Fig. 1). Our recent kinetic isotope effect studies demonstrated that hydride and proton transfers in MR are concerted, but at the time the identity of the active site acid was unknown (26). Subsequent studies have demonstrated that residue His-186, a ligand to the NADH coenzyme and $a/b$ unsaturated carbonyl compounds, does not function as a proton donor in the oxidative half-reaction (24). In this study, we have attempted to identify active site residues that might participate in proton donation to 2-cyclohexen-1-one. The crystal structure of MR has revealed that Tyr-196 of OYE1, which is known to donate a proton in the oxidative half-reaction of OYE1 (23), is replaced by Cys-191. However, mutagenesis studies have indicated that Cys-191 does not donate a proton to substrate in the oxidative half-reaction of MR (17). The crystal structure of MR indicates that the only remaining candidates for side-chain proton donors in the active

FIG. 6. Stopped-flow traces for the oxidation of wild-type and T32A MR enzymes by codeinone. A, kinetic trace observed at 454 nm for the reaction of the two-electron-reduced T32A MR with codeinone. The absorbance changes were monitored over 0.5 s in the main panel and for 200 s in the inset. The observed kinetic transient is triphasic with rates of 274 s⁻¹, 22 s⁻¹, and 2 s⁻¹ and amplitudes of 0.06, 0.04, and 0.03, respectively. B, kinetic trace observed at 464 nm for the reaction of the two-electron-reduced wild-type MR with codeinone. The absorbance changes were monitored for 1 s. The observed kinetic transient is biphasic with rates of 125 s⁻¹ and 11 s⁻¹ and amplitudes of 0.13 and 0.06, respectively. Reaction conditions: reduced enzyme (20 μM) was mixed with codeinone (2 mM) in 50 mM potassium phosphate buffer, pH 7.0, at 25 °C under anaerobic conditions.
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Site are Tyr-72 and Tyr-356. However, the role of these two residues cannot be inferred from the structure. Both side-chain hydroxyl groups line the active site, but the density for codeine is difficult to unambiguously and significantly that codeine binds in more than one orientation (17); the Tyr-72 OH– and Tyr-356 OH– codeine a carbon distances range from −4 to −7 Å and −3 to −7 Å, respectively, across the range of codeine orientations modeled into the density. In this light of these data we exchanged residues Tyr-72 and Tyr-356 for phenylalanine. Care also needs to be exercised in drawing inferences from the structure of the oxidized enzyme-codeine complex, because it is the reduced enzyme-codeine complex that is the catalytically relevant form. The analysis presented herein, however, reveals that both steady-state and half-reaction kinetics are not substantially perturbed, consistent with the notion that neither of these residues act as key proton donors in a concerted hydride/proton transfer reaction. Our data suggest that solvent acts as a proton donor in the oxidative half-reaction of MR, which is in stark contrast to the mechanism proposed for OYE (23). That said, the possibility remains that Tyr-196 has a more indirect role in the proton transfer reaction in OYE1 by assisting protonation via a solvent water molecule. Likewise, with the Y72F and Y356F MR enzymes we cannot rule out the possibility that proton and hydride are decoupled. Additional work using single and double isotope effect experiments, as described for wild-type MR (26), are required to demonstrate if this is the case.

Our studies with the T32A enzyme have helped to establish the general importance of this residue in the OYE family. Massey and co-workers have demonstrated that mutation of this residue in OYE1 drives the mid point reduction potential of the FMN more negative from −233 mV to −263 mV. These measurements were performed in the presence of reducing coenzymes, whereas the potentiometry work described herein is in the absence of nicotinamide coenzyme. That said, our observations are qualitatively similar to those reported for OYE1, although the change in potential is larger (~50 mV). In the absence of nicotinamide coenzyme, we infer that the mid-point potential for the flavin semiquinone species in both wild-type and T32A enzyme is substantially more negative than ~242 mV and ~290 mV, respectively, because the titration of each enzyme with dithionite does not give rise to any significant semiquinone signature in the flavin absorption spectrum. This contrasts with wild-type OYE1, where ~60% thermodynamic stabilization of the flavin semiquinone is observed, although with the T37A mutant of OYE spectral signature for the flavin semiquinone is lost (41). A key role of Thr-32 (MR) and Thr-37 (OYE1) is to modulate the reduction potential of the FMN and thereby maintain a relatively fast rate of hydride transfer from nicotinamide coenzyme to the flavin. The natural substrates for OYE1 and MR are not known (we infer that morphinone reduction is an adventitious activity exploited by Pseudomonas putida M10 for the purposes of degrading quinate compounds (27)), and thus the kinetics of the oxidative half-reaction with the physiological substrates are not accessible. Although with 2-cyclohexenone, the rate of flavin oxidation in the oxidative half-reaction is less favorable in wild-type enzyme compared with the T32A (MR) and T37A (OYE1) enzymes, one can only infer that the flavin reoxidation rates are sufficiently rapid so as not to compromise the physiological functions of the enzymes.

CONCLUDING REMARKS

We have shown that proton transfer in the oxidative half-reaction of wild-type MR with 2-cyclohexen-1-one, a generic substrate for the OYE family of enzymes, is coupled to hydride transfer and that the proton is most likely derived from solvent water and not an active site amino acid side chain. The highly conserved tryptophan residue has no major role in the reduction of unstaturated carbonyl compounds but is important in pathway selection in some members in the reduction of nitroaromatic substrates. Thr-32 plays a key role in modulating the FMN reduction potential and thereby fine-tunes the kinetics of the reductive and oxidative-half reactions. Our studies have revealed some similarities, but also major differences in the mechanisms of OYE family members, and emphasize the need for caution in inferring mechanism in structurally similar proteins in the absence of supporting solution data.

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Role of Active Site Residues and Solvent in Proton Transfer and the Modulation of Flavin Reduction Potential in Bacterial Morphinone Reductase
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