Morphinone reductase (MR) catalyzes the NADH-dependent reduction of αβ unsaturated carbonyl compounds in a reaction similar to that catalyzed by Old Yellow Enzyme (OYE1). The two enzymes are related at the sequence and structural levels, but key differences in active site architecture exist which have major implications for the reaction mechanism. We report detailed kinetic and solution NMR data for wild-type MR and two mutant forms in which residues His-186 and Asn-189 have been exchanged for alanine residues. We show that both residues are involved in the binding of the reducing nicotinamide coenzyme NADH and also the binding of the oxidizing substrates 2-cyclohexen-1-one and 1-nitrocyclohexene. Reduction of 2-cyclohexen-1-one by FMNH₂ is concerted with proton transfer from an unknown proton donor in the active site. NMR spectroscopy and flavin reoxidation studies with 2-cyclohexen-1-one are consistent with His-186 being unprotonated in oxidized, reduced, and ligand-bound MR, suggesting that His-186 is not the key proton donor required for the reduction of 2-cyclohexen-1-one. Hydride transfer is decoupled from proton transfer with 1-nitrocyclohexene as oxidizing substrate, and unlike with OYE1 the intermediate nitronate species produced after hydride transfer from FMNH₂ is not converted to 1-nitrocyclohexane. The work highlights key mechanistic differences in the reactions catalyzed by MR and OYE1 and emphasizes the need for caution in inferring mechanistic similarities in structurally related proteins.

§ Royal Society Leverhulme Trust senior research fellow.

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Hanan Latif Messiha‡, Andrew W. Munro‡§, Neil C. Bruce‡, Igor Barsukov‡, and Nigel S. Scrutton‡

From the ‡Department of Biochemistry, University of Leicester, University Road, Leicester LE1 7RH and the §Centre for Novel Agricultural Products, Department of Biology, University of York, York YO10 5YW, United Kingdom

Enzyme family (1, 2). This family includes the isoforms of OYE (3), estrogen-binding protein from Candida albicans (4), pentahydrithiol 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). MR is also related to the more complex bile acid-inducible flavoenzymes Bii H and Bii C from Escherichia coli (10), the bacterial Fe/S flavoenzymes tri- and dimethylamine dehydrogenases (11), the histamine dehydrogenase from Nocardiodes simplex (13), and the NADH oxidase of Thermoanaerobium brockii (14). These latter enzymes utilize diverse substrates, but the catalytic framework has clearly evolved from a common progenitor (2). Structures for a number of these enzymes are available including OYE1 (15), PETN reductase (16), MR (17), and the more complex member trimethylamine dehydrogenase (18, 19).

The products of the reactions catalyzed by MR with morphine and codeinone substrates are hydroxymorph and hydrocodeone, respectively. These products are valuable semisynthetic opiate drugs. Hydroxymorphine is a powerful analgesic (seven times more potent than morphine (20)), and hydrocodeone is a mild analgesic and antitussive (21). The synthesis of both compounds is complicated because of difficulty in specifically oxidizing the C-6 hydroxy group of morphine and the often limiting supply of thebaine (a precursor for the synthesis of hydrocodeone). A desire to find alternative routes to their synthesis has led to the development of novel recombinant biocatalytic routes for hydroxymorphone and hydrocodeone synthesis (22). Biological synthesis exploits the ability of Escherichia coli transformed with the genes encoding MR and morphine dehydrogenase and fed with morphine or codeine to accumulate hydroxymorphone and hydrocodeone, respectively (23, 24).

The crystal structure of MR has been determined at 2.2 Å resolution in complex with the oxidizing substrate codeinone (17). The structure reveals a dimeric enzyme comprising two 8-fold βα barrel domains, each bound to FMN. The active site structures of MR, OYE1, and PETN reductase are highly conserved, reflecting the ability of these enzymes to catalyze "generic" reactions such as the reduction of 2-cyclohexen-1-one (25). The active site acid identified in OYE1 (Tyr-196), and conserved in PETN reductase (Tyr-186), 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). The mechanism of flavin reduction and oxidation in MR has been studied by stopped-flow and steady-state kinetic methods (26, 27). Recent studies of 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 (27). The oxidative half-reaction of MR is fully rate-limiting in steady-state turnover with the substrate 2-cyclohexen-1-one and NADH at saturating concentrations (27). The kinetic isotope effect studies for hydride transfer from reduced flavin to the α/β unsaturated bond of 2-cyclohexen-1-one are again consistent with transfer by quantum mechanical tunneling. Additionally, 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, are concerted (27).

The source of the proton in the reduction of 2-cyclohexen-1-one by MR is uncertain. In OYE1, Tyr-196 performs this role (28), but this residue is not conserved in MR (17). In this study we report the isolation and detailed properties of two mutant forms of MR altered at residues His-186 and Asn-189 which have provided new information on the mechanism of the reductive and oxidative half-reactions in MR. Specifically, we demonstrate that (i) both residues are key determinants for the binding of coenzyme and α/β unsaturated carbonyl compounds; (ii) His-186 is unprotonated in oxidized, reduced, and ligand-bound enzyme, thus ruling out a role as the key proton donor in the oxidative half-reaction of the enzyme; and (iii) hydride transfer can be decoupled from proton transfer using the alternative substrate 1-nitrocyclohexene. The work highlights important mechanistic features of MR in its reaction with reducing coenzyme and α/β unsaturated carbonyl compounds and also major differences in reaction chemistry compared with those reactions catalyzed by OYE1.

**EXPERIMENTAL PROCEDURES**

**Materials**—All materials were 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 *E. coli* expressing the enzyme from the cloned morB gene as described previously (17, 29). MR mutant enzymes H186A and N189A did not bind to the affinity column (Mimetic Yellow 2) and were purified using Q-Sepharose resin only, using conditions described previously (17). The mutant MR enzymes were isolated using the QuickChange mutagenesis protocol (Stratagene) and the plasmid template pMORBS1 (1). The mutant genes were sequenced completely to ensure that spurious changes had not arisen during the mutagenesis reaction.

**Kinetic Studies**—All kinetic studies were performed under strict anaerobic conditions (<5 ppm O2) within a glove box environment (Belle Technology) to prevent oxidase activity of MR. Reduced 20 μM stock of MR was then mixed with 2-cyclohexen-1-one or 1-nitrocyclohexene at different concentrations, and flavin oxidation was monitored at 462 nm. Equations used to analyze the reductive and oxidative half-reactions have been described elsewhere (26). In pH dependence studies of the oxidative half-reaction, stopped-flow studies were performed in 55 mM MES, 25 mM Tris, and 25 mM ethanolamine, which allows changes in pH without changing the ionic strength (30). Multiple wavelength studies of MR were performed using XSCAN software (Applied Photophysics), and data were analyzed using Prokin software (Applied Photophysics).

**Ligand Binding Studies**—Ligand binding studies were performed for wild-type MR and the N189A and H186A mutant enzymes. For each enzyme, 10 μM enzyme was titrated with progesterone (2 mM and 10 mM stock solutions) in 50 mM potassium phosphate buffer, pH 7.0, at 25 °C in a 1-ml quartz cuvette, and the absorption spectra of the enzyme were measured after each titration using a Jasco V-550 spectrophotometer until no significant absorbance changes were detected. Readings were monitored at a wavelength where the progesterone and the enzyme had little or no absorbance (at λ = 518 nm), and the absorbance of the free enzyme at λ = 518 nm was subtracted from the individual data for each ligand concentration. The change in absorbance (ΔA) at λ = 518 nm was analyzed as a function of progesterone concentration, and the data were fitted to the quadratic equation (Equation 1) where the dissociation constant (Kd) of the enzyme-ligand complex was calculated.

\[
\Delta A = 4 \cdot [L]_T \cdot (E)_T^5 / (2 \cdot [E])_T^5
\]

where \( \Delta A \) is the maximum absorbance change, \([L]_T\) is the total ligand concentration, \([E]_T\) is the total concentration of binding sites, and \(K_d\) is the dissociation constant.

**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 ml of titration buffer) were electrochemically titrated according to the method of Dutton (31) 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. 5 μM 2-hydroxy-1,4-naphthaquinone, 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 (ThermoRussell Ltd.) that had been calibrated previously using the Fe3+/Fe2+ /EDTA couple as a standard (+108 mV). The calomel electrode was corrected by +244 ± 2 mV relative to the standard hydrogen electrode. The electrode was allowed to stabilize between each addition of oxidant or reductant, and spectra (300–700 nm) were recorded using a Cary 50 Probe UV-visible scanning 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 5–7-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 (32). Plots of the absorbance against redox potential were analyzed by extension of the Nernst equation and the Beer-Lambert law using Equation 2,

\[
A_{462} = \frac{(a \cdot b0 \cdot 10^{E0 \cdot (E-E_{1/2})})}{T + 10^{E0 \cdot (E-E_{1/2})}}
\]

where \(A_{462}\) is the absorbance value at 462 nm at the electrode potential \(E\), and \(a\) and \(b\) are the absorbance values of the fully oxidized and reduced enzyme at 462 nm, respectively. \(E_{1/2}\) is the midpoint potential. Data manipulation and analysis were performed using Grafit software package version 5.0. All redox potentials are given relative to the standard hydrogen electrode.

**Nuclear Magnetic Resonance spectroscopy**—For NMR spectroscopy, wild-type and H186A enzymes were grown in modified 2M9 medium. 15N-Labeled enzyme and partially deuterated 15N-labeled (85% deuterated) samples were prepared. For NMR studies, the purified enzymes were filtered and eluted twice through an NAP-5 column (Amersham Biosciences) that had been equilibrated previously with deuterated 50 mM potassium phosphate buffer, pH 7.0 (made from 50 mM potassium phosphate) and deuterium ox还原

formation and decay of the enzyme-NADH charge-transfer species were monitored at 552 nm (26). In studies of the oxidative half-reaction, MR was titrated with sodium dithionite to produce the two-electron-reduced form of MR. Reduced 20 μM enzyme was then mixed with 1-cyclohexene-1-one or 1-nitrocyclohexene at different concentrations, and flavin oxidation was monitored at 462 nm. Equations used to analyze the reductive and oxidative half-reactions have been described elsewhere (26). In pH dependence studies of the oxidative half-reaction, stopped-flow studies were performed in 55 mM MES, 25 mM Tris, and 25 mM ethanolamine, which allows changes in pH without changing the ionic strength (30). Multiple wavelength studies of MR were performed using XSCAN software (Applied Photophysics), and data were analyzed using Prokin software (Applied Photophysics).
phosphite buffer in D$_2$O; the pH was adjusted to 6.6 by phosphoric acid then lyophilized and dissolved in the required amount of D$_2$O. $^{15}$N-Labeled enzymes were concentrated to 400 mM, and the partially deuterated $^{15}$N-labeled enzymes were concentrated to 800 mM.

NMR spectra were acquired at 600 MHz on a Bruker DRX600 spectrometer at 30 °C. The long range HMQC experiments for detection of His resonances and some of the HSQC spectra were recorded using a CryoProbe (Bruker) to improve the sensitivity. Two types of experiment were recorded: (i) two-dimensional $^{15}$N HSQC (33, 34) performed on the $^{15}$N-labeled enzymes (400 mM), and (ii) long range $^{15}$N HMQC optimized for detection of $^{15}$N,$^1$H,$^1$H,$^1$H,$^1$H,$^1$H,$^1$H,$^1$H,$^1$H,$^1$H correlrelations between carbon-bound protons and $^{15}$N nuclei in His aromatic rings (35) performed on the partially deuterated $^{15}$N-labeled enzymes (800 μM) and on $^{15}$N-labeled enzymes in some experiments. All experiments employed water flip-back pulses (36) in combination with the WATERGATE method for water suppression (37) to minimize saturation of the water signal. $^{15}$N HSQC spectra were acquired with sweep widths of 7,878 × 12,165 Hz ($^1$H, $^{15}$N) using 1,024 × 220 complex data points and processed to 2,048 × 1024 real data points. Long range $^{15}$N HMQC spectra were acquired with sweep widths of 7,788 × 12,165 Hz ($^1$H, $^{15}$N) using 512 × 100 complex data points and processed to 2,048 × 1,024 real data points. The $^{15}$N offset was set to 175 ppm to maximize the intensities of His resonances. The spectra were processed and analyzed using XWINNMR 3.5 software (Bruker) and were referenced to the external standard at 0.000 ppm. The $^{15}$N reference was calculated using $^{15}$N/H γ ratios of 0.101329118.

In a second series of experiments, the change in the two-dimensional $^{15}$N HSQC spectra was recorded for 500 mM enzymes titrated with 2-cyclohexen-1-one and progesterone. A stock solution of 500 mM 2-cyclohexen-1-one in deuterated potassium phosphate buffer pH 7.0 was used to titrate the enzymes across a concentration range of 0.5–10 mM. For titrations with progesterone, a stock solution of 10 mM progesterone (in deuterated methanol) was used. Changes in two-dimensional $^{15}$N HSQC spectra for wild-type MR as a result of enzyme reduction were also recorded where 500 mM wild-type enzyme was reduced with an excess of sodium dithionite under anaerobic conditions and introduced into the NMR tube, which was then sealed inside an anaerobic glove box.

RESULTS

Active Site Structure and Implications for Catalysis—His-186 and Asn-189 form part of the active site of MR and are located close to the redox active region of the FMN isoalloxazine ring (i.e. the flavin N5–N1 subnucleus). The residues are conserved in other related enzymes, having a histidine residue at the corresponding position of His-186 and an asparagine or histidine at the corresponding position of Asn-189 (Fig. 1A). A reduction mechanism for αβ unsaturated carbonyl compounds such as 2-cyclohexen-1-one and progesterone, a stock solution of 500 mM 2-cyclohexen-1-one in deuterated potassium phosphate buffer pH 7.0 was used to titrate the enzymes across a concentration range of 0.5–10 mM. For titrations with progesterone, a stock solution of 10 mM progesterone (in deuterated methanol) was used. Changes in two-dimensional $^{15}$N HSQC spectra for wild-type MR as a result of enzyme reduction were also recorded where 500 mM wild-type enzyme was reduced with an excess of sodium dithionite under anaerobic conditions and introduced into the NMR tube, which was then sealed inside an anaerobic glove box.

and mutant enzymes revealed that a semiquinone signal was not observed during reductive or oxidative titrations, suggesting that the potential of the oxidized/semiquinone couple is much lower than the semiquinone/hydroquinone couple. Fitting of absorption data measured at the flavin peak (~460 nm) to Equation 2 gave potentials of $-242 ± 6$ mV (wild-type MR), $-214 ± 7$ mV (H186A), and $-236 ± 6$ mV (N189A) for the $E_{1/2}$ potential of the flavin (Fig. 2B).

Steady-state kinetic studies of the H186A and the N189A enzymes were performed using 2-cyclohexen-1-one as oxidizing substrate under anaerobic conditions to prevent adventitious oxidase activity. To measure the apparent kinetic constants of the H186A mutant enzyme with the substrate 2-cyclohexen-1-one, the initial velocities of the reaction were determined at 150 μM β-NADH and various concentrations of 2-cyclohexen-1-one. The initial velocities measured were very low and within values obtained with control experiments performed in the absence of 2-cyclohexen-1-one; initial velocities were also independent of 2-cyclohexen-1-one concentrations (up to 100 mM 2-cyclohexen-1-one), suggesting that the mutant is essentially inactive under steady-state turnover conditions. Activity could not be rescued for the H186A enzyme by the addition of imid-
The apparent kinetic constants of the N189A mutant enzyme for 2-cyclohexen-1-one were derived by fitting data to a steady-state rate expression that incorporates substrate inhibition (Equation 3).

\[ v = \frac{V_{\text{max}} K_m}{[S] + K_i \cdot [S]} \]  

A \( k_{\text{cat}} \) value of 0.089 ± 0.004 s\(^{-1}\) (0.80 ± 0.01 s\(^{-1}\)) for wild-type MR; (17)), \( K_m \) value of 2.2 ± 0.2 mM (4.2 ± 0.2 mM for the wild-type enzyme; (17)), and \( K_i \) value of 13.8 ± 1.4 mM were obtained. The apparent kinetic constants of the N189A enzyme were also measured for \( \beta\)-NADH using 1 \( \mu\)M enzyme at a fixed concentration of 2-cyclohexen-1-one (6 mM). A hyperbolic dependence was observed (Fig. 2C, inset) and fitting to the Michaelis-Menten equation yielded a \( k_{\text{cat}} \) value of 0.083 ± 0.003 s\(^{-1}\) (0.88 ± 0.01 s\(^{-1}\)) for wild-type (17)) and \( K_m \) value of 119.3 ± 9.1 \( \mu\)M (8.9 ± 0.6 \( \mu\)M for wild-type (17)).

**Stopped-flow Kinetic Studies of the Reductive Half-reaction in H186A and N189A Enzymes**—The reductive half-reactions of the H186A and N189A enzymes were studied by multiple wavelength and single wavelength stopped-flow spectroscopy to ascertain the effect of each mutation on flavin reduction. Comparable studies for wild-type MR have been reported elsewhere (26, 27). For the H186A enzyme, multiple wavelength analysis of the kinetics of flavin reduction on mixing 20 \( \mu\)M enzyme with 200 \( \mu\)M \( \beta\)-NADH was found to be slow (Fig. 3A), and, unlike wild-type enzyme (26), there was no evidence at long wavelength for a NADH-oxidized enzyme charge-transfer complex. Reduction of the flavin was not complete, and higher concentrations of \( \beta\)-NADH (at least 30-fold higher than the enzyme concentration) were required to achieve full reduction (Fig. 3A, inset). In single wavelength mode, absorption changes were not observed at 552 nm on mixing H186A with NADH, thus confirming the observations made using photodiode array spectroscopy. This wavelength has been used previously for wild-type MR to provide evidence for the formation and decay of an oxidized enzyme-NADH charge-transfer species. The observed rate of flavin reduction for the H186A mutant was linearly dependent on \( \beta\)-NADH concentration and unaffected by the presence of imidazole in the reaction buffer (Fig. 3B). Although with H186A a limiting rate could not be defined, it is clear that the observed rates for flavin reduction in H186A are compromised compared with wild-type enzyme (at 4.5 mM NADH, the observed rate constant is 1.8 s\(^{-1}\) versus a limiting rate of 22 s\(^{-1}\) for H186A and wild-type, respectively). It is important to emphasize that the value of 1.8 s\(^{-1}\) for H186A MR is not a limiting value, and saturation behavior is not observed with respect to NADH concentration (Fig. 3B). We infer that the effect of the H186A mutation is to increase substantially the dissociation constant for the enzyme-NADH complex.

For the N189A mutant enzyme, under pseudofirst order conditions (reduction of 20 \( \mu\)M N189A mutant enzyme with 200 \( \mu\)M \( \beta\)-NADH), the rate of formation of the oxidized enzyme-NADH charge-transfer species was too rapid to enable capture of the long wavelength absorption increases using a photodiode array detector (first acquisition at 1.28 ms) (Fig. 3C). Similar results were obtained in multiple wavelength mode with a stoichiometric mix of 20 \( \mu\)M enzyme and 20 \( \mu\)M NADH, although the extent of flavin reduction is less in this case (Fig. 3C). Absorbance changes associated with charge-transfer formation and decay were, however, detected in single wavelength mode (shorter acquisition time of 1 ms from mixing) under similar reaction conditions. Single wavelength absorption transients collected at 552 nm showed a positive and negative absorption change (Fig. 3D). The rates of charge-transfer formation were too fast.
to be measured accurately. The rate of charge-transfer decay was found to be similar to the rate of flavin reduction measured at 466 nm, showing that flavin reduction and decay of the charge-transfer intermediate are kinetically the same process. The rate of flavin reduction measured at 466 nm was determined using different β-NADH concentrations. Unlike for the wild-type enzyme, the transients obtained for flavin reduction in the N189A mutant enzyme (Fig. 3E) were biphasic. Observed rates for both the fast and slow phases were found to be dependent on β-NADH concentration (up to 400 μM) and then independent of β-NADH at higher concentrations (Fig. 3F). The origin of the two phases is uncertain but might reflect different binding modes for the reducing coenzyme. The amplitudes of each phase contribute 50% of the total absorbance change.
of 22 s\(^{-1}\) for wild-type enzyme. The dissociation constant (\(K_d\)) for the enzyme-\(\beta\)-NADH complex for the N189A mutant is high (\(>200 \mu M\)) compared with a value of 6 \(\mu M\) for wild-type enzyme. As with H186A, the data confirm that Asn-189 is a key binding determinant for reducing coenzyme in MR.

**Stopped-flow Kinetic Studies of the Oxidative Half-reaction of H186A and N189A MR with 2-Cyclohexen-1-one**—The oxidative half-reactions of the H186A and N189A enzymes with the substrate 2-cyclohexen-1-one were studied by stopped-flow photodiode array spectroscopy under anaerobic condition. Spectral changes associated with the reoxidation of dithionite-reduced enzyme by 40 mM 2-cyclohexen-1-one and analysis by global fitting indicated that a single step describes the reoxidation process (Fig. 4, A and B). For the H186A mutant enzyme, the observed rates of flavin reoxidation were very slow (\(\sim 0.004 \text{ s}^{-1}\) compared with 0.9 s\(^{-1}\) for the limiting rate of reoxidation seen for wild-type enzyme (17)) and were similar to rates from control experiments performed using the stopped-flow apparatus in the absence of substrate. Moreover, observed rates were independent of 2-cyclohexen-1-one concentration (Fig. 4C), suggesting that the reoxidation is not substrate mediated but attributable to adventitious oxidants in the solution (e.g., residual oxygen in buffers). Clearly, mutation of His-186 to alanine has a major effect on the oxidative half-reaction. This is consistent with the lack of activity in steady-state turnover experiments with the H186A enzyme. Again, attempts to rescue activity in the oxidative half-reaction with 50 mM imidazole were unsuccessful. For the N189A mutant, analysis of the concentration dependence of the observed rates of flavin reoxidation showed a hyperbolic dependence (Fig. 4D) giving a limiting rate constant, \(k_{\text{lim}}\), of 0.093 ± 0.005 s\(^{-1}\) and a dissociation constant for the reduced enzyme-2-cyclohexen-1-one complex of 80 ± 8 mM. These compare with wild-type values of 0.9 s\(^{-1}\) for \(k_{\text{lim}}\) and 5.7 mM for \(K_d\) (17).

**Ligand Binding in Wild-type MR and the H186A and N189A Enzymes**—To investigate the role of His-186 and Asn-189 in substrate binding, ligand binding studies were performed with the wild-type, H186A, and the N189A mutant enzymes. 2-Cyclohexen-1-one cannot be used in such studies because of the lack of any absorbance change of the flavin elicited on binding of this substrate to the enzyme. The inhibitor progesterone, however, is a substrate analog and in PETN reductase is known to bind to the enzyme in a way similar to 2-cyclohexen-1-one with the carbonyl functional group bound to two histidine residues that correspond to His-186 and Asn-189 in MR (16, 38). The absorbance changes accompanying the titration of the wild-type and mutant MR enzymes with progesterone are shown in Fig. 5 (A–C). For each enzyme, the change in absorbance at 518 nm was plotted as a function of progesterone concentration, and the data were fitted to Equation 1 (Fig. 5, D–F). The \(K_d\) values calculated were 0.79 ± 0.07 \(\mu M\) for the wild-type enzyme, 48.2 ± 12 \(\mu M\) for the H186A mutant, and 9.2 ± 0.5 \(\mu M\) for the N189A mutant enzyme. Weaker binding is observed with the H186A enzyme, and the error obtained from fitting the H186A data to Equation 1 is large. Fitting to a standard hyperbolic expression produced a \(K_d\) value of 32.3 ± 1.7 \(\mu M\) (Fig. 5E).

**NMR Spectroscopic Studies of the Wild-type and H186A MR**—The kinetic data presented above established that the H186A enzyme is compromised substantially in the oxidative half-reaction. To rule out a role for His-186 in proton donation to the substrate, the protonation state of this residue was investigated by NMR spectroscopy using long range \(^1H, ^{15}N\) HMBC NMR experiments optimized for histidine ring observation. Wild-type MR has seven histidine residues; it was anticipated that assignment of His-186 could be made from single bond \(^1H, ^{15}N\) HSQC and long range \(^1H, ^{15}N\) HMBC NMR experiments carried out on wild-type and H186A enzymes.
Long range $^1$H, $^{15}$N HMQC experiments optimized for signals originating from histidine rings allow one to correlate nonexchangeable carbon-bound H$^\alpha$ and H$^\beta$ protons with N$^\alpha$ and N$^\beta$ atoms through two-bond $^3$J(H$^\alpha$, N$^\beta$), $^3$J(H$^\beta$, N$^\beta$) and $^3$J(H$^\alpha$, N$^\beta$) couplings. This leads to the detection of H$^\alpha$/N$^\alpha$, H$^\beta$/N$^\beta$, and H$^\beta$/N$^\beta$ cross-peaks in a clearly identifiable rectangular pattern (35). Additionally, a weaker cross-peak H$^\alpha$/N$^\beta$ caused by the correlation through a smaller $^3$J(H$^\alpha$, N$^\beta$) coupling can be observed for small proteins or highly mobile histidine side chains. Given the size of MR, to minimize signal losses caused by increased relaxation, random fractional deuteration at an average 85% deuterium content was used. Additionally, the protein sample was dissolved in 100% D$_2$O to remove contributions from solvent exchangeable protons, which is particularly strong for histidine ring systems because of the short distance between H$^\alpha$ and H$^\beta$ protons. Seven histidine ring spin systems were identified in the long range HMQC spectrum of partially deuterated $^{15}$N-labeled wild-type enzyme, corresponding to seven histidine residues in the protein (Fig. 6A). Four spin systems (marked 1, 2, 5 and 6) only exhibit H$^\alpha$/N$^\beta$ and H$^\beta$/N$^\beta$ cross-peaks, making it impossible to assign nitrogen chemical shifts to the specific position. Spin systems 1 and 2 correspond to a positively charged histidine with both sites protonated, as evidenced by the relatively low values of both $^{15}$N chemical shifts. Spin system 6 corresponds to a neutral histidine ring. Cross-peaks of spin system 5 are severely broadened in the nitrogen dimension, and chemical shift values are intermediate between those for protonated and nonprotonated nitrogens; this is typical for a fully exposed His ring with fast exchange of nitrogen-bound protons with solvent. Three spin systems 3, 4, and 7 show an additional cross-peak H$^\alpha$/N$^\alpha$ that allows one to assign nitrogen chemical shifts to the specific position in the ring and thus to determine the positions of the protons. All three spin systems correspond to neutral rings, protonated on N$^\alpha$ for spin systems 3 and 4, and on N$^\beta$ for spin system 7.

Spin systems identified in the long range $^1$H, $^{15}$N HMQC spectrum were correlated with single bond $^1$H/$^{15}$N cross-peaks of histidine rings as shown in Fig. 6A. These cross-peaks were registered in $^1$H, $^{15}$N HSQC spectrum for a fully protonated, $^{15}$N-labeled wild-type MR (500 $\mu$M) dissolved in H$_2$O for observation of the exchangeable HN protons. Five cross-peaks are observed in the spectral region characteristic for histidine side chain resonances (Fig. 6A). $^{15}$N chemical shifts of four cross-peaks match with some of the spin systems identified in the long range HMQC experiment. The match is unambiguous for spin systems 1, 4, and 6, whereas peak 7 can be connected to either spin system 2 or 7 in the long range HMQC spectrum. An additional cross-peak is observed in the same region with the nitrogen chemical shift not matching any of the histidine spin systems. This cross-peak can thus be assigned as FMN H5/N3 correlation, which is the only additional moiety that has a $^{15}$N chemical shift in this region of the spectrum. The HN protons of three other histidine side chains are in fast exchange with solvent and have no observable cross-peaks in the single bond $^1$H, $^{15}$N HSQC spectrum.

The spin system of His-186 was identified through the H186A mutation. Because of the low expression level for the mutant in the deuterated medium, fully protonated H186A mutant enzyme sample (~900 $\mu$M) dissolved in D$_2$O was used. A large number of backbone HN protons have slow solvent...
FIG. 6. NMR spectra of wild-type MR enzyme (black) and the H186A enzyme (red). The spectra in A are the single bond \([1\text{H}, ^{15}\text{N}]\) HSQC spectra of 500 \(\mu\text{M}\) \(^{15}\text{N}\)-labeled enzymes acquired with sweep widths of 17,985 × 12,165 Hz \([1\text{H}, ^{15}\text{N}]\) using 1,024 × 230 complex data points and processed to 2,048 × 1,024 real data points. The spectra in B are the long range \([1\text{H}, ^{15}\text{N}]\) HMQC spectra for the \(^{15}\text{N}\)-labeled partially deuterated enzymes (800 \(\mu\text{M}\) for wild-type and 900 \(\mu\text{M}\) for H186A mutant) acquired with sweep widths of 7,788 × 12,165 Hz \([1\text{H}, ^{15}\text{N}]\) using 512 × 100 complex data points and processed to 2,048 × 1,024 real data points. The spin systems identified in B are correlated with the cross-peaks of histidine rings in A with lines. The inset in A is an illustration of the histidine ring (the double bond and the protons are not shown because they are changing with the protonation state). The spectra were acquired at 600 MHz on a Bruker DRX600 spectrophotometer at 30 °C in deuterated 50 mM potassium phosphate buffer, pD 7.0 (pH 6.6), processed and analyzed using XWINNMR 3.5 software.
exchange rates, leading to the incomplete exchange with solvent even after several days at room temperature. Intense signals from these protons, present in the long range HMQC experiment, lead to spectral artifacts, as can be seen in Fig. 6B. These artifacts were easily identifiable from broad line shapes and did not interfere with the detection of the histidine ring cross-peaks. The mutation causes the disappearance of spin system 6 in the long range HMQC spectrum, whereas other spin systems were observed in similar positions (in red) marked with arrows (Fig. 6B). The corresponding cross-peak 6 is also missing in the single bond HSQC spectrum. In this spectrum, chemical shift changes are observed for cross-peak 4, in agreement with the shift changes of the corresponding spin system in the long range HMQC spectrum and for the FMN cross-peak. The observed spectral changes upon mutation allow assigning spin system 6 to the aromatic ring of His-186. The nitrogen chemical shifts observed for this residue clearly indicate that His-186 ring is neutral. The position of the proton in the aromatic ring cannot be identified because of the missing H^6/N^6 cross-peak in the long range HMQC spectrum.

The observation of two distinct cross-peaks for neutral imidazole rings is consistent with a stable tautomeric state and slow exchange of the H^9 protons with the solvent under the conditions used (30 °C, pD 7.0/pH 6.6), as expected for buried structural histidines. Additional evidence for the negligible exchange contribution is derived from the comparison of the long range HMQC spectra at 30 °C and 20 °C (Fig. 7B). The positions of the resonances remain unchanged, whereas the intensities of the cross-peaks decrease significantly because of the increase of the overall rotational correlation time. The change of pD from 7.0 to 6.5 (pH 6.6 to 6.1) has no effect on the 15N chemical shifts of all imidazole rings apart from the exposed ring 5 (Fig. 7C), demonstrating that the rings 3, 4, 6, and 7 remain neutral above pD 6.5 (pH 6.1). The 15N chemical shifts of the exposed ring 5 decrease, in agreement with the transition toward the fully protonated state. The line widths of the reso-
nances increase noticeably between pH 7.0 (pD 6.6) and pD 6.5 (pH 6.1) as can be seen in Fig. 7, D and E. A further decrease in pD to 6.0 (pH 5.6) results in dramatic broadening of all resonances (Fig. 7F) making it impossible to register a long range HMQC spectrum. This observation suggests that the protein structure is destabilized at low pD, which can also lead to aggregation. The likely cause of such destabilization is the titration of some of the histidine side chains, indicating that the protonated state is unfavorable for some buried histidines. This observation agrees well with the decrease of the enzyme activity at pH 5.6.

**NMR Studies of the Effect of Substrate Binding and Enzyme Reduction on His-186**—Given that His-186 is likely involved in substrate binding, the His-186 signal in the NMR spectrum should be affected in the presence of substrates. The protonation state might also be affected during enzyme reduction and ligand binding. For this reason, the single bond HSQC spectra of wild-type MR and the H186A enzyme were acquired in the absence and presence of substrates. The change in the single bond HSQC spectra of 500 μM wild-type MR and 500 μM H186A enzyme were recorded in the presence of 2-cyclohexen-1-one (a range of 0.5–10 mM concentrations was used). In the presence of 2-cyclohexen-1-one, the enzyme was unstable and precipitated over the course of a few hours preventing the collection of spectra over long periods of time. The spectra that were obtained were also very broad (data not shown). For this reason, progesterone, which is an alternative αβ unsaturated carbonyl substrate and thought to bind in a manner similar to 2-cyclohexen-1-one through interactions with His-186 and Asn-189, was used to probe the effects of binding αβ unsaturated carbonyl substrates on the properties of His-186. The structure of the enzyme-progesterone complex in the highly homologous PETN reductase indicates that the carbonyl group of progesterone interacts with the corresponding histidine residue (16), similar to that found with the enzyme-2-cyclohexen-1-one complex (38). Progesterone was added to wild-type MR and the H186A enzyme (500 μM) in a ratio of 1:0.5 and 1:1.2 enzyme:progesterone.

Progesterone binding leads to specific localized changes in the HSQC spectrum of wild-type MR (Fig. 8A). In the spectral region corresponding to the aromatic HN protons of histidine side chains and of the FMN ring system, addition of progesterone only affects the cross-peaks of His-186 and FMN. At a 1:0.5 enzyme:substrate ratio (data not shown) these two cross-peaks are not detectable because of strong exchange broadening; the signals reappear at a 1:1.2 enzyme:substrate ratio (shown in red in Fig. 8A). Two cross-peaks are observed for each HN group of His-186 and FMN in the complex, indicating the presence of two substrate-bound forms of MR with a population ratio ~1:3, as estimated from the relative cross-peak intensities. The smaller cross-peaks are located close to the corresponding cross-peaks of the free enzyme, reflecting a relatively small change in the environment of the HN groups for the minor form upon complex formation. The major form shows larger changes in the environment of both groups on substrate binding, as follows from the larger chemical shift differences between the free and the bound signals. Corresponding changes were observed in the long range HMQC spectra for spin system 6 (data not shown), confirming the correlation between the cross-peaks in the single bond and long range HSQC experiments. The protonation state of the His-186 ring does not change on complex formation as follows from the values of the 15N chemical shifts. In the single bond HSQC spectrum 15N chemical shifts of the major His-186 and FMN peaks in the complex have similar values, which prevents the use of the long range HSQC spectrum for the direct identification of the His-186 cross-peak. However, the peaks can be assigned from the intensity ratio of the minor and major components. This ratio is equal to the population difference between the two components and should be the same for both sets of cross-peaks. Similar intensity ratios of ~1:3 can only be obtained if the peaks are assigned as shown in Fig. 8A.

Additional evidence for the assignment of His-186 comes from titration of the H186A mutant with progesterone (Fig. 8B). In this case, a progressive chemical shift change is observed for the FMN cross-peak with no indication of multiple states. The direction of the chemical shift changes is similar to those observed for the wild-type enzyme, supporting the assignments based on the cross-peak intensities. The rate of exchange of progesterone between the free and the bound states can be estimated from the chemical shift differences in the free and bound forms. The spectral changes upon progesterone addition for the wild-type enzyme, with strong broadening at subequimolar ratios, corresponds to the intermediate progesterone exchange between the free and the bound states. This condition is satisfied when the exchange rate is comparable with the chemical shift difference between the free and the bound states. These changes are ~300 Hz for both FMN and His-186 cross-peaks, leading to the estimated exchange rate of ~300 s⁻¹. In the case of the H186A mutant, no detectable broadening of the cross-peak was observed at subequimolar substrate concentrations, implying a significantly faster exchange of progesterone between the free and the bound forms for the mutant.

Enzyme reduction by sodium dithionite had an effect on the same groups of cross-peaks in the HSQC spectrum of the wild-type enzyme similar to that observed in binding the enzyme with substrate. In the spectral region corresponding to the aromatic HN protons of histidine side chains and of the FMN ring (Fig. 9) only FMN and His-186 cross-peaks were affected. The changes in the position of the His-186 cross-peak were in a direction similar to the changes caused by complex formation. Two conformational forms were observed in the reduced enzyme, as evidenced by the presence of two cross-peaks for both His-186 and FMN in the HSQC spectrum. The population ratio between the forms is 1:3–1:4, as follows from the relative cross-peak intensities. Both forms correspond to reduced MR, as the chemical shifts of the FMN moiety are significantly different from those of both forms of the oxidized state. In the minor form, the environment of His-186 is similar to that of the oxidized state, resulting in a similar position of the HSQC cross-peak. No change in the reduced state was observed after 24 h in the sealed NMR sample tube. These studies reveal that His-186 is unprotonated in the oxidized and reduced states, thus ruling out a role for His-186 as an active site acid during reduction of the olefinic bond of 2-cyclohexen-1-one.

**Dependence of Reaction Rate on Solution pH for the Oxidative Half-reaction of Wild-type MR**—The oxidative half-reaction of wild-type MR was studied as a function of solution pH (range 5.5–9.0), at constant ionic strength, with 2-cyclohexen-1-one as substrate. The limiting rate constant (klim) for flavin oxidation and the dissociation constant (Kd) for the reduced enzyme-2-cyclohexen-1-one complex are independent of solution pH in the range 6–9; the values calculated were in the region of 0.83–1.1 s⁻¹. At pH 5.5, the klim value decreased to 0.22 s⁻¹. However, at this pH value MR is relatively unstable, and the enzyme had a tendency to precipitate, reflected in smaller amplitudes in stopped-flow kinetic transients. The Kd values across the pH range for 2-cyclohexen-1-one were in the range of 7.2–13.2 mM. The data suggest that His-186 remains unprotonated in the pH range studied because protonation of this residue in the lower pH regime would be expected to perturb the measured kinetic parameters.
Reaction of MR with 1-Nitrocyclohexene—1-Nitrocyclohexene has been shown to be a good substrate for OYE (28, 39). The reaction is stepwise, involving an initial hydride transfer to form a nitronate intermediate, followed by proton transfer to form nitrocyclohexane. Similar studies have not been performed previously with MR. 1-Nitrocyclohexene offers the pos-
 FIG. 9. The single bond [1H, 15N] HSQC spectra of 15N-labeled wild-type MR in the oxidized and reduced states. Spectra for 500 μM oxidized enzyme and 500 μM dithionite-reduced enzyme are shown in black and red, respectively. The spectra were acquired at 600 MHz on a Bruker DRX600 spectrophotometer at 30 °C in 10% deuterated 50 mM phosphate buffer, pH 7.0 (pH 6.6), with sweep widths of 17,985 × 12,165 Hz [1H, 15N] using 1,024 × 230 complex data points, processed to 2,084 real data points, and analyzed using XWIN-NMR 3.5 software.

TABLE I

|                          | OYE1a | H191N | H191N/H194H |
|--------------------------|-------|-------|-------------|
| Reductive half-reaction   |       |       |             |
| With NADPH               |       |       |             |
| $k_{\text{cat}}$ NADPH   | 5.1 s⁻¹ | 5.4 s⁻¹ | 78 s⁻¹      |
| With NADH                |       |       |             |
| $k_{\text{cat}}$ NADH    | 0.9 M⁻¹ s⁻¹ | 0.03 s⁻¹ | 4 s⁻¹       |
| Oxidative half-reaction   |       |       |             |
| With 2-cyclohexen-1-one  |       |       |             |
| $k_{\text{cat}}$ 2-cyclohexen-1-one | 102 s⁻¹ | 1.4 s⁻¹ | ~100 M⁻¹ s⁻¹ |
| With 1-nitrocyclohexane  |       |       |             |
| $k_{\text{cat}}$ 1-nitrocyclohexane | 6.1 × 10² M⁻¹ s⁻¹ | NDb | ND |

a Data for OYE1 were taken from Refs. 28 and 40.

b ND, not determined.

sibility of decoupling hydride and proton transfer in MR, which is concerted in reactions with 2-cyclohexen-1-one (27). In multiple wavelength stopped-flow measurements, 20 μM MR was reduced with sodium dithionite, and spectral changes accompanying reoxidation of the flavin by 1 mM 1-nitrocyclohexene were analyzed by global analysis and numerical integration methods and found to fit best to a two-step kinetic model, $A \rightarrow B \rightarrow C$ (Fig. 10A). Spectrum A represents dithionite-reduced enzyme, and spectrum B is an oxidized enzyme species. A smaller spectral change then gives rise to species C, the origin of which is uncertain.

The dependence of each kinetic phase on 1-nitrocyclohexene concentration was measured by single wavelength stopped-flow spectroscopy at 462 nm (Fig. 10A, inset). The observed rate constant for the fast (major) phase is hyperbolically dependent on 1-nitrocyclohexene concentration giving a limiting value of 7.78 ± 0.24 s⁻¹ and a dissociation constant of 1,090 ± 70 μM. The second observed rate constant (for the minor kinetic phase) was independent of 1-nitrocyclohexene at lower concentrations, but some dependence was seen at higher 1-nitrocyclohexene concentrations. 1-Nitrocyclohexene is volatile, which presents some technical difficulties in scrubbing solutions free of oxygen. For this reason, the substrate might contain traces of oxygen even after it has been left to equilibrate and diluted with buffer. This might explain the increase in the observed rate constant for the slow phase when using higher concentrations of the substrate. It is clear that 1-nitrocyclohexene is a good substrate for MR: the oxidative half-reaction of the enzyme with 1-nitrocyclohexene is faster than with 2-cyclohexen-1-one (see Table II).

A NADH-generating system consisting of NAD, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase, was used to monitor the spectral changes accompanying reduction of 1-nitrocyclohexene ($\lambda_{\text{max}} = 270$ nm) by MR. The reaction proceeds with an increase in absorbance in the 230 nm region (Fig. 10B) representing formation of the nitronate intermediate (Fig. 10C). Unlike with OYE1, however, conversion of this intermediate to the protonated form of nitrocyclohexane ($\lambda_{\text{max}} = 205$ nm) was not observed. With MR, the nitronate intermediate was at a maximum concentration at 23 min under the conditions used. Thereafter, the absorption peak declined (Fig. 10B). The decline of the nitronate absorption peak is attributed to degradation of the nitronate species. With the Y169F mutant of OYE1 in which protonation of the nitronate species is prevented, the nitronate species was formed, but conversion to nitrocyclohexane was prevented (39). However, the absorption peak for the nitronate intermediate also declined with a rate similar to that observed with wild-type MR, suggesting a nonenzymic degradation.
With the N189A MR enzyme, the nitronate intermediate was produced, as observed with wild-type enzyme. The rate of formation of the nitronate species is slower in the N189A enzyme with 1-nitrocyclohexene than with wild-type enzyme. This is consistent with the lack of activity with 2-cyclohexen-1-one. Subsequent studies of the properties of the single mutant H191N and the double mutant H191N/N194H of OYE1 indicated a role for these residues in binding 2-cyclohexen-1-one and phenolic ligands and smaller effects on enzyme reduction by reducing coenzyme (40). Likewise, crystallographic studies of PETN reductase in complex with steroid substrates 2,4-dinitrophenol and picric acid have also confirmed the importance His-181 and His-184 in forming H bonds with the carbonyl and hydroxy groups of these ligands (16, 38), but no quantitative solution data are available to assess the importance of these interactions in ligand binding. We chose to isolate the H186A and N189A MR enzymes because, unlike with studies of the H191N and H191N/N194H OYE1 enzymes (40), the introduction of alanine residues restores potential hydrogen bonding interactions with the ligand. This therefore enables quantitative assessment of the role of these interactions in binding and kinetics. Tables I and II

**FIG. 10. Reaction of MR with 1-nitrocyclohexene.** A, multiple wavelength stopped-flow analysis of the oxidative half-reaction of wild-type MR with 1-nitrocyclohexene. Main panel, the spectral intermediates obtained from global fitting of time-dependent data. The solid line represents species A (dithionite-reduced enzyme), the dashed line is species B (an oxidized enzyme species), and the dotted line is species C (also an oxidized enzyme species that forms slowly). Inset, dependence of the observed rates for the fast phase (species A to B; filled circles) and slow phase (species B to C; unfilled circles) on 1-nitrocyclohexene concentration. Conditions: 20 μM dithionite-reduced MR and 1 mM 1-nitrocyclohexene were mixed at 25 °C in 50 mM potassium phosphate buffer, pH 7.0, and 400 spectra were recorded over a period of 4 s. B, absorbance peak of the nitronate form of nitrocyclohexane respectively; the spectrum obtained after 45 min. The black spectra in A represents species A (dithionite-reduced enzyme), the line C (also an oxidized enzyme species that forms slowly). With the N189A MR enzyme, the nitronate intermediate was produced, as observed with wild-type enzyme. The rate of formation of the nitronate species is slower in the N189A enzyme with 1-nitrocyclohexene, consistent also with the lack of activity with 2-cyclohexen-1-one.

**DISCUSSION**

Crystal structures of OYE1, PETN reductase, and MR have enabled detailed comparison of active site architecture, in particular the role of residues in ligand binding. The structure of OYE1 bound to parahydroxy benzoate has been solved, and this structure indicates the key roles played by His-191 and Asn-194 in binding this ligand (15). Subsequent studies of the properties of the single mutant H191N and the double mutant H191N/N194H of OYE1 indicated a role for these residues in binding 2-cyclohexen-1-one and phenolic ligands and smaller effects on enzyme reduction by reducing coenzyme (40). Likewise, crystallographic studies of PETN reductase in complex with steroid substrates 2,4-dinitrophenol and picric acid have also confirmed the importance His-181 and His-184 in forming H bonds with the carbonyl and hydroxy groups of these ligands (16, 38), but no quantitative solution data are available to assess the importance of these interactions in ligand binding. We chose to isolate the H186A and N189A MR enzymes because, unlike with studies of the H191N and H191N/N194H OYE1 enzymes (40), the introduction of alanine residues removes potential hydrogen bonding interactions with the ligand. This therefore enables quantitative assessment of the role of these interactions in binding and kinetics. Tables I and II

**TABLE II**

|                  | MR          | H186A      | N189A      |
|------------------|-------------|------------|------------|
| **Reductive half-reaction** |             |            |            |
| With NADH       |             |            |            |
| $k_{\text{lim}}$ | 22 s$^{-1}$ | 360 M$^{-1}$ s$^{-1}$ | 70 s$^{-1}$ (fast phase); 15 s$^{-1}$ (slow phase) |
| $K_d$ NADH      | 6 × 10$^{-6}$ M |                      | $>200 \times 10^{-6}$ M |
| **Oxidative half-reaction** |             |            |            |
| With 2-cyclohexen-1-one |             |            |            |
| $k_{\text{lim}}$ | 0.9 s$^{-1}$ | No detectable activity | 0.093 s$^{-1}$ |
| $K_d$ 2-cyclohexen-1-one | 5.7 × 10$^{-3}$ M | No detectable activity | 80 × 10$^{-3}$ M |
| With 1-nitrocyclohexene |             |            |            |
| $k_{\text{lim}}$ | 7.8 s$^{-1}$ | ND$^a$     | ND         |
| $K_d$ 1-nitrocyclohexene | 1.09 × 10$^{-3}$ M | ND$^a$     | ND         |

$^a$ ND, not determined.
provide a detailed comparison of stopped-flow kinetic data for OYE1 and MR enzymes.

Mutagenesis of His-186 and Asn-189 has major effects on the dissociation constants for the enzyme-coenzyme complex and the reduced enzyme-2-cyclohexen-1-one complex (Table II). The most striking effect is observed with the H186A enzyme, in which the reductive half-reaction becomes second order with respect to NADH concentration. In the N189A enzyme saturation of the enzyme by NADH is observed, but only at elevated concentrations of coenzyme. These are more pronounced effects compared with similar data obtained with OYE1, but in the latter the H bonding potential to the ligand is maintained in the mutant enzymes. The most striking effect of the mutations in MR are seen in the oxidative half-reaction. In this case, exchange of His-186 for alanine leads to the loss of enzyme activity, presumably attributable to the poor binding of 2-cyclohexen-1-one to the enzyme. A modest increase in the dissociation constant for the reduced enzyme-2-cyclohexen-1-one complex is also seen in the N189A enzyme. Poor geometrical alignment of 2-cyclohexen-1-one in the active site of the N189A enzyme most likely accounts for the ~10-fold decrease in the limiting rate for flavin reoxidation compared with wild-type MR (Tables I and II). The weaker apparent binding of the substrate in the dissociation constant for oxidized enzyme-progesterone complexes in the H186A and N189A enzymes, respectively. Potentiometric analysis of both mutant enzymes revealed that mutation of His-186 to alanine has only a minor effect on the redox potential of the enzyme flavin (~214 ± 7 mV compared with a value of ~242 ± 6 mV for the wild-type enzyme), whereas the mutation of Asn-189 to alanine has essentially no effect on the redox potential (~236 ± 6 mV). The latter is perhaps surprising given the close proximity of Asn-189 to the N1/C2 carbonyl region of the FMN isoalloxazine ring but is consistent with recent studies in which the nature of the charge was altered close to the N1/C2 carbonyl region in MR without adverse effect on the FMN reduction potential (32).

The lack of a tyrosine residue in MR that corresponds to Tyr-196 in OYE1 (known to function as a proton donor) is a key difference in the active site structure of MR and OYE1. In MR, the position is occupied by Cys-191, but mutagenesis has ruled this residue out as a proton donor for the oxidative half-reaction (17). The lack of activity in the oxidative half-reaction for the H186A enzyme raises the possibility that this residue out as a proton donor for the oxidative half-reaction. However, this possibility is ruled out on the basis of the reported NMR data, which indicate clearly that His-186 is in the neutral form in oxidized, reduced, and progesterone-bound enzyme. Moreover, studies of the pH dependence of the oxidative half-reaction with 2-cyclohexen-1-one demonstrate that the limiting rate of flavin oxidation and the reduced enzyme-2-cyclohexen-1-one dissociation constant are not sensitive to changes in pH in the accessible range, consistent with His-186 remaining in the neutral form across the pH range. It is clear, therefore, that other residues in the active site of MR (e.g. Tyr-72 or Tyr-356), or indeed water, must supply a proton in a concerted reaction with hydride transfer from FMNH$_2$ to 2-cyclohexen-1-one. This emphasizes the different geometry for hydride and proton transfer in OYE1 and MR.

1-Nitrocyclohexene is comparatively a good substrate for MR with the limiting rate constant for flavin oxidation being ~9-fold greater than that with 2-cyclohexen-1-one (Table I). Reactions with OYE1 demonstrated a second order dependence on 1-nitrocyclohexene concentration, but reduced MR has a higher affinity for 1-nitrocyclohexene and saturation kinetic behavior is observed (Table II). In OYE1, the reduction of 1-nitrocyclohexene by the enzyme proceeds via stepwise reduction (39) where a hydride is transferred from the reduced enzyme to the β-carbon of the substrate producing the nitrate form of nitrocyclohexane. This is followed by the transfer of a proton from Tyr-196 to the nitronate species, to form the protonated form of nitrocyclohexane. In the Y196F mutant of OYE1, the nitrate species is formed, but further conversion to nitrocyclohexane does not occur (39). By contrast, with wild-type MR only the nitrate species forms, indicating protonation by an active site residue to form nitrocyclohexane is prevented. The nitrate species decays with time, and the similar rates for this process observed with the Y169F OYE1 enzyme, wild-type and N189A MR enzymes suggest that this is not enzyme-catalyzed. The inability of the H186A MR enzyme to reduce 1-nitrocyclohexene likely reflects the poor binding affinity of the mutant for substrate.

That the protonated form of nitrocyclohexane is not formed in the reaction of wild-type MR with 1-nitrocyclohexene might reflect competition with an alternative protonation pathway (Scheme 1). The nitrate species can be protonated by solvent at the nitro group (i.e. not the α carbon of the unsaturated bond as seen in reactions mediated by Tyr-196 in OYE1). This leads to the formation of nitric acid, which is characterized by an absorption peak with a maximum at 230 nm similar to that of nitrate species. The nitrate form of nitrocyclohexane is known to undergo aci-nitro tautomerization in a fast reaction at pH 7.0 in 0.5 M phosphate buffer to produce nitric acid (Scheme 1) (39), consistent with this proposal. Nitric acid formation most likely also accounts for the inability of the N194H mutant of OYE1 to form nitrocyclohexane from the nitrate intermediate (39). In this case, the N194H mutations would lead to poor alignment of the nitrate species in the enzyme active site such that Tyr-196 is not optimally positioned to catalyze proton transfer to the α carbon.

Concluding Remarks—His-186 and Asn-189 are key binding determinants for reducing coenzyme and oxidizing substrates in the reaction catalyzed by MR. His-186 is neutral across the accessible pH range, indicating that it does not act as a proton donor in the reduction of αβ unsaturated carbonyl compounds. Decoupling of hydride and proton transfer is observed in reactions of MR with 1-nitrocyclohexene, but unlike with OYE1 the product nitrocyclohexane is not formed. Alternative proton transfer pathways most likely account for the lack of formation of nitrocyclohexane in MR through the formation of nitric acid. The study highlights key mechanistic differences in the reactions catalyzed by MR and OYE1 and emphasizes the need for caution in inferring mechanistic similarities in structurally related proteins.

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