H-tunneling in the Multiple H-transfers of the Catalytic Cycle of Morphinone Reductase and in the Reductive Half-reaction of the Homologous Pentaerythritol Tetrinitrate Reductase*

Received for publication, June 6, 2003, and in revised form, August 4, 2003 Published, JBC Papers in Press, August 26, 2003, DOI 10.1074/jbc.M305983200

Jaswir Basran‡, Richard J. Harris‡, Michael J. Sutcliffe§§, and Nigel S. Scrutton¶¶
From the Departments of ‡Biochemistry and §§Chemistry, University of Leicester, University Road, Leicester LE1 7RH, United Kingdom

The mechanism of flavin reduction in morphinone reductase (MR) and pentaerythritol tetrinitrate (PETN) reductase, and flavin oxidation in MR, has been studied by stopped-flow and steady-state kinetic methods. The temperature dependence of the primary kinetic isotope effect for flavin reduction in MR and PETN reductase by nicotinamide coenzyme indicates that quantum mechanical tunneling plays a major role in hydride transfer. In PETN reductase, the kinetic isotope effect (KIE) is essentially independent of temperature in the experimentally accessible range, contrasting with strongly temperature-dependent reaction rates, consistent with a tunneling mechanism from the vibrational ground state of the reactive C–H/D bond. In MR, both the reaction rates and the KIE are dependent on temperature, and analysis using the Eyring equation suggests that hydride transfer has a major tunneling component, which, unlike PETN reductase, is gated by thermally induced vibrations in the protein. The oxidative half-reaction of MR is fully rate-limiting in steady-state turnover with the substrate 2-cyclohexenone and NADH at saturating concentrations. The KIE for hydride transfer from reduced flavin to the α/β unsaturated bond of 2-cyclohexenone is independent of temperature, contrasting with strongly temperature-dependent reaction rates, again consistent with ground-state tunneling. A large solvent isotope effect (SIE) accompanies the oxidative half-reaction, which is also independent of temperature in the experimentally accessible range. Double isotope effects indicate that hydride transfer from the flavin N5 atom to 2-cyclohexenone, and the protonation of 2-cyclohexenone, are concerted and both the temperature-independent KIE and SIE suggest that this reaction also proceeds by ground-state quantum tunneling. Our results demonstrate the importance of quantum tunneling in the reduction of flavins by nicotinamide coenzymes. This is the first observation of (i) three H-nuclei in an enzyme reaction being transferred by tunneling and (ii) the utilization of both passive and active dynamics within the same native enzyme.

Enzymes are phenomenal catalysts that can achieve rate enhancements of up to ~21 orders of magnitude over the uncatalyzed reaction rate (1). Our quest to understand the physical basis of this catalytic power is challenging and has involved sustained and intensive research efforts by many workers in the physical and life sciences (for recent reviews see Refs. 2–5). Recent years have witnessed new activity in this area and extended our theoretical understanding beyond the shortcomings of transition state theory (6) to include roles for protein “motion” (7, 8), low barrier hydrogen bonds (e.g. Refs. 9–11), active site preorganization (e.g. reviewed in Refs. 4 and 12), and quantum mechanical tunneling (for recent reviews see Refs. 13–15). New theoretical frameworks incorporating quantum mechanical tunneling and protein motion are emerging to address the catalytic potency of enzymes. These invoke motion in the protein and/or substrate to drive the reaction (16–19). The reaction itself (i.e. the breaking and making of bonds) is normally modeled using a hybrid quantum mechanical/molecular mechanical (QM/MM) formulism, in which those atoms involved in the reaction are treated quantum mechanically and the rest of the system treated classically using molecular mechanics (e.g. Ref. 20). An alternative approach, the “quantum Kramers” method (15, 21, 22), which treats the whole system using a simplified quantum mechanical formulism, has been applied so far only to small organic systems. Additionally, methodology has been developed for identifying computationally residues important in creating reaction-promoting vibrations in enzymes (23). It has also been suggested that dynamics of the enzyme should be divided into two types, passive (reorganization energy) and active (gating or vibrational enhancement), and that tunneling is gated via active dynamics (i.e. a vibration modulating the hydrogen transfer coordinate becomes thermally active resulting in increased tunneling probability) (14, 24).

Hydride transfer from a reducing nicotinamide coenzyme to a flavin cofactor is a common reaction in biology, but the potential importance of H-tunneling in these reactions has not been explored. H-tunneling has been characterized extensively in NAD+-dependent alcohol dehydrogenases (e.g. Refs. 25–27), which prompted us to study more broadly a potential role for H-tunneling in flavoproteins that operate with nicotinamide coenzymes. Herein, we have studied hydride and proton transfer in morphinone reductase (MR) and pentaerythritol tetrinitrate (PETN) reductase. Crystallographic structures of these enzymes have established a close relationship to Old Yellow Enzyme (OYE) (28–30), reflected also in the ability of the OYE family members to reduce a number of two-cyclic enones and to form complexes with steroids. Like OYE, MR is a dimer, but the
nature of the subunit interactions is different from those seen in OYE. PETN reductase is a monomer and resembles in fold a single subunit of OYE and MR, based on the archetypal 8-fold β/α barrel topology. The active sites of all three enzymes are remarkably conserved despite differences in their catalytic properties. NADPH-dependent PETN reductase was purified and cloned from a strain of Enterobacter cloacae (strain PB2), which was isolated on the basis of its ability to utilize nitrite ester explosives such as PETN and glycerol trinitrate as sole nitrogen source (31, 32). MR was identified in a strain of Pseudomonas putida (strain M10) isolated from industrial waste liquors (33). MR catalyzes the NADH-dependent saturation of the carbon-carbon bond of both morphinone and codeinone to produce hydromorphone (a powerful analgesic) and hydrocodone (an antitussive), which are valuable semi-synthetic opiate drugs (34, 35). The half-reactions of MR and PETN reductase have been investigated using stopped-flow methods (29, 36, 37), enabling elucidation of the kinetic mechanisms for each half-reaction. We demonstrate in this report that hydride transfer from nicotinamide coenzyme to flavin in both enzymes occurs by quantum tunneling but that the nature of the tunneling reaction is different. Despite the similar active site architectures, this likely reflects differences in the dynamics of the enzyme scaffold in MR and PETN reductase. We show additionally that hydride transfer from reduced flavin to the substrate 2-cyclohexenone in MR also occurs by tunneling and that this reaction is concerted with proton transfer from an unidentified active site acid to the substrate unsaturated bond.

EXPERIMENTAL PROCEDURES

Chemicals and Enzymes—Complex bacteriological media were from Oxoid. Mimetic Orange 2 and Mimetic Yellow 2 affinity chromatography resins were from Affinity Chromatography Ltd. Q-Sepharose resin was from Amersham Biosciences. PETN reductase was prepared from Escherichia coli JM109/pONR1 and purified as described previously (32) but also incorporating a final chromatographic step using Q-Sepharose (28). MR was purified from a recombinant strain of E. coli transformed with plasmid pMORB2, which expresses the enzyme from the cloned mor B gene as described previously (33), but also incorporating a final chromatographic step using Q-Sepharose (29). NADPH and NADH were from Sigma. 2-Cyclohexenone was from Acros Organics. Deuterium oxide (99.9% deuterium) was from Goss Scientific Instruments Ltd. The following extinction coefficients were used to calculate the concentration of substrates and enzymes: NADPH (ε 260 = 6.22 × 10^4 M^-1 cm^-1); PETN reductase and MR (ε 260 = 11.3 × 10^4 M^-1 cm^-1); and 2-cyclohexenone (ε 260 = 11.0 × 10^4 M^-1 cm^-1).

Deuterated Compounds—A-side NADPH was synthesized enzymatically as described previously (38) and ethanol-precipitated by using the method of Pollock and Barber (39). A further purification step was performed using a Q-Sepharose column. The column was equilibrated with 10 mM ammonium hydrogen carbonate, pH 9 (buffer A), and A-side NADPH (~20 ng) applied to the column. The column was washed with buffer A and then developed with a linear gradient of 10 mM to 400 mM ammonium hydrogen carbonate, pH 9; NADPH eluted at 260 nm (1 cm light path). Assays were conducted in 50 mM potassium phosphate buffer, pH 7.0, at the stated temperatures. Rapid reaction kinetic experiments were performed by mixing enzyme in the appropriate buffer with an equal volume of reducing cofactor in the same buffer at the desired concentration. For studies of the oxidative half-reaction, the sequential mixing mode of the stopped-flow apparatus was used. Enzyme was rapidly mixed with a stoichiometric amount of reducing cofactor to enable reduction of the enzyme-bound FMN and after a suitable pre-determined aging period, the reduced enzyme solution was rapidly mixed with 2-cyclohexenone and reoxidation monitored at 464 nm. In both half-reactions, the concentration of substrate was always at least 10-fold greater than that of enzyme, thereby ensuring pseudo-first-order conditions. For each substrate concentration, at least five replica measurements were collected and averaged.

The kinetics of the reductive half-reaction of MR and PETN reductase were investigated at 464 nm, essentially as described previously (36, 37). Observed rate constants for flavin absorption changes occurring in the reductive half-reactions of both enzymes were obtained from fits of the data to a standard double-exponential expression, where k_{obs1} (95% total absorption change) and k_{obs2} (5% total absorption change) are observed rate constants for fast and slow phases, respectively. The faster of the two phases is attributed to flavin reduction, as indicated by photodiode array experiments of the reductive half-reaction. The slow phase represents a minor spectral change, the origin of which is uncertain. Observed rates for the fast phase (k_{obs1}) for both half-reactions were fitted using the general hyperbolic expression (Equation 1), consistent with the kinetic schemes presented under “Results” and with previous reported studies (see Refs. 36 and 37 for further details).

In Equation 1, k_{lim} is the limiting rate for flavin reduction (reductive half-reaction) or flavin oxidation (oxidative half-reaction). At low temperatures (5 °C), flavin reduction was essentially independent of reducing nicotinamide coenzyme concentration, consistent with previous studies (36, 37). The pH dependence of the rate of flavin reduction was measured in H2O in the range pH 5.5–9 using KMB buffer (55 mM MES, 25 mM Tris, 25 mM ethanolamine). The reductive half-reaction of MR, absorption changes at 464 nm were reported upon flavin oxidation by 2-cyclohexenone as described previously (29). Transients were biphasic, with the fast phase (k_{obs1}; 90% of the total absorption change) reporting upon flavin oxidation. The origin of the slow phase (k_{obs2}; 10% of the total absorption change) is uncertain. Observed rates for the fast phase were hyperbolically dependent on substrate concentration, and data were fitted using Equation 1.

Steady-state Kinetic Analysis—Steady-state kinetic measurements were performed using a Jasco V530 UV/VIS spectrophotometer with a 1-cm light path. Assays were conducted in 50 mM potassium phosphate buffer, pH 7.0, at 25 °C in a total volume of 1 ml. For determination of Km values, [S] and [E] were equimolar, and the Bio-Rad 113F Coomassie Blue dye (Bio-Rad) was added to the reaction mixture contained 150 μM NADH, 250 mM MR, and the concentration of 2-cyclohexenone was varied. Initial velocity data as a function of 2-cyclohexenone were analyzed by fitting to the standard Michaelis-Menten rate equation. The pH dependence of the steady-
state reaction was measured in H₂O and ²H₂O in the range pH 5.5–9 using KMB buffer. In both the pH and temperature dependence studies the concentration of 2-cyclohexenone was kept constant (and saturating) at 50 mM. MR activity was measured by following the decrease in absorption at 350 nm due to oxidation of NADH, and initial rates of reaction were calculated using an \( \epsilon = 5650 \text{ M}^{-1} \text{ cm}^{-1} \).

**Enzyme-monitored Turnover**—Steady-state measurements were also performed using the enzyme monitored turnover method as described by Gibson et al. (40) for reactions catalyzed by glucose oxidase. Reactions were performed in an Applied Photophysics SX18-MR reaction analyzer. Solution conditions are described under “Results.” Data analysis was essentially as described elsewhere (40).

**RESULTS**

**Temperature Dependence of the Reductive half-reaction of MR and Kinetic Isotope Effects**—The mechanism of flavin reduction in MR was determined previously and shown to involve the rapid formation of an E-NADH\(^{\text{CT}}\) charge-transfer intermediate prior to flavin reduction (36) (Scheme 1). Formation and decay of the charge-transfer species can be monitored at 552 nm and flavin reduction at 462 nm. The decay of the charge-transfer species is kinetically indistinguishable from flavin reduction, indicating the two processes are linked (36). Spectral changes observed in the reductive half-reactions of MR with a 10-fold excess of NADH were previously shown to fit to a two-step kinetic model: A \( \rightarrow \) B \( \rightarrow \) C, consistent with these assignments, where A is the oxidized enzyme, B is an enzyme- coenzyme charge-transfer intermediate, and C is the enzyme containing the reduced (dihydropyridine) form of the flavin cofactor (36), although fitting to more complex reversible kinetic models was not explored. In performing more extensive kinetic studies to search for tunneling regimes in the hydride transfer reaction from NADH to FMN, it is essential to know if the measured rate constants in stopped-flow experiments support (i) the approach to an equilibrium position for a reversible chemical step or (ii) an essentially irreversible reaction. With this in mind, we have conducted additional stopped-flow measurements using photodiode array spectroscopy and analyzed globally the spectral changes by fitting to reversible kinetic models.

Data were collected for reactions of MR with protiated and deuterated coenzyme at both 5 °C and 36 °C (i.e. the extremes of the temperature range used in analysis of the temperature dependence of the reductive half-reaction described below). A typical dataset is shown in Fig. 1 for the reaction of 20 \( \mu \)M MR with 200 \( \mu \)M NADH at 5 °C. Attempts to fit to a fully reversible model, A \( \leftrightarrow \) B \( \leftrightarrow \) C were unsuccessful based on a number of criteria. However, the data were readily fitted to the A \( \rightarrow \) B \( \rightarrow \) C kinetic model (rate constant for A \( \rightarrow \) B is 154 s\(^{-1}\); rate constant for B \( \rightarrow \) C is 14.8 s\(^{-1}\)) as described previously by Craig et al. (36), and the determined rate constants agree closely with those determined from single wavelength analysis of kinetic transients at 462 nm (flavin reduction) and 552 nm (charge transfer formation and decay). For the fully reversible kinetic model A \( \leftrightarrow \) B \( \leftrightarrow \) C, the criteria used to assess the fit could only be satisfied when the rate constant for the conversion of C \( \rightarrow \) B was initially estimated as a very small value (i.e. \(< 0.1 \text{ s}^{-1}\)); the final rate constants are A \( \rightarrow \) B (149 s\(^{-1}\)), B \( \rightarrow \) C (15.2 s\(^{-1}\)), B \( \rightarrow \) A (4.5 s\(^{-1}\)), and C \( \rightarrow \) B (0.1 s\(^{-1}\)) (Fig. 1), again consistent with studies performed at a single wavelength (36). The predicted spectra for the enzyme forms (Fig. 1B) obtained by fitting to a reversible scheme in which the rate of conversion of C \( \rightarrow \) B is very small (0.1 s\(^{-1}\)) are essentially identical to those obtained when fitting to the A \( \rightarrow \) B \( \rightarrow \) C model (36). This indicates that the rate of reverse hydride transfer from FMNH\(^{2+}\) to NAD\(^{+}\) is negligible. Qualitatively, similar results were obtained for reactions performed at 36 °C and in studies with NAD\(^{2H}\). These observations are consistent with the known reduction potential of NADH (−320 mV) and MR (−237 mV) (41), and they indicate that reductive transients measured under single-wavelength conditions at 464 nm support essentially irreversible reduction of the FMN by NADH. More recent studies with the C191A mutant of MR have provided evidence for an additional E-NADH intermediate that accumulates in the dead time (−1 ms) of the stopped-flow instrument, prior to the formation of the E-NADH\(^{\text{CT}}\) charge-transfer complex, giving rise to Scheme 2 for the reductive half-reaction of MR (29). This modified

\[
\begin{align*}
E + \text{NADH} & \rightarrow E\text{-NADH}^{\text{CT}} \\
& \rightarrow E\text{-NADH}^{\text{CT}}^- \\
& \rightarrow E\text{-NAD}^+ + \text{NADH} \\
& \rightarrow E\text{-NAD}^+ + \text{NADH} \\
\end{align*}
\]

**Scheme 1**

\( k_1, k_2, k_3 \) are rate constants for the forward and reverse reactions.
scheme for the reductive half-reaction of MR is consistent with work on Old Yellow Enzyme (42) and estrogen-binding protein (43) and probably holds also for wild-type MR (although unequivocal evidence is lacking (29)). Notwithstanding the increased complexity of this scheme, measurements of flavin reduction at 464 nm would still support the essentially irreversible rate of reduction of the FMN cofactor by NADH.

For wild-type MR at 5 °C, the rate of flavin reduction is independent of NADH concentration in the range 100–1100 μM coenzyme (36), but at higher temperatures a hyperbolic dependence of the rate of flavin reduction on NADH concentration is observed (Fig. 2). The value of K is dependent on temperature (45 ± 7 at 15 °C, 101 ± 7 at 25 °C, and 320 ± 24 at 35 °C). In fitting data, it was assumed that flavin reduction is essentially irreversible (i.e., the ordinate intercept in Fig. 2A approximates to zero). This is consistent with global fitting of photodiode array for the reductive half-reaction (see above). The limiting rate constant, \( k_{\text{lim}} \), for flavin reduction in MR is independent of solution pH from pH 5.5 to 9.0. A primary KIE of 3.9 ± 0.1 (25 °C) is observed for the limiting rate of flavin reduction calculated by fitting to Equation 1 (Fig. 2A), and there is no significant solvent isotope effect on flavin reduction (SIE = 1.05 ± 0.02). These results suggest that the solvent isotope effect observed in steady-state turnover of MR (see below) must arise from effects on step(s) that take place after flavin reduction (i.e., in the oxidative half-reaction).

Eyring plots for the limiting rate of flavin reduction, \( k_{\text{lim}} \), in protiated solvent were constructed by performing stopped-flow studies at each temperature with 5 mM coenzyme (Fig. 2B), thus ensuring that the NADH concentration was always at least 10-fold greater than the value of K. Data were fitted to the Eyring equation (Equation 2), and the parameters \( \Delta H^* \) and \( A^H.A^D \) were obtained.

\[
\ln(k_{\text{lim}}(T)) = \ln(k_0) + \Delta S^*/R - \Delta H^*/RT \quad \text{(Eq. 2)}
\]

The definitions of the terms \( A^H \) and \( A^D \) are given in our previous work (44). The data indicate that the KIE is dependent on temperature, with \( \Delta A^H \left( \Delta H^{\text{ID}} - \Delta H^{\text{FD}} \right) = (43.5 - 35.3) \text{ kJ mol}^{-1} \right) = 8.2 ± 0.27 \text{ kJ mol}^{-1} \). The value of \( A^H.A^D < 1 \) (0.126 ± 0.005), is consistent with a tunneling mechanism for transfer of the hydride ion from NADH to FMN that is gated by vibrations coupled to the reaction coordinate (see “Discussion”) (45).

Temperature Dependence of the Reductive Half-reaction of PETN Reductase and Kinetic Isotope Effects—The mechanism of action of PETN reductase is similar to that of MR, and detailed stopped-flow studies with NADPH have revealed the existence of an E-NADPH\(^{\text{CT}} \) charge-transfer species prior to flavin reduction (37). The kinetic model shown in Scheme 1 (but with NADPH as coenzyme) is consistent with the published kinetic data. Given the potential importance of tunneling in the reductive half-reaction of MR, we also investigated the temperature dependence of this half-reaction in PETN reductase. Like with MR, charge-transfer formation and decay can be observed at long wavelength (560 nm), and flavin reduction is monitored conveniently at 464 nm. As with MR, fitting of spectral data-sets for the reductive half-reaction (20 μM PETN reductase mixed with 200 μM NADPH at 5 °C) to a fully reversible kinetic model \( A \leftrightarrow B \leftrightarrow C \) indicated that hydride transfer is essentially irreversible (i.e., C → B, 0.1 s\(^{-1} \)) to satisfy the fitting criteria (data not shown). All other microscopic rate constants determined from this fit (A → B, 109 s\(^{-1} \); B → C 11 s\(^{-1} \); and B → A, 2 s\(^{-1} \)) are consistent with data obtained from single wavelength studies (37). Our analyses again demonstrate that hydride transfer is essentially irreversible, which is consistent with the known redox potential of PETN reductase (37). These data also confirm that single wavelength studies of the reductive half-reaction performed at 464 nm support the essentially irreversible rate of hydride transfer from NADPH to FMN.

As with MR, decay of the charge-transfer species monitored at 560 nm is linked kinetically to flavin reduction at 464 nm (37). Our previous studies also indicated that the flavin reduction rate is independent of NADPH concentration at 5 °C (37), but as with MR we have shown herein that at higher temperatures a hyperbolic dependence is observed (Fig. 3). The value of \( K \) determined by fitting to Equation 1 is dependent on temperature (27 ± 3 at 15 °C; 73 ± 4 at 25 °C; and 186 ± 14 at...
35 °C). As with MR, the limiting rate constant for flavin reduction is independent of solution pH between the pH values 5.5 and 9. An Eyring plot of the limiting rate of flavin reduction indicates that the KIE (-4.1) for hydride transfer from coenzyme to FMN is essentially independent of temperature (A°D = 4.1 ± 0.3; ΔH°D = 0.20 ± 0.01 kJ mol⁻¹); fitting to the Eyring equation yielded values for ΔH°D and ΔH°H of 36.6 ± 0.9 and 36.4 ± 0.9 kJ mol⁻¹, respectively. The data is strikingly similar to that obtained for C–H bond breakage catalyzed by a number of amine oxidizing enzymes in which reaction rates are strongly dependent on temperature, but the KIE is independent of temperature over the experimentally accessible range (44, 46–48). H-transfer in these enzymes occurs by a quantum tunneling mechanism, probably from the vibrational ground state of the reactive C–H/C–D bond (see Ref. 13 for a recent review), and this interpretation is consistent with hybrid quantum mechanical/molecular mechanical simulations of these reactions (49).

Stopped-flow Methods for the Kinetics of Hydride Transfer from FMNH₂ to 2-Cyclohexeno in the Oxidative Half-reaction of MR—The kinetics of the oxidative half-reaction of MR determined at 25 °C have been reported elsewhere (29). Also, using the fitting criteria discussed for the reductive half-reaction, fitting of spectral data associated with the oxidative half-reaction to a fully reversible model indicates that the reverse rate of hydride transfer is at least ~150-fold slower than the forward rate. This is consistent with the lack of an ordinate intercept when fitting data in plots of observed rate of flavin oxidation versus 2-cyclohexenone concentration to Equation 1 (29). Thus, the transfer of a hydride ion from FMN to 2-cyclohexenone can be analyzed essentially as an irreversible reaction.

Herein, our studies were extended over the accessible temperature range (4–40 °C) to enable comparison with steady-state turnover data (see below) and to assess the effect of temperature on the binding of 2-cyclohexenone. In these experiments, enzyme (5 μM) was reduced with a stoichiometric amount of NADH, and, following an appropriate time to effect full reduction, the reduced enzyme was mixed with 2-cyclohexenone in a double mixing sequential stopped-flow experiment. The experimental approach is different to that reported previously, in which the kinetics of the oxidative half-reaction were analyzed by single mixing of MR (which had been titrated with sodium dithionite) with 2-cyclohexenone (29). Notwithstanding, at a range of temperatures studied in the double mixing method described in this report, the dependence of the observed flavin reoxidation rate on 2-cyclohexenone concentration is hyperbolic (Fig. 4), consistent with our previous findings at 25 °C (29). Limiting rate constants, klim, and reduced enzyme-substrate dissociation constants, K, at 4 and 40 °C are given in the legend to Fig. 4. The lack of major change in K over the accessible temperature range established that a study of the temperature dependence of the limiting rate of flavin oxidation is possible at a 2-cyclohexenone concentration of 50 mM throughout the temperature range.

In the oxidative half-reaction of MR a hydride ion is transferred from the N5 atom of FMN to 2-cyclohexenone (Fig. 5). Saturation of the double bond also requires a proton, but the identity of the proton donor in MR is as yet uncertain (29). The exchange of protium or deuterium on the flavin N5 atom with protons from bulk solvent is a potential problem one encoun-

![Fig. 3. A, plots of observed flavin reduction rate versus coenzyme concentration for the reductive half-reaction of PETN reductase. Conditions: 50 mM potassium phosphate buffer, pH 7.0; 25 °C. Filled circles, data for NADPH; fitting to Equation 1 yields values for kobs1 (54 ± 0.4 s⁻¹) and K (73 ± 4 μM). Open circles, data for NADPH; fitting to Equation 1 yields values for kobs1 (10 ± 0.3 s⁻¹) and K (98 ± 12 μM). B, Eyring plot for the limiting rate of flavin reduction in PETN reductase. NADPH (filled circles) and NADPH (open circles). ln(A°) = 12.7 ± 0.4, ln(A°D) = 11.3 ± 0.4, ΔH°H = 36.4 ± 0.9 kJ mol⁻¹, and ΔH°D = 36.6 ± 0.9 kJ mol⁻¹. Inset: plot of ln(KIE) versus 1/T. Rate constants are observed rate constants measured at 5 mM coenzyme. Each data point is the average of at least five measurements. All errors for temperature dependence plots are ±5% of the measured value. Owing to the temperature dependence of K, klim values measured above 30 °C were obtained by fitting plots of the rate of flavin reduction versus substrate concentration to Equation 1. Below this temperature, klim values were obtained by performing reactions with saturating coenzyme (5 mM NADPH).](image)

![Fig. 4. Plot of klim versus 2-cyclohexenone concentration for the oxidative half-reaction of MR at 40 °C determined from a double mixing stopped-flow experiment. Fitting to Equation 1 yields a K of 1.9 ± 0.1 mM and a klim of 4.0 ± 1 s⁻¹. Comparable data collected at 4 °C yields a K of 2.5 ± 0.2 mM and a klim of 0.92 ± 0.01 s⁻¹.](image)
bers in studies of the oxidative half-reaction that employ KIEs as probes of H-transfer. A series of double mixing stopped-flow studies were performed to investigate how rapidly deuterium at the N5 position of enzyme-bound FMN exchanges with protons in bulk solvent. MR (5 μM) was mixed with 5 μM NADH (NADH2), and the time required to fully reduce the flavin was determined from absorption changes at 464 nm in single mix experiments at 4, 24, and 40 °C (Fig. 6). Using the sequential mixing mode of the stopped-flow apparatus, and following a suitable aging time to effect complete reduction of the enzyme, the kinetics of the OHR were then followed by rapidly mixing the reduced enzyme with 50 mM 2-cyclohexene (Fig. 6). In a series of “wash-out” experiments, the reduced enzyme was also allowed to age for increasing lengths of time to allow exchange of deuterium on the flavin N5 atom, before enzyme was mixed with 2-cyclohexene. These experiments indicated that the KIE value remained constant up to an aging time of ~100 s at 24 °C. Thereafter, the KIE diminished as the aging time was extended beyond 100 s, reflecting exchange of deuterium with protons from bulk solvent. At 24 °C and with an aging time of 10 s, the KIE measured for the oxidative half-reaction was 3.8 ± 0.4. Although the exchange kinetics were faster at higher temperatures, an aging time of 10 s did not lead to significant exchange with protons from bulk solvent. Using this approach, a KIE of 3.7 ± 0.3 was measured at 4 °C (Fig. 6). The sequential mixing method provides a guide as to the value of the KIE for hydride transfer in the oxidative half-reaction at different temperatures and serves to illustrate that deuterium is not rapidly lost from the N5 atom of the flavin following reduction by coenzyme. However, the method is not sufficiently robust to provide accurate and highly reproducible rates for hydride/deutered transfer as a function of temperature (and thus as a probe of tunneling). For this reason, we chose to study the oxidative half-reaction under steady-state conditions.

Steady-state Analysis of MR, Double Isotope Effects, and Temperature Dependence of Kinetic Isotope Effects—Steady-state assays of MR have established that the limiting rate of hydride transfer in the oxidative half-reaction is comparable to kcat, suggesting this is the overall rate-limiting step in steady-state turnover (29). The enzyme monitored turnover method (40) using diode array and single wavelength detection in the stopped-flow instrument was used to confirm that the oxidative half-reaction is rate-limiting. In enzyme-monitored turnover experiments, the reduction level of the flavin is monitored prior to, during, and after the steady-state phase by absorption measurements at 464 nm. In these experiments there is a rapid and almost complete bleaching of the flavin absorption at 464 nm on mixing enzyme with NADH and 2-cyclohexene (Fig. 7A). This is followed by a steady-state phase and then finally an increase in absorbance as the oxidized enzyme is regenerated owing to depletion of the reducing cofactor. The spectral forms of MR obtained at different time points during the course of this reaction are shown in the inset of Fig. 7A. These confirm that the reduced form of the enzyme is the predominant species under steady-state turnover conditions. At the start of data acquisition (point 1 on the trace; 3.8 ms after mixing) the oxidized E-NADHCT charge transfer species has already formed owing to the relatively high concentration of NADH used (formation of the E-NADHCT charge transfer species is second order with respect to NADH concentration) and the relatively long time delay from mixing to data acquisition in this experiment. Following rapid reduction of the flavin by NADH, a steady-state phase is established during which the predominant species is the two-electron form of MR (point 2 on the trace). As NADH is depleted, the oxidized enzyme is formed once again (point 3 on the trace).

The data were analyzed by using the method of Gibson et
al. (40), and a series of parallel lines were obtained when the reciprocal of the turnover number was plotted versus the reciprocal of the NADH concentration (Fig. 7B), consistent with a ping-pong reaction that is the result of shared binding sites for NADH and 2-cyclohexenone in the OYE family of enzymes (28–30). The inset to Fig. 7B shows a secondary plot of the ordinate intercept versus 2-cyclohexenone concentration. The true turnover number ($k_{\text{cat}} = 2.5 \pm 0.1 \text{ s}^{-1}$) for the MR-catalyzed reaction is obtained from the ordinate intercept of this secondary plot, and the true $K_m$ for 2-cyclohexenone ($3.0 \pm 0.2 \text{ mM}$) is derived from this plot by dividing the value of the gradient by the ordinate intercept. The true $K_m$ for NADH ($6.2 \pm 1.4 \mu$M) was calculated by dividing the slope of any line in Fig. 7B by the intercept of that line. The kinetic parameters measured using the enzyme monitored turnover method are similar to apparent values published previously for MR obtained by conventional enzyme assay (29) using the initial rate method. In addition, the limiting rate for flavin oxidation by 2-cyclohexenone ($2.9 \text{ s}^{-1}$) measured in the stopped-flow apparatus is similar to the turnover number ($2.5 \text{ s}^{-1}$) indicating that flavin oxidation is rate-limiting in steady-state turnover.

Having established that flavin oxidation is rate-limiting in steady-state turnover, we performed a detailed study of the oxidative half-reaction as a function of temperature and isotopic substitution to (i) demonstrate that hydride transfer in the oxidative half-reaction is fully rate-limiting, (ii) probe for tunneling in this reaction, and (iii) obtain evidence for a concerted hydride and proton transfer in the oxidative half-reaction. The oxidative half-reaction of PETN reductase is not rate-limiting in steady-state turnover (37), and thus our studies were restricted to MR.

Steady-state assays were initially performed at 25 °C using NADH or NAD$^2$H as reducing coenzyme and in protiated and deuterated solvent. Plots of initial velocity versus 2-cyclohexenone concentration (range 0–30 mM) at a fixed coenzyme concentration (150 μM) were hyperbolic, and fitting to the Michaelis-Menten equation yielded apparent $K_m$ values for 2-cyclohexenone of 5.5 ± 0.1 mM (for NADH in protiated solvent), 4.3 ± 0.5 mM (for NAD$^2$H in protiated solvent), 5.4 ± 0.4 mM (NADH in deuterated solvent), and 3.5 ± 0.4 mM (NAD$^2$H in deuterated solvent). Turnover numbers were then determined using a fixed concentration of 2-cyclohexenone (50 mM) and reducing coenzyme concentration (150 μM) to obtain values for the primary KIE for hydride transfer from the flavin N5 atom to 2-cyclohexenone, the SIE for protonation of 2-cyclohexenone, and the double isotope effect for the oxidative half-reaction (Table I). The primary KIE observed for hydride transfer from the N5 atom to 2-cyclohexenone, the SIE for protonation of 2-cyclohexenone, and the double isotope effect for the oxidative half-reaction are consistent with this reaction being fully rate-limiting in multiple turnover assays. Moreover, a large SIE is seen on the turnover number indicating that a protonation event accompanies reduction of 2-cyclohexenone, consistent with the chemical scheme shown in Fig. 5. The double isotope effect method is useful in demonstrating that two H-transfer reactions are concerted, i.e. if the chemical step is fully rate-limiting (as is the case for MR) and the two isotopes are on the same step, the second isotope effect should remain unchanged. A more detailed treatment is given in Ref. 50. This prediction holds (within error) for the oxidative half-reaction of MR, consistent with a concerted reaction involving the simultaneous transfer of a hydride and proton (Table I).

The temperature dependence of the KIE values in protiated solvent indicate that the KIE is essentially independent of temperature ($\Delta \Delta H^\ddagger = -0.52 \pm 0.05 \text{ kJ mol}^{-1}$) over the accessible temperature range (Fig. 8), and the $A^\text{H}:A^\text{D}$ ratio (3.7 ± 2.1) is greater than unity. The data are consistent with a concerted hydride and proton transfer reaction that occurs by quantum tunneling from the vibrational ground states of the reactive bonds, in a reaction that is not gated by vibrations coupled to the reaction coordinate (see “Discussion”). Comparable studies with deuterated solvent and NAD$^2$H were not performed owing to the very slow turnover rates with NAD$^2$H at low temperature, but temperature-dependent studies with NADH in deuterated solvent indicate that the solvent isotope effect is also independent of temperature ($\Delta \Delta H^\ddagger = -0.90 \pm 0.05 \text{ kJ mol}^{-1}$) with the $A^\text{H}:A^\text{D}$ ratio (3.1 ± 2.0) greater than unity, again consistent with a tunneling mechanism. Steady-state pH dependence studies demonstrated that the oxidative half-reaction in both H$_2$O and D$_2$O is independent of solution pH (in the pH range pH 7–9) and that the solvent isotope effect remains constant (~2) in this pH range. Also, the turnover number of MR was independent of solution viscosity in reactions performed over a range of glycerol concentrations (1–35%...
Isotope effects obtained from steady-state reactions of MR with 2-cyclohexenone

| Condition | kcat | H2O   | NADH | kcat | 2H2O  | NADPH |
|-----------|------|-------|------|------|-------|-------|
| NADH/NADPH; protiated solvent (a/b) | 2.78 ± 0.11 (n=8) | 0.79 ± 0.02 (n=8) | 1.22 ± 0.086 (n=8) | 0.34 ± 0.043 (n=10) |
| NADH/NADPH; deuterated solvent (c/d) | 3.52 ± 0.23 | 3.56 ± 0.7 | 2.28 ± 0.25 | 2.32 ± 0.35 | 8.18 ± 1.36 |

| SIE | NADH/NADPH; protiated solvent (a/b) | NADH/NADPH; deuterated solvent (c/d) |
|-----|-------------------------------------|-------------------------------------|
| H2O/H2O; NADH | 3.52 ± 0.23 | 3.56 ± 0.7 |
| H2O/H2O; NADPH | 2.28 ± 0.25 | 2.32 ± 0.35 | 8.18 ± 1.36 |

**DISCUSSION**

Our earlier studies with methylene dehydrogenase established that enzymatic H-transfer can proceed solely by quantum tunneling (44), without the need to (partially) ascend the energy barrier separating reactants from products. The reaction of methylene dehydrogenase with methyleneamine was originally modeled using the vibrationally enhanced ground-state tunneling model of Bruno and Bialek (16) at a time when the environmentally coupled hydrogen tunneling model (19), which explicitly recognizes reorganization energy (so-called “passive dynamics”) and active dynamics (gating motion) (24), was not available. We have continued to provide (along with others) experimental evidence for enzyme catalysis based on dissipative tunneling models in which H-transfer occurs entirely by quantum mechanical tunneling (e.g. Refs. 24, 25, 46–48), and these models are distinct from the earlier tunnel-correction models (52) used to interpret anomalous kinetic isotope effect data with other enzyme systems (see Ref. 53 for a review).

A major step forward has been the realization that tunneling is driven by thermally induced vibrations in the protein scaffold (a thermally fluctuating energy surface), as described by the theoretical model of Kuznestov and Ulstrup (19), and illustrated in Fig. 9. This model (24) is given by Equation 3.

$$k_{tunnel} = (const.) \times \left[ \exp \left( -\frac{\Delta G^0 + \lambda T}{4RT} \right) \right] \times (F.C. \ Term) \quad (Eq. 3)$$

where $k_{tunnel}$ is the tunneling rate constant; $const.$ an isotope-independent term; the term in brackets is an environmental energy term relating the driving force of the reaction, $\Delta G^0$ to the reorganization energy; $\lambda$ ($R$ is the gas constant and $T$ the temperature in K); $F.C. \ Term$ is the Frank-Condon nuclear overlap along the hydrogen coordinate and arises from the overlap between the initial and the final states of the hydrogen's wave function. In the simplest limit, in which only the lowest vibrational level is occupied, $F.C. \ Term$ will be temperature-independent; otherwise, it will be temperature-dependent. Temperature-dependent “gating” dynamics can modulate the tunneling overlap, and the KIE is dependent on the energetic cost of gating, the KIE (Equation 4) can be derived from Equation 3 (24),

$$KIE = \frac{k_{cat \, H2O}}{k_{cat \, 2H2O}} = \frac{1}{1 + \frac{\Delta \nu \, E^* \, T}{\Delta H^0}} \quad (Eq. 4)$$

**Fig. 8.** Eyring plots for steady-state reactions of MR in protiated and deuterated solvent. MR with NADH (closed circles) and NADPH (open circles) in protiated solvent; ln(A^H2O) = – 2.3 ± 0.4, ln(A^H2O) = 1.0 ± 0.4, ΔH^H2O = 17.8 ± 0.9 kJ mol^-1, and ΔH^H2O = 17.1 ± 0.9 kJ mol^-1. MR with NADH in deuterated solvent (filled triangles). ln(A^H2O) = 1.4 ± 0.3, ΔH^H2O = 16.7 ± 0.7 kJ mol^-1. Inset: plot of ln(KIE) versus 1/T.

**Fig. 9.** Environmentally coupled tunneling model of enzyme-catalyzed reactions. Reactant (R = ES) and product (P = EP) energy curves for distortion of the protein structure. The tunneling does not occur until the geometry of the protein is distorted, so that the R and P curves intersect, the intersection point (asterisk) is the optimum geometry required for the reaction to occur. Thus, thermally induced conformational change in the protein is a prerequisite for the tunneling reaction. Top, hydrogen Gibbs free energy surface at different positions on the R and P curves; left panel, equilibrium configuration of the reactants; center panel, transition configuration; right panel, equilibrium configuration of the products. (Adapted from Refs. 19 and 14.)
H-tunneling in Morphinone Reductase and PETN Reductase

We have compared the high resolution crystal structures of MR (29) and PETN reductase (28) in an attempt to provide insight into why gating is potentially more important in MR (a more detailed analysis in the future will involve QM/MM, variational transition state theory, and molecular dynamics studies). A key factor could be double stranded anti-parallel β-sheet D, against which the NADP+/H coenzyme is thought to bind (29), which harbors arginine residues important in the recognition of the 2'-phosphate of NADPH (PETN reductase) and a glutamate residue required to form a H-bond with the 2'-OH group of NADH (MR). These types of side chain-coenzyme interactions are consistent with work on other nicotinamide-dependent enzymes (e.g. Refs. 51, 55). The position of this sheet diverges at Leu-133 (PETN reductase)/Val-138 (MR) and converges again at Ile-141 (PETN reductase)/Gly-146 (MR). Another difference in this region is the insertion of a glycine residue (Gly-133) in MR immediately before the start of β-sheet D. Taken together, these differences suggest that MR might be more mobile at physiological temperatures in this region than PETN reductase, thus, active dynamics (gating) is more likely an important feature in MR than in PETN reductase. This is consistent with the temperature factors for MR (all Cα temperature factors > 40; Protein Data Bank accession code 1GWJ) and PETN reductase (all Cα temperature factors <20; PDB accession code 1GVQ) in this region. Of course, caution must be exercised in interpreting the experimental results in the light of crystal structures, which do not contain the nicotinamide coenzyme. Structural studies with coenzyme bound are now a priority for future work, and these will form a platform for more detailed computational analysis using hybrid QM/MM and related methods.

Our kinetic studies have additionally indicated that hydride transfer from reduced flavin to the substrate 2-cyclohexenone in MR also occurs by tunneling. The KIE for this reaction is temperature-independent, consistent with the lack of vibrational assistance. This reaction is concerted with proton tunneling from an unidentified active site acid to the substrate unsaturated bond. Thus, MR invokes both active (i.e. vibrationally gated) hydride transfer (reductive half-reaction) and passive (i.e. no vibrational assistance) hydride and proton tunneling (oxidative half-reaction). This illustrates, for the first time, how both active and passive tunneling can be invoked within the same enzyme.

Concluding Remarks—Hydride transfer from a reducing nicotinamide coenzyme to a flavin cofactor is a common reaction in biology, and examples of such systems include adrenodoxin reductase, cytochrome P450 reductase, ferredoxin reductase, nitric oxide synthase, and sulfite reductase. Despite their general similarity, the potential importance of H-tunneling in these reactions has not been explored hitherto. Our studies have shown that two such systems, PETN reductase and MR, invoke H-tunneling. Despite the similar overall architecture of the active sites, hydride transfer in the reductive half-reaction of MR, but not in that of PETN reductase, is gated by protein dynamics. Also, hydride transfer from reduced flavin to the substrate 2-cyclohexenone in MR occurs by tunneling with no significant gating component. Furthermore, this reaction is concerted with proton tunneling from an unidentified active site acid to the substrate unsaturated bond. This is the first observation of (i) three H-nuclei in an enzymic reaction all being transferred by quantum mechanical tunneling and (ii) both passive and active dynamics associated with H-tunneling in the same native enzyme (although both tunneling regimes have been identified in wild-type versus mutant enzymes, e.g. Ref. 14). More generally, our work reinforces the key role of quantum tunneling reactions in enzymic H-transfer.
REFERENCES

1. Lad, C., Williams, N. H., and Wolfenden, R. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 5607–5611
2. Neet, K. E. (1998) J. Biol. Chem. 273, 25527–25528
3. Cannon, W. R., and Benkovic, S. J. (1998) J. Biol. Chem. 273, 26257–26260
4. Warshel, A. (1998) J. Biol. Chem. 273, 27035–27038
5. Cleland, W. W., Frey, P. A., and Gerlt, J. A. (1998) J. Biol. Chem. 273, 25529–25532
6. Kraut, J. (1988) Science 242, 533–540
7. Cameron, C. E., and Benkovic, S. J. (1997) Biochemistry 36, 15792–15800
8. Rajagopalan, P. T., Lutz, S., and Benkovic, S. J. (2002) Biochemistry 41, 12618–12628
9. Frey, P. A., Whitt, S. A., and Tobin, J. B. (1994) Science 264, 1927–1930
10. Gerlt, J. A., and Gassman, P. G. (1993) Biochemistry 32, 11943–11952
11. Cleland, W. W., and Kreevoy, M. M. (1994) Science 264, 1887–1890
12. Cannon, W. R., Singleton, S. F., and Benkovic, S. J. (1996) Nat. Struct. Biol. 3, 821–833
13. Sutcliffe, M. J., and Scrutton, N. S. (2002) Eur. J. Biochem. 269, 3096–3102
14. Knapp, M. J., and Klinman, J. P. (2002) Eur. J. Biochem. 269, 3113–3121
15. Antoniou, D., Caratzoulas, S., Kalyanaraman, C., Minner, J. S., and Schwartz, S. D. (2002) Eur. J. Biochem. 269, 3103–3112
16. Bruno, W. J., and Bialek, W. (1992) Biophys. J. 63, 689–699
17. Borgis, D., and Hynes, J. T. (1996) J. Phys. Chem. 100, 1118–1128
18. Antoniou, D., and Schwartz, S. D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12380–12385
19. Kuznetsov, A. M., and Uletrup, J. (1999) Can. J. Chem. 77, 1085–1096
20. Gao, J., and Thompson, M. (eds) (1998) Methods and Applications of Combined Quantum Mechanical and Molecular Mechanical Methods, American Chemical Society, Washington, D. C.
21. Antoniou, D., and Schwartz, S. D. (1999) J. Chem. Phys. 110, 7359–7364
22. Antoniou, D., and Schwartz, S. D. (1999) J. Chem. Phys. 110, 465–472
23. Minner, J. S., and Schwartz, S. D. (2003) J. Phys. Chem. B 107, 366–371
24. Knapp, M. J., Rickert, K., and Klinman, J. P. (2002) J. Am. Chem. Soc. 124, 3865–3874
25. Kohn, A., Cannio, R., Bartolucci, S., and Klinman, J. P. (1999) Nature 399, 486–489
26. Bahnsen, B. J., Colby, T. D., Chin, J. K., Goldstein, B. M., and Klinman, J. P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12797–12802
27. Bahnsen, B. J., Park, D. H., Kim, K., Flapp, B. V., and Klinman, J. P. (1993) Biochemistry 32, 5503–5507
28. Barna, T., Khan, H., Bruce, N. C., Barsukov, I., Scrutton, N. S., and Mood, P. C. (2001) J. Mol. Biol. 310, 433–447
29. Barna, T., Messia, H. L., Petosa, C., Bruce, N. C., Scrutton, N. S., and Mood, P. C. (2002) J. Biol. Chem. 277, 30976–30983
30. Fox, K. M., and Karplus, P. A. (1994) Structure 2, 1089–1105
31. Binks, P. R., French, C. E., Nicklin, S., and Bruce, N. C. (1996) Appl. Environ. Microbiol. 62, 1214–1219
32. French, C. E., Nicklin, S., and Bruce, N. C. (1996) J. Bacteriol. 178, 6623–6627
33. French, C. E., and Bruce, N. C. (1994) Biochem. J. 301, 97–103
34. Melson, K., and Morrelli, H. (1972) Clinical Pharmacology: Basic Principles in Therapeutics, Macmillan Publishing Co., New York
35. Moffat, A., Jackson, J., Moss, M., and Widdop, B. (1986) Clarke’s Isolation and Identification of Drugs, The Pharmaceutical Press, London
36. Craig, D. H., Moody, P. C. E., Bruce, N. C., and Scrutton, N. S. (1998) Biochemistry 37, 7598–7607
37. Khan, H., Harris, R. J., Barna, T., Craig, D. H., Bruce, N. C., Munro, A. W., Moody, P. C., and Scrutton, N. S. (2002) J. Biol. Chem. 277, 21906–21912
38. Viola, R. E., Cook, P. F., and Cleland, W. W. (1979) Annu. Rev. Biochem. 46, 334–340
39. Pellock, V. V., and Barber, M. J. (2001) Biochemistry 40, 1430–1440
40. Gibson, Q. H., Swaboda, B. E. P., and Masssey, V. (1964) J. Biol. Chem. 239, 3927–3934
41. Craig, D. H., Barna, T., Moody, P. C., Bruce, N. C., Chapman, S. K., Munro, A. W., and Scrutton, N. S. (2001) Biochem. J. 359, 313–323
42. Massey, V., and Schopfer, L. M. (1986) J. Biol. Chem. 261, 1215–1222
43. Buckman, J., and Miller, S. M. (1998) Biochemistry 37, 14326–14336
44. Basran, J., Sutcliffe, M. J., and Scrutton, N. S. (1999) Biochemistry 38, 3218–3222
45. Jonsson, T., Glickman, M. H., Sun, S. J., and Klinman, J. P. (1996) J. Am. Chem. Soc. 118, 10319–10320
46. Harris, R. J., Meskes, R., Sutcliffe, M. J., and Scrutton, N. S. (2000) Biochemistry 39, 1189–1198
47. Basran, J., Patel, S., Sutcliffe, M. J., and Scrutton, N. S. (2001) J. Biol. Chem. 276, 6254–6262
48. Basran, J., Sutcliffe, M. J., and Scrutton, N. S. (2001) J. Biol. Chem. 276, 24581–24587
49. Faulder, P. P., Tresadern, G., Chohan, K. K., Scrutton, N. S., Sutcliffe, M. J., Hillier, I. H., and Burton, A. N. (2001) J. Am. Chem. Soc. 123, 8604–8605
50. Cleland, W. W. (1991) in Enzyme Mechanism from Isotope Effects (Cook, P. F., ed) pp. 247–265, CRC Press, Boca Raton, FL
51. Scrutton, N. S., Berry, A., and Perham, R. N. (1990) Nature 343, 38–43
52. Bell, R. P. (1980) The Tunnel Effect in Chemistry, pp. 51–140, Chapman and Hall, London
53. Bahnsen, B. J., and Klinman, J. P. (1995) Methods Enzymol. 249, 373–397
54. Deleted in proof
55. Becanegra, J. A., Scrutton, N. S., and Perham, R. N. (1993) Biochemistry 32, 2737–2740