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Evidence To Support the Hypothesis That Promoting Vibrations Enhance the Rate of an Enzyme Catalyzed H-Tunneling Reaction

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The transfer of hydrogen is one of the most ubiquitous enzyme catalyzed reactions. There is major interest in understanding mechanisms of H-transfer catalyzed by enzymes and a general recognition of the importance of quantum tunneling in enzymatic H-transfers. In transition state theory, the height of the potential energy barrier is of key importance in determining the rate of an enzymatic reaction. For tunneling reactions, the width of the potential energy barrier, effectively the donor−acceptor distance, is the key determinant.[1,2] Certain vibrational modes of the protein and/or substrate (termed promoting vibrations) may decrease the donor−acceptor distance, maximizing wave function overlap and thereby increasing the tunneling rate constant. While there is evidence to support the existence of promoting vibrations from experimental[3−9] and computational[10−13] studies, others have argued against dynamical contributions to enzyme catalysis.[14,15] Currently, direct evidence for promoting vibrations leading to rate enhancement is lacking.

The flavoprotein pentaerythritol tetranitrate reductase (PETNR) is an excellent model system for understanding enzymatic H-transfer.[3,16] The reductive half-reaction (RHR) in PETNR involves stereoselective hydride transfer from the C4 *pro-R* hydrogen of reduced β-NADPH to the N5 atom of flavin mononucleotide (FMN) and is thought to proceed predominantly by a tunneling mechanism.[3] Prior to flavin reduction, NADPH binds to oxidized PETNR forming a charge-transfer (CT) complex (a binary complex). FMN reduction can be monitored as a pseudo-first-order reaction using single-turnover approaches.[3] In the homologous flavoprotein morphinone reductase (MR) ∼99% of the analogous hydride transfer, from NADH to FMN, has been shown to proceed by tunnelling[13] and to invoke a promoting vibration.[3,5] In PETNR, hydride transfer can proceed from both NADH and NADPH and the mechanism of binding is effectively the same for both coenzymes.[16] Despite the similarity in coenzyme structure and chemistry, there is a significant difference in the observed rate of FMN reduction, ∼15× faster with NADPH compared to NADH (Table 1).[16] This study examines the origin of this rate enhancement.

While the H-transfer distance is generally considered the primary determinant of the rate for a tunneling reaction (see below), it is important to consider the effect of any difference in driving force, $\Delta G°$, with NADPH and NADH. We have measured the two-electron midpoint reduction potential of the FMN cofactor in PETNR with $NADPH_4$ ($E_m = -185 \pm 5$ mV; 25 °C) and $NADH_4$ ($E_m = -135 \pm 5$ mV; 25 °C) bound in the active site [Supporting Information (SI) Figure S1]. If the observed rate difference is attributable to effects of $\Delta G°$, then the $\Delta G°$ contribution to the observed rate should be less for NADPH compared to NADH.

Table 1. Temperature/Pressure Dependence of FMN Reduction in PETNR with NADPH and NADH

	PETNR−NADPH	PETNR−NADH
k_{obs} (s^{-1})[a,b]	33.5 ± 0.2	2.0 ± 0.02
1° KIE [a,b]	7.0 ± 0.04	8.12 ± 0.09
2° KIE [b]	1.17 ± 0.01[a]	1.18 ± 0.02
$\Delta H^{‡H}$ (kJ mol^{-1})	32.3 ± 1.02	34.2 ± 0.62
A_H'/A_D'	0.51 ± 0.04	5.2 ± 0.46
$\Delta\Delta H^{‡}$ (kJ mol^{-1})	6.5 ± 2.8	−1.1 ± 2.1
$\Delta\Delta\beta^{‡}$ (cm^3 mol^{-1} $kbar^{-1}$)	2.6 ± 6.5	−1.9 ± 3.2

[a] Data reported in Pudney et al.[21,16] [b] Data reported at 25 °C.

However, the observed trend is opposite to this, which argues against differences in driving force with NADPH and NADH being the origin of the difference in the observed rate of hydride transfer.

Temperature and pressure dependence studies of the primary kinetic isotope effect (1° KIE) are able to probe the contribution of promoting vibrations to a reaction.[17,18] A significant temperature dependence on the KIE is considered to reflect a promoting vibration with a small force constant (a "soft" vibrational mode), and a temperature independent KIE reflects either a promoting vibration with a large force constant (a "hard" vibrational mode) or the lack of a promoting vibration coupled to the H-transfer coordinate.[6] Pressure (p) dependence studies are an additional tool for identifying the contribution of promoting vibrations and can be used to independently corroborate temperature dependence studies.[19,5] Numerical modeling[19] suggests that the key hallmark of a "soft" promoting vibration with respect to pressure is the presence of curvature in a plot of KIE vs p, and a "hard" promoting vibration gives a linear decrease in a plot of KIE vs p. This in turn is reflected in the magnitude of the compressibility of the KIE, $\Delta\Delta\beta^{‡}$ (SI eq 1), which effectively monitors curvature in a plot of KIE vs p.[19]

We next studied the temperature ($\Delta\Delta H^{‡}$) and pressure dependence ($\Delta\Delta\beta^{‡}$) of the 1° KIE for FMN reduction in PETNR with both NADH and NADPH (Figure 1 and Table 1). SI Tables S1,2 and Figure S2 show the temperature/pressure dependence of the observed rates ($\Delta H^{‡}$ and $\Delta\beta^{‡}$). Figure 1A shows a temperature dependent KIE with NADPH ($\Delta\Delta H^{‡} = 6.5 \pm 2.8$ kJ mol^{-1}) and a temperature independent KIE with NADH ($\Delta\Delta H^{‡} = -1.1 \pm 2.1$ kJ mol^{-1}). The temperature dependence of the KIE with NADPH has been previously measured, although the lower value obtained in the previous study appears to be due to isotopic fractionation.[20] The data in Figure 1 are consistent with a "soft" promoting vibration associated with the reduction of PETNR by NADPH.

With NADH, we infer that the contribution to the reaction from the promoting vibration is much weaker due to the much "harder" promoting vibration. We have also measured the pressure dependence of the KIE with NADPH and NADH (Figure 1B). There is (Figure 1B) significant positive curvature in the KIE with NADPH

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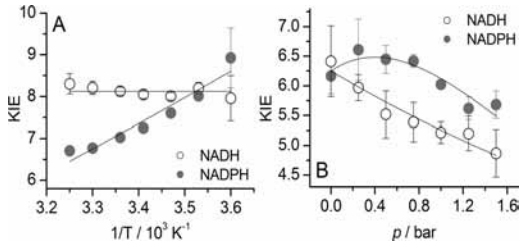

Figure 1. Temperature dependence (A) and pressure dependence at 25 °C (B) of the observed 1° KIEs for FMN reduction in PETNR with NADH and NADPH. The solid lines are fits to the Eyring equation (A) and to eq 1 in the Supporting Information (B). Fitting parameters are given in Table 1. Conditions: 20 μM PETNR, 25 mM NADH/5 mM NADPH.

and a linear decrease with NADH, $\Delta\Delta\beta^{\ddagger} = 2.6 \pm 6.5$ and -1.9 ± 3.2 cm^3 mol^{-1} kbar^{-1}, respectively. As such, the pressure dependence data agree qualitatively with the temperature analysis, suggesting that a "soft" promoting vibration is a significant feature of the reaction coordinate with the "fast" coenzyme (NADPH) while a "hard" or absent promoting vibration is associated with the "slow" coenzyme (NADH).

For enzymatic tunneling reactions, the donor−acceptor transfer distance is considered to be the key determinant of the H-transfer rate. Usually, a promoting vibration is induced by making active site or distal mutations[22] or by using different substrates[23] and is accompanied by a decrease in reaction rate (a consequence of the change in H-transfer distance). Consequently, it is difficult to conclude if promoting vibrations give rise to rate enhancements. In an attempt to address this issue, we have investigated the geometry of the PETNR−NADH and −NADPH reactive complexes using a number of methods.

First, we determined the crystal structure of PETNR bound to the unreactive NADH mimic 1,4,5,6-tetrahydro NADH ($NADH_4$), shown in SI Figure S3; data collection statistics are given in SI Table S3. $NADPH_4$ is less stable than the $NADH_4$ complex in solution,[24,25] preventing crystallographic analysis of the $NADPH_4$−PETNR complex. Consequently, we performed molecular dynamics (MD) simulations with PETNR and NADPH (SI Figures S3,4). A structural overlay of PETNR bound to NADPH and $NADH_4$ is shown in Figure S3. The key moiety is the nicotinamide, with H-transfer proceeding from the C4 *pro-R* hydrogen. The alignment suggests that PETNR binds the nicotinamide moieties of NADH and NADPH in near identical configurations and that the donor−acceptor distance is essentially the same [3.73 Å (PETNR-NADH) vs 3.64 Å (PETNR-NADPH)]. Binding and kinetic studies indicate that binary charge-transfer (CT) complex formation directly precedes H-transfer.[26,16] The magnitude of the absorbance of the CT complex depends on the orientation of the species involved, with better $\pi-\pi$ overlap between the FMN isoalloxazine and nicotinamide ring of NAD(P)H giving increased broad absorbance centered at ~555 nm.[27,4] The PETNR−NADH and PETNR−NADPH complexes have similar extinction coefficients in the CT absorbance band at 555 nm.[16] This suggests that the nicotinamide ring is similarly oriented in both complexes.

Although the crystallographic, modeling, and spectroscopic data suggest that the geometry of the (minimum energy) binary complex is similar, this is not the tunneling ready configuration (TRC), the active site configuration from which tunneling proceeds.[21] The TRC is approached by thermal activation of the binary complex to reach a configuration in which acceptor−donor states are degenerate.[17] The respective ground state complexes and TRCs are likely to have very similar geometries, a notion that is also supported through measurement of 2° KIEs.[28,21] The magnitude of the 2° KIE reflects the geometry of the TRC,[28,21] and the 2° KIE in PETNR is the same within error at 25 °C with both NADPH (1.17 ± 0.01; ref 21) and NADH (1.18 ± 0.01; reported here). We therefore infer that the tunneling distance in the TRC is comparable in both the PETNR−NADH and PETNR−NADPH complexes.

Through our study of two essentially isostructural enzyme−coenzyme complexes we conclude that the difference in observed rate between two near identical coenzymes bound to the same enzyme species is attributable to a vibrational mode present predominantly in the PETNR−NADPH complex. This provides evidence to support the hypothesis that promoting vibrations can enhance the rate of enzymatic H-tunneling reactions.

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Supporting Information Available: Full experimental and computational details, potentiometric titrations of PETNR with $NAD(P)H_4$, temperature/pressure dependence of rate constants, temperature/pressure fitting functions, data collection statistics for the crystal structure of $NADH_4$ bound to PETNR, rmsd for MD simulations and structural alignment of PETNR−$NADH_4$/NADPH. This information is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Hay, S.; Pudney, C. R.; Scrutton, N. S. *FEBS J.* **2009**, *276* (15), 3930.
(2) Nagel, Z. D.; Klinman, J. P. *Nat. Chem. Biol.* **2009**, *5* (8), 543.
(3) Basran, J.; Harris, R. J.; Sutcliffe, M. J.; Scrutton, N. S. *J. Biol. Chem.* **2003**, *278* (45), 43973.
(4) Hay, S.; Pudney, C. R.; McGory, T. A.; Pang, J.; Sutcliffe, M. J.; Scrutton, N. S. *Angew. Chem., Int. Ed.* **2009**, *48* (8), 1452.
(5) Hay, S.; Sutcliffe, M. J.; Scrutton, N. S. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104* (2), 507.
(6) Johannissen, L. O.; Hay, S.; Scrutton, N. S.; Sutcliffe, M. J. *J. Phys. Chem. B* **2007**, *111* (10), 2631.
(7) Knapp, M. J.; Rickert, K.; Klinman, J. P. *J. Am. Chem. Soc.* **2002**, *124* (15), 3865.
(8) Kohen, A.; Cannio, R.; Bartolucci, S.; Klinman, J. P. *Nature* **1999**, *399* (6735), 496.
(9) Maglia, G.; Allemann, R. K. *J. Am. Chem. Soc.* **2003**, *125* (44), 13372.
(10) Caratzoulas, S.; Mincer, J. S.; Schwartz, S. D. *J. Am. Chem. Soc.* **2002**, *124* (13), 3270.
(11) Hammes-Schiffer, S. *Biochemistry.* **2002**, *41* (45), 13335.
(12) Masgrau, L.; Roujeinikova, A.; Johannissen, L. O.; Hothi, P.; Basran, J.; Ranaghan, K. E.; Mulholland, A. J.; Sutcliffe, M. J.; Scrutton, N. S.; Leys, D. *Science.* **2006**, *312* (5771), 237.
(13) Pang, J. Y.; Hay, S.; Scrutton, N. S.; Sutcliffe, M. J. *J. Am. Chem. Soc.* **2008**, *130* (22), 7092.
(14) Olsson, M. H. M.; Siegbahn, P. E. M.; Warshel, A. *J. Am. Chem. Soc.* **2004**, *126* (9), 2820.
(15) Warshel, A.; Villa-Freixa, J. *J. Phys. Chem. B* **2003**, *107* (44), 12370.
(16) Pudney, C. R.; Hay, S.; Scrutton, N. S. *FEBS J.* **2009**, *276* (17), 4780.
(17) Hay, S.; Pudney, C.; Hothi, P.; Johannissen, L. O.; Masgrau, L.; Pang, J.; Leys, D.; Sutcliffe, M. J.; Scrutton, N. S. *Biochem. Soc. Trans.* **2008**, *36*, 16.
(18) Nagel, Z. D.; Klinman, J. P. *Chem. Rev.* **2006**, *106* (8), 3095.
(19) Hay, S.; Scrutton, N. S. *Biochemistry.* **2008**, *47* (37), 9880.
(20) Hay, S.; Pudney, C. R.; Hothi, P.; Scrutton, N. S. *J. Phys. Chem. A.* **2008**, *112* (50), 13109.
(21) Pudney, C. R.; Hay, S.; Sutcliffe, M. J.; Scrutton, N. S. *J. Am. Chem. Soc.* **2006**, *128* (43), 14053.
(22) Klinman, J. P. *Chem. Phys. Lett.* **2009**, *471*, 179.
(23) Hothi, P.; Hay, S.; Roujeinikova, A.; Sutcliffe, M. J.; Lee, M.; Leys, D.; Cullis, P. M.; Scrutton, N. S. *ChemBioChem* **2008**, *9* (17), 2839.
(24) Lowry, O. H.; Rock, M. K.; Passonneau, J. V. *J. Biol. Chem.* **1961**, *236* (10), 2756.
(25) Wu, J. T.; Wu, L. H.; Knight, J. A. *Clin. Chem.* **1986**, *32* (2), 314.
(26) Khan, H.; Harris, R. J.; Barna, T.; Craig, D. H.; Bruce, N. C.; Munro, A. W.; Moody, P. C. E.; Scrutton, N. S. *J. Biol. Chem.* **2002**, *277* (24), 21906.
(27) Ewald, A. H.; Scudder, J. A. *J. Phys. Chem.* **1972**, *76* (2), 249.
(28) Hay, S.; Pang, J. Y.; Monaghan, P. J.; Wang, X.; Evans, R. M.; Sutcliffe, M. J.; Allemann, R. K.; Scrutton, N. S. *ChemPhysChem* **2008**, *9* (11), 1536.

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