Enzyme Catalysis

DOI: 10.1002/ange.200805502

Barrier Compression Enhances an Enzymatic Hydrogen-Transfer Reaction**

Sam Hay, Christopher R. Pudney, Tom A. McGrory, Jiayun Pang, Michael J. Sutcliffe, and Nigel S. Scrutton**

Although enzymes are efficient catalysts that can achieve unparalleled rate enhancements over the uncatalyzed reaction,[1] the precise origin(s) of their catalytic power still remain unresolved after more than a century of research.[2–4] The dominant paradigm of enzyme catalysis remains transition state theory (TST),[5] in which the rate of the reaction is determined by the height of the reaction barrier, that is, the free energy of the transition state above the reactant state. It is often possible to probe experimentally the height (energy) of the reaction barrier by measuring the temperature dependence of the reaction rate and analyzing these data in terms of Arrhenius or Eyring theory.[5] However, it is likely that more than half of all known enzyme-catalyzed reactions involve one or more hydrogen (H^+, H, or $H^{\bullet-}$) transfers, and it is now becoming apparent that in many cases these H transfers occur in part, or in full, by quantum mechanical hydrogen tunneling.[6–11] Unlike classical over-the-barrier (TST) reactions, the rate of a tunneling reaction—which proceeds through the reaction barrier rather than over it—is governed by the overall shape, and particularly the width, of the barrier.[6,7,9,11–13] It is not possible to experimentally deduce any real information about the shape of the reaction barrier from the temperature dependence of the reaction rate. Clearly, an alternative experimental probe of barrier shape, and particularly barrier width, is desirable when studying these reactions.

As an alternative to temperature, hydrostatic pressure is a tractable experimental condition with which to probe enzymatic reactions in solution. Northrop pioneered the use of combining steady-state isotope and pressure effects to study enzymatic H-transfer reactions, and analyzed the results in terms of equilibrium perturbations to TST.[14–16] We recently extended this approach to study the pre-steady-state (single-turnover) kinetics of a hydride transfer during the reductive half-reaction (RHR) of the flavoprotein morphinone reductase (MR).[12] This H transfer has been shown to occur by deep (> 99 %) tunneling[17] and has a putative fast promoting vibration[18–21] thought to transiently compress the reaction (tunneling) barrier width, which leads to a depressed and highly temperature-dependent primary kinetic isotope effect (KIE).[12,22–24] Consequently, the combined temperature and pressure dependence of the rate and KIE for this reaction was originally analyzed[12,25] in terms of a Marcus-like H-tunneling model.[8,18–21,26] High-pressure X-ray crystallography has shown that, over several kilobars of pressure change, atoms within proteins are typically displaced by ≈0.1–1 Å (see, for example, references [27,28], and references therein). The key assumption in the modeling of the MR data was that hydrostatic pressure "squeezes" the enzyme and consequently compresses the reaction barrier. This squeezing—an untested and major assumption of the physical model—is investigated in the current study.

H transfer during the RHR of MR with the coenzyme nicotinamide adenine dinucleotide (NADH) is concomitant with flavin mononucleotide (FMN) reduction and can be directly observed in a stopped-flow instrument. The RHR proceeds in three steps and H transfer is kinetically resolved from the preceding step involving coenzyme binding and formation of the MR–NADH binary complex:[12,29]

$$\mathrm{MR} + \mathrm{NADH} \xrightarrow{\text{binding}} [\mathrm{MR}\cdot\mathrm{NADH}]^{\mathrm{CT}} \xrightarrow{\text{reduction/H transfer}} \mathrm{MR_{red}}\cdot\mathrm{NAD^+} \xrightarrow{\text{product release}} \mathrm{MR_{red}} + \mathrm{NAD^+} \quad (1)$$

The binary complex—that is, the reactant state—has a characteristic π–π charge-transfer (CT) absorbance (Figure 1 A), as the FMN isoalloxazine and NADH nicotinamide rings are roughly coplanar within the active site[30] (Figure 1 B). We have previously examined the effect of pressure on the transient CT absorbance of the MR–NADH binary complex during stopped-flow experiments and were unable to measure a significant change in CT absorbance with pressure.[12] However, this method is imprecise and it is also possible to study the binary complex by mixing MR with the nonreactive NADH analogue 1,4,5,6-tetrahydroNADH ($NADH_4$), because although this also binds and forms a CT complex, it cannot go on to reduce the FMN[30] (Figure 1). This method is advantageous as more sensitive static absorbance measurements can be performed.

In the current study, we measured the absorption spectra of the MR–$NADH_4$ CT complex every 50 bar (1 bar = 100 kPa) from 1 bar to 2 kbar. A small but significant increase

[*] T. A. McGrory, Dr. J. Pang, Prof. M. J. Sutcliffe
Manchester Interdisciplinary Biocentre, School of Chemical Engineering and Analytical Science, University of Manchester
131 Princess Street, Manchester M1 7DN (UK)
Fax: (+44) 161-306-5201
E-mail: mike.sutcliffe@manchester.ac.uk
Dr. S. Hay, Dr. C. R. Pudney, Prof. N. S. Scrutton
Manchester Interdisciplinary Biocentre, Faculty of Life Sciences, University of Manchester
131 Princess Street, Manchester M1 7DN (UK)
Fax: (+44) 161-306-8918
E-mail: nigel.scrutton@manchester.ac.uk

[**] This work was funded by the UK Biotechnology and Biological Sciences Research Council (BBSRC). N.S.S. is a BBSRC Professorial Research Fellow.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200805502.

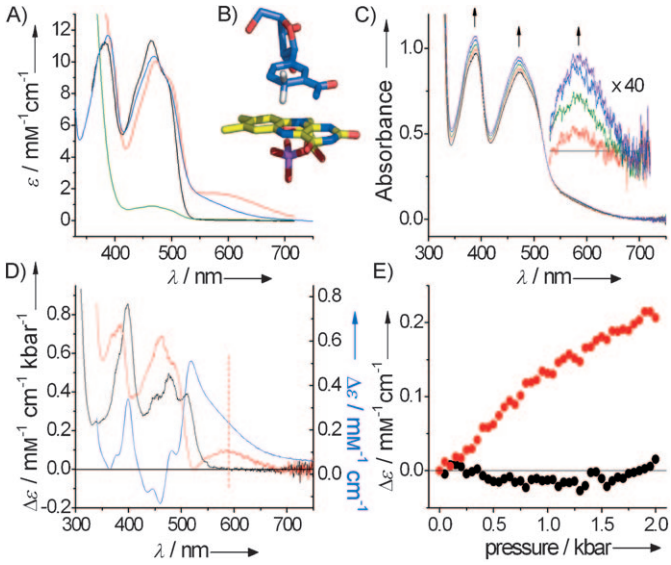

Figure 1. Effect of pressure on the CT absorbance of the MR binary complex. A) Absorption spectra of oxidized MR (black), the spectrally deconvoluted CT complex with NADH (red), and reduced MR (green); from Ref. [12]. The MR–$NADH_4$ binary complex formed with 22 mM $NADH_4$ is shown in blue for comparison. B) FMN isoalloxazine (yellow) and $NADH_4$ nicotinamide (blue) moieties within the MR active site (pdb 2R14[30]). C) Absorption spectra of the MR–$NADH_4$ binary complex measured at 1 bar and 0.5, 1, 1.5, and 2.0 kbar. The arrows show the change with increasing pressure, and offset difference spectra (relative to 1 bar) of the CT absorbance around 590 nm are also shown. D) Difference spectra (1 kbar minus 1 bar) of MR (black) and $NADH_4$-bound MR (red) compared to the 1 bar $NADH_4$ bound-minus-unbound spectrum (blue). The origin of the MR and MR–$NADH_4$ absorbance increase at ≈460 nm is uncertain but may result from electrostriction of water molecules within the active site (see, for example, Ref. [32]). E) Pressure dependence of the 590 nm absorbance (red line in (C)) of MR (black) and the binary complex of $NADH_4$ with MR (red). Note: raw spectra are also shown in the Supporting Information.

in the CT absorbance with increasing pressure was observed (Figure 1 C–E). A previous experimental study has shown that increasing pressure causes a progressive shortening of the CT bond in π–π complexes and, as the CT bond is shortened, the CT spectra shift to red wavelengths and increase in absorbance.[31] The MR–$NADH_4$ binary complex shows a small CT absorbance peak shift with increasing pressure, with the difference spectra (relative to 1 bar) showing a maximum at ≈590 nm (Figure 1 C,D). The apparent dissociation constant for the MR–$NADH_4$ complex at this temperature (25 °C) is 0.35 ± 0.05 mM (see the Supporting Information) and the experiments in Figure 1 were performed with 22 mM $NADH_4$ (>50× the K_d value), so it is unlikely that the observed change in absorbance with pressure reflects the binding of more or less $NADH_4$ at high pressure. Together, these data suggest that, at elevated pressure, the MR–$NADH_4$ binary complex is stable and the CT bond between the FMN isoalloxazine and NADH nicotinamide moieties becomes shorter.

Pressure acts on chemical systems by shifting preexisting equilibria toward the species with the smaller volume.[14,32] As a result, pressure can be used to probe physiological transitions that occur infrequently at atmospheric pressure. The MR–NADH binary complex probably exists in multiple conformations with differing volumes and reaction barrier widths,[30] that is, as a multidimensional free-energy surface.[33] If the conformations with the smaller volumes also have shorter CT bonds, then pressure will effectively squeeze the FMN isoalloxazine and NADH nicotinamide rings together by restricting the conformations of the binary complex to those conformations with shorter CT bonds (and smaller volumes). If the binary complex is the reactant state for the H-transfer reaction—we cannot rule out the presence of another intermediate state that is not observed—then pressure will cause a decrease in the average reaction barrier width, because squeezing the isoalloxazine and nicotinamide rings together will bring the nicotinamide C4 (donor) and isoalloxazine N5 (acceptor) heavy atoms closer together. As the volume of the active site changes with pressure, this squeezing may also manifest as compressibility ($d(-\Delta V^{\neq})/dp$), which we have observed during the RHR of MR with NADH.[12]

It is possible to run constant-pressure molecular dynamics (MD) simulations (in which the volume, rather than pressure, can fluctuate) to specifically investigate the role of hydrostatic pressure on a system. MD simulations of the MR–NADH binary complex were run at fixed (constant) pressures of 1 bar, 1 kbar, and 2 kbar. The simulations showed that the protein is stable over this range of pressures because, except for a slight increase in the helical content, the overall secondary structure does not appear to significantly change as the pressure is increased. Analysis over 10 ns trajectories of the NADH nicotinamide C4–FMN isoalloxazine N5 separation shows a roughly Gaussian distribution between 3 and 6 Å. This distribution both narrows and shifts to shorter distances at elevated pressures (Figure 2 and the Supporting Information).

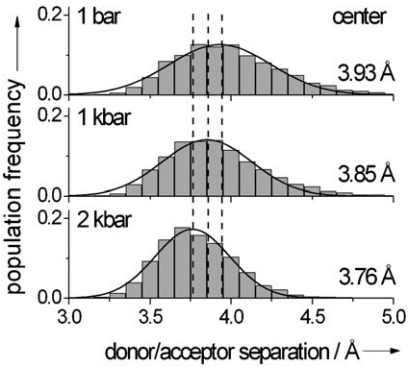

Figure 2. MD analysis of NADH-bound MR showing the NADH C4–FMN N5 separation over 10 ns trajectories binned at 0.1 Å resolution. The vertical dotted lines show the center of the respective Gaussian fits (solid lines) with their central positions listed.

Interestingly, while the average C4–N5 separation decreases with pressure, the minimum separation does not appear to significantly change, consistent with the hypothesis that pressure simply causes a shift in equilibrium, thus favoring those binary complex conformations with shorter

CT bonds and reaction barrier widths. In other words, pressure does not physically squeeze the microscopic reaction barrier in MR but appears to reduce the *average* barrier width (that is, the macroscopic barrier) by restricting the conformational space available to the FMN isoalloxazine and NADH nicotinamide moieties, through disfavoring those configurations with a larger C4–N5 separation (and presumably larger atomic volumes).

In the case of the MR/NADH reaction, we have previously shown that pressure increases the apparent rate of catalysis by increasing the rate of H transfer by about twofold per kilobar.[12] Regardless of whether there is more or less hydride tunneling at high pressure (relative to over-the-barrier TST transfer), hydrostatic pressure does appear to cause barrier compression in this enzyme, which is accompanied by an increase in the catalyzed rate of reaction. If, in keeping with current dogma, promoting vibrations[18–21] (environmental coupling) also cause barrier compression then they should, at least in MR, cause qualitatively similar effects as hydrostatic pressure, that is, an increase in the rate of catalysis.

Experimental Section

All materials were obtained from Sigma–Aldrich (St. Louis, MO), except NADH (Melford Laboratories, Chelsworth, UK). MR was purified as described previously[22,29] and the enzyme concentration was determined by $\varepsilon(462\text{ nm}) = 11.3\text{ mM}^{-1}\text{cm}^{-1}$. $NADH_4$ was prepared as described previously[30] and its concentration was determined by $\varepsilon(289\text{ nm}) = 16.8\text{ mM}^{-1}\text{cm}^{-1}$.[34]

High-pressure static absorbance measurements were performed with a Hi-Tech Scientific HPSF-56 high-pressure stopped-flow spectrophotometer (TgK Scientific, Bradford on Avon, UK). All measurements were made in Tris-HCl (50 mM)/2-mercaptoethanol (2 mM), pH 8.0.

MD simulations were performed by using AMBER8[35] with the AMBER 03 force field.[36] The initial system setup has been described in detail previously.[17] A different equilibration procedure was applied, however, in which the system was heated to 298 K under constant-volume condition, then the constant-pressure condition was turned on to equilibrate the system at the desired pressure of 1 bar, 1 kbar, or 2 kbar for 2 ns. The production trajectories were then collected for 10 ns. These trajectories were analyzed with PTRAJ implemented in AMBER8. The NADH C4–FMN N5 distance trajectories were also analyzed by binning the data at 0.1 Å intervals and fitting these data to a Gaussian (see the Supporting Information).

Received: November 11, 2008
Published online: January 14, 2009

Keywords: enzyme catalysis · hydrogen transfer · molecular dynamics · quantum chemistry · reaction barrier

[1] C. Lad, N. H. Williams, R. Wolfenden, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 5607.
[2] W. W. Cleland, P. A. Frey, J. A. Gerlt, *J. Biol. Chem.* **1998**, *273*, 25529.
[3] S. J. Benkovic, S. Hammes-Schiffer, *Science* **2003**, *301*, 1196.
[4] M. Garcia-Viloca, J. Gao, M. Karplus, D. G. Truhlar, *Science* **2004**, *303*, 186.
[5] S. Gladstone, K. J. Laidler, H. Eyring, *The Theory of Rate Processes*, McGraw-Hill, New York, **1941**.
[6] Z. D. Nagel, J. P. Klinman, *Chem. Rev.* **2006**, *106*, 3095.
[7] D. Antoniou, J. Basner, S. Nunez, S. D. Schwartz, *Chem. Rev.* **2006**, *106*, 3170.
[8] R. A. Marcus, N. Sutin, *Biochim. Biophys. Acta Rev. Bioenerg.* **1985**, *811*, 265.
[9] S. Hay, C. Pudney, P. Hothi, L. O. Johannissen, L. Masgrau, J. Pang, D. Leys, M. J. Sutcliffe, N. S. Scrutton, *Biochem. Soc. Trans.* **2008**, *36*, 16.
[10] D. G. Truhlar, J. L. Gao, C. Alhambra, M. Garcia-Viloca, J. Corchado, M. L. Sanchez, J. Villa, *Acc. Chem. Res.* **2002**, *35*, 341.
[11] S. Hammes-Schiffer, *Acc. Chem. Res.* **2006**, *39*, 93.
[12] S. Hay, M. J. Sutcliffe, N. S. Scrutton, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 507.
[13] J. Basran, S. Patel, M. J. Sutcliffe, N. S. Scrutton, *J. Biol. Chem.* **2001**, *276*, 6234.
[14] D. B. Northrop, *J. Am. Chem. Soc.* **1999**, *121*, 3521.
[15] D. B. Northrop, *Philos. Trans. R. Soc. London Ser. B* **2006**, *361*, 1341.
[16] D. B. Northrop, *Biochim. Biophys. Acta Protein Struct. Mol. Enzymol.* **2002**, *1595*, 71.
[17] J. Pang, S. Hay, N. S. Scrutton, M. J. Sutcliffe, *J. Am. Chem. Soc.* **2008**, *130*, 7092.
[18] D. Antoniou, S. D. Schwartz, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 12360.
[19] A. M. Kuznetsov, J. Ulstrup, *Can. J. Chem.* **1999**, *77*, 1085.
[20] D. Borgis, J. T. Hynes, *J. Phys. Chem.* **1996**, *100*, 1118.
[21] W. J. Bruno, W. Bialek, *Biophys. J.* **1992**, *63*, 689.
[22] J. Basran, R. J. Harris, M. J. Sutcliffe, N. S. Scrutton, *J. Biol. Chem.* **2003**, *278*, 43973.
[23] C. R. Pudney, S. Hay, M. J. Sutcliffe, N. S. Scrutton, *J. Am. Chem. Soc.* **2006**, *128*, 14053.
[24] S. Hay, C. R. Pudney, M. J. Sutcliffe, N. S. Scrutton, *Angew. Chem.* **2008**, *120*, 547; *Angew. Chem. Int. Ed.* **2008**, *47*, 537.
[25] S. Hay, M. J. Sutcliffe, N. S. Scrutton in *Quantum Tunnelling in Enzyme Catalyzed Reactions* (Eds.: R. K. Allemann, N. S. Scrutton), Royal Society of Chemistry, in press.
[26] M. J. Knapp, K. Rickert, J. P. Klinman, *J. Am. Chem. Soc.* **2002**, *124*, 3865.
[27] C. E. Kundrot, F. M. Richards, *J. Mol. Biol.* **1987**, *193*, 157.
[28] B. Barstow, N. Ando, C. U. Kim, S. M. Gruner, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 13362.
[29] D. H. Craig, P. C. E. Moody, N. C. Bruce, N. S. Scrutton, *Biochemistry* **1998**, *37*, 7598.
[30] C. R. Pudney, S. Hay, J. Y. Pang, C. Costello, D. Leys, M. J. Sutcliffe, N. S. Scrutton, *J. Am. Chem. Soc.* **2007**, *129*, 13949.
[31] A. H. Ewald, *Trans. Faraday Soc.* **1968**, *64*, 733.
[32] P. Masson, C. Balny, *Biochim. Biophys. Acta Gen. Subj.* **2005**, *1724*, 440.
[33] S. J. Benkovic, G. G. Hammes, S. Hammes-Schiffer, *Biochemistry* **2008**, *47*, 3317.
[34] G. Branlant, B. Eiler, J. F. Biellmann, *Anal. Biochem.* **1982**, *125*, 264.
[35] D. A. Case, T. E. Cheatham, T. Darden, H. Gohlke, R. Luo, K. M. Merz, A. Onufriev, C. Simmerling, B. Wang, R. J. Woods, *J. Comput. Chem.* **2005**, *26*, 1668.
[36] Y. Duan, C. Wu, S. Chowdhury, M. C. Lee, G. M. Xiong, W. Zhang, R. Yang, P. Cieplak, R. Luo, T. Lee, J. Caldwell, J. M. Wang, P. Kollman, *J. Comput. Chem.* **2003**, *24*, 1999.

