Enzyme Catalysis

DOI: 10.1002/anie.200900086

Solvent-Slaved Protein Motions Accompany Proton but Not Hydride Tunneling in Light-Activated Protochlorophyllide Oxidoreductase**

Derren J. Heyes, Michiyo Sakuma, and Nigel S. Scrutton**

The link between dynamics and catalysis remains a key question in studies of enzyme mechanism.[1–4] Of particular interest are the mechanisms by which localized structural changes in an enzyme active site are coupled to more distant perturbations of enzyme structure and ultimately to solvent dynamics.[2,5–7] Studies of the perturbation of solvent and protein dynamics are often hindered for thermally activated enzymes because of limitations imposed by mixing enzyme and substrate to initiate catalysis. The light-driven enzyme protochlorophyllide oxidoreductase (POR) allows synchronous triggering of enzyme chemistry with laser light in a 'dark-assembled' enzyme-substrate complex, and is therefore a good model for studies of dynamics linked to enzyme catalysis.[8]

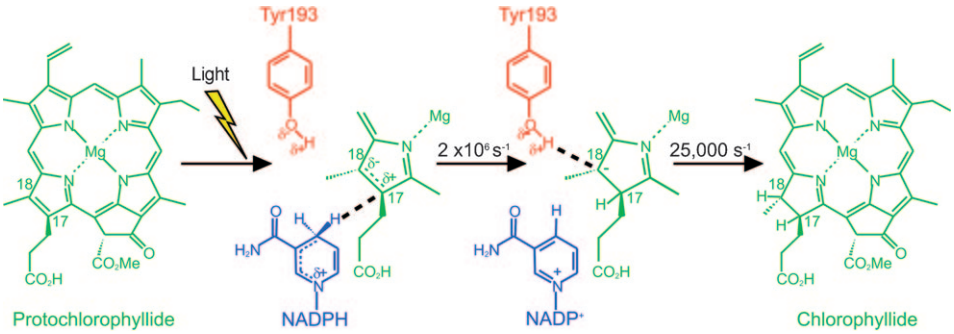

Figure 1. The light-activated reduction of protochlorophyllide (Pchlide) catalyzed by POR. Upon activation by light, a hydride is transferred from NADPH to the C17 position of Pchlide and a proton is transferred from Tyr193 to the Pchlide C18 position. Rate constant at 298 K is shown for each step. [16]

POR catalyzes the light-dependent *trans* addition of hydrogen across the C17–C18 double bond of protochlorophyllide (Pchlide) to produce chlorophyllide (Chlide) (Figure 1), a reaction that is important in chlorophyll biosynthesis and assembly of the photosynthetic apparatus.[8,9] POR catalyzes hydride transfer from the *pro-S* face of NADPH to the C17 of Pchlide,[10] and proton transfer to C18 of Pchlide.[11] Low temperature absorption and emission spectroscopy in the visible range indicates that the reaction cycle comprises an initial light-driven reaction[12] followed by a series of subsequent dark reactions.[13,14] The initial light-driven step involves hydride transfer to form a charge-transfer complex, which facilitates the subsequent protonation of the C18 position of Pchlide from the protein during the first of the 'dark' reactions.[15] Both H-transfer (hydride- and proton-transfer) reactions occur by quantum mechanical tunneling.[16] Proton tunneling has a strong requirement for motion coupled to the H-transfer (proton-transfer) coordinate to facilitate the tunneling reaction by compression of the tunneling barrier. Hydride transfer also depends on 'compressive' motion at physiological temperatures, but this motion is lessened at cryogenic temperatures as these motions are 'frozen out'.[16] The remaining 'dark' reactions involve ordered product release and cofactor binding steps linked to conformational change.[13,14,17] Ultrafast measurements have identified spectral changes on the picosecond timescale that likely represent conformational change prior to Pchlide reduction.[18–20] The excitation with a laser pulse generates an optimal conformation of the active site that leads to enhanced catalytic efficiency.[20]

Protein motions can be intimately linked to the solvent environment. It therefore follows that studies of solvent effects on catalysis can yield important information on the dynamics of an enzyme system.[21–25] Different classes of protein motions might be controlled by solvent fluctuations to varying degrees.[23–25] Solvent 'slaved' motions are influenced by the dielectric fluctuations of the overall bulk solvent and become significantly reduced below the solvent glass transition temperature. Hydration-shell coupled motions are largely independent of bulk solvent motions and follow the dynamics of the hydration layers. 'Non-slaved' motions are not influenced by the bulk solvent and follow the thermally-activated internal vibrations of the protein. Perturbation of solvent viscosity has been used to probe protein rearrangements following dissociation of carbon monoxide from myoglobin,[26] and configurational or conformational gating of protein electron-transfer reactions.[27–30] However, comparable studies on enzymatic hydride tunneling and proton tunneling reactions are limited.[31–35]

[*] Dr. D. J. Heyes, M. Sakuma, 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: derren.heyes@manchester.ac.uk
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.200900086.

We analyzed the sequential hydride and proton tunneling reactions catalyzed by POR to include studies of bulk solvent on the dynamics of H-transfer (proton and hydride). Catalysis by the POR-NADPH-Pchlide ternary complex was triggered using a 6 ns laser pulse at 450 nm using established methods.[16] The formation of an intermediate with an absorbance band centered at 696 nm occurs with a rate constant of circa $2\times10^6\ s^{-1}$ at 298 K and represents hydride tunneling from NADPH to C17 of Pchlide.[16] A slower exponential decay of this 696 nm absorbance band represents proton tunneling (ca. 25000 s^{-1} at 298 K[16]). The rate constants for both the hydride and proton-transfer reactions are essentially independent of the solution pH (5–10), confirming that the proton does not originate from the bulk solvent (Supporting Information, Figure S1).

Rate constants (Figure 2a) for hydride transfer were not affected by the solution viscosity, which was tuned using different concentrations of glycerol (0 to 60%). Measurements were limited to a maximum glycerol concentration of 60%, as the amplitude of the absorbance change of the intermediate at 696 nm, probing the hydride tunneling mechanism, become insignificant at higher concentrations. As solvent molecules are much smaller than the enzyme under study, the effect of viscosity on the observed rate of a reaction can be described by combining the Kramers and the Eyring equations to describe the contribution of the protein friction to the total friction of the system according to Equation (1):[22,28]

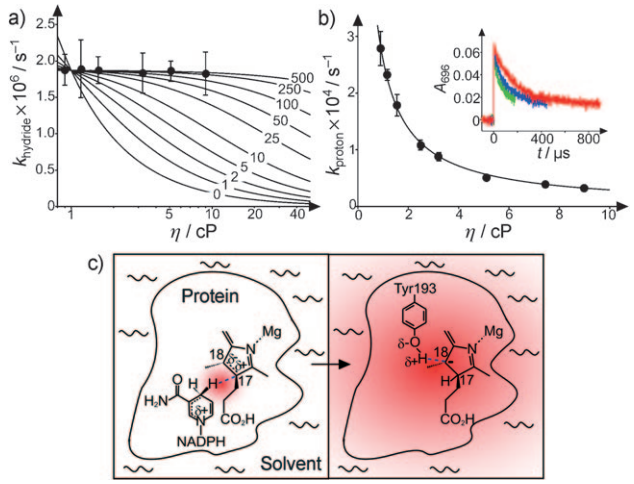

Figure 2. Viscosity-dependence of H-transfer (proton and hydride) rate constants in POR. a) Dependence of hydride transfer rate constants on solvent viscosity. $k_{hydride}$ is the rate constant for hydride transfer. The solid lines represent hydride-transfer rates vs. η calculated according to Equation (1) for values of σ ranging from 0 to 500 cP. b) Dependence of proton-transfer rate constants on solvent viscosity. k_{proton} is the rate constant for proton transfer. Inset: kinetic transients for proton transfer at 696 nm (green,1.56 cP; blue, 2.49 cP, red, 5.11 cP). c) Scheme showing the extent of dynamic motions (shown by red gradient) associated with hydride (left hand panel) and proton transfer (right hand panel).

$$k_{obs}=\frac{k_B T}{h}\left(\frac{1+\sigma}{\eta+\sigma}\right)\exp\left(\frac{-\Delta G^{\neq}}{RT}\right) \quad (1)$$

where σ, in units of viscosity, is the contribution of the protein friction and η is the absolute viscosity. As $\Delta G^{\neq}$ is assumed to be independent of viscosity,[28] we modeled the expected viscosity dependence for various values of σ between 0 and 500 cP (Figure 2a) using a $\Delta G^{\neq}$ value of 37.4 kJ mol^{-1} that was determined from previous temperature dependence measurements of the reaction.[16] The modeling showed that σ must be greater than circa 100 cP for the hydride-transfer reaction in POR (Figure 2a). By contrast, the rate constant for proton tunneling is strongly dependent on glycerol concentration and becomes significantly slower at higher viscosities (Figure 2b). The rate decreases from 28100 s^{-1} at 0.90 cP to 3100 s^{-1} at 8.99 cP (298 K). These effects are not glycerol specific: the rate constant for proton tunneling decreases in a similar fashion when sucrose is used as viscosogen, confirming the influence of the solvent viscosity on the reaction. The contribution of the protein friction to the total friction of the system was determined by fitting data using Equation (1) ($\sigma=(0.15\pm0.05)$ cP; $\Delta G^{\neq}=(47.8\pm0.03)$ kJ; Figure 2b).

We also investigated whether changes in solvent dielectric (ε) could affect the rate constants for hydride and proton tunneling. Thus, the dependence of measured rate constants on glycerol concentration was reanalyzed in terms of the solvent dielectric. As the solvent dielectric is typically altered by the addition of organic solvents to the buffer solution, the rate constants for both reactions were also measured in 5, 10, and 20% (v/v) ethanol (Supporting Information, Table S1). The rate constant for hydride transfer is not sensitive to changes in solvent dielectric (ε ranging from 60 to 80), but the rate constant for proton tunneling decreases at higher ethanol concentrations and is thus dependent on the dielectric of the bulk solvent.

The rate constant for proton transfer and the isotope effect for this proton transfer is strongly dependent on temperature.[16] This temperature dependence conforms to an environmentally coupled tunneling model for proton transfer and the need for coupled motion to narrow the tunneling barrier.[16,36] Herein we extend this analysis of proton and deuteron transfer by combining the temperature (278–323 K) and viscosity dependence (0.90–8.99 cP) of POR-catalyzed proton transfer in both protiated and deuterated buffer systems. The viscosity of the buffer is temperature dependent and was previously taken into account.[33] Data were analyzed using the Eyring equation (Supporting Information; Figure S2, Table S2). Rate constants for proton transfer are dependent on temperature at each of the chosen solvent viscosities. The isotope effect is temperature sensitive at each viscosity with an extrapolated Eyring prefactor ratio, which is less than one for this step, indicating a tunneling mechanism. Unlike the rate constant, the isotope effect, shows little or no dependence on the solvent viscosity (Supporting Information, Figure S3). Within an environmentally coupled tunneling model this little or no dependence on the viscosity indicates that motions coupled to the proton-transfer coordinate (i.e. the tunneling coordinate) are unaffected by the solvent

viscosity. The effect of viscosity is therefore felt only on the thermally equilibrated motions (Marcus-like dynamics) along the collective reaction coordinate required to produce degenerate reactant and product wells to enable quantum mechanical tunneling. Therefore, these thermally equilibrated motions are clearly slaved to the solvent dynamics.

Theory dictates that the effect of solvent viscosity on reaction rates are explained by considering the contribution of the protein friction to the total friction of the system (i.e. the solvent viscosity controls the rate of protein conformational change[22,28]). Studies of solvent dynamics on enzyme catalyzed proton or hydride tunneling reactions are in their infancy,[31–35] but are already proving useful in probing networks of extended dynamics in enzyme systems. With POR, the effects of viscosity are substantially different for hydride and proton tunneling, despite the close spatial origin of these reactions in the active site. For hydride tunneling, the lack of viscosity or dielectric dependence indicates that both non equilibrated (promoting) motions and equilibrated (Marcus-like) motions do not constitute a network of long-range protein motions influenced by the overall bulk solvent. We infer that these motions accompanying hydride transfer are localized in the active site and are non-slaved to solvent (Figure 2c). This is consistent with cryogenic spectroscopy studies of POR in which hydride transfer proceeds well below the glass transition temperature of proteins (<200 K).[16] By contrast proton transfer is very sensitive to changes in both the viscosity and dielectric of the solvent. This sensitivity indicates that a network of long-range and thermally equilibrated motions are required to drive the proton tunneling reaction (Figure 2c). These motions are solvent slaved and are likely to be similar to the conformational rearrangements that are prevalent in many gated protein electron-transfer reactions, where the rates of reaction are also viscosity-dependent.[26–30] The fact that proton transfer is associated with solvent slaved motions is consistent with low temperature spectroscopic analyses of the proton-transfer step. Unlike the hydride-transfer reaction, the proton transfer only occurs close to or above the glass transition temperature of proteins.[13,14] We emphasize that the independence of the measured isotope effect for proton/deuteron transfer on viscosity indicates that the non-equilibrated motions (promoting motions) coupled to the proton-transfer coordinate are non-slaved and thus represent a more localized dynamical contribution. The localized nature of this motion is generally consistent with other enzyme systems.[7,33,37]

We conclude that studies of solvent vicosity and dielectric can provide new and important insight into the importance of solvent and protein dynamics in enzymatic H-transfer (proton or hydride). In particular, the ability to study solvent coupling to both Marcus-like (equilibrated) and promoting (non equilibrated) motions in an enzyme system has been elucidated. This study of solvent coupling is achieved by studying the temperature and viscosity (or dielectric) dependence of isotope effects, and reaction rates within the context of the environmentally coupled tunneling model for enzymatic proton or hydride transfer.[36]

Experimental Section

All chemicals were obtained from Sigma–Aldrich, except NADPH (Melford Laboratories), D_2O, and [D_8]-glycerol (both Goss Scientific). Recombinant POR from the thermophilic cyanobacteria *Thermosynechococcus elongatus* and purified Pchlide were produced as described.[14] Solvent isotope effects were measured using deuterated buffer systems made up with D_2O and [D_8]-glycerol (Goss Scientific). POR was deuterated by exchange into a deuterated buffer containing Tris (50 mM, pH 7.5), NaCl (100 mM), and DTT (1 mM). Laser photoexcitation experiments were carried out as described previously.[16] The dependence of reaction rate on solution pH was performed in MTE buffer (50 mM MES, 25 mM Tris, 25 mM Ethanolamine) at 298 K. For viscosity studies, glycerol or sucrose solutions were prepared by weight and calculation of solution viscosity was done as described previously.[33]

Received: January 6, 2009
Published online: April 16, 2009

Keywords: enzymes · proton transfer · hydride transfer · reaction mechanisms · solvent dynamics

[1] J. Villà, A. Warshel, *J. Phys. Chem. B* **2001**, *105*, 7887.
[2] S. J. Benkovic, S. Hammes-Schiffer, *Science* **2003**, *301*, 1196.
[3] M. Garcia-Viloca, J. Gao, M. Karplus, D. G. Truhlar, *Science* **2004**, *303*, 186.
[4] D. D. Boehr, D. McElheny, H. J. Dyson, P. E. Wright, *Science* **2006**, *313*, 1638.
[5] A. Kohen, R. Cannio, S. Bartolucci, J. P. Klinman, *Nature* **1999**, *399*, 496.
[6] E. Z. Eisenmesser, O. Millet, W. Labeikovsky, D. M. Korzhnev, M. Wolf-Watz, D. A. Bosco, J. J. Skalicky, L. E. Kay, D. Kern, *Nature* **2005**, *438*, 117.
[7] L. Masgrau, A. Roujeinikova, L. O. Johannissen, P. Hothi, J. Basran, K. E. Ranaghan, A. J. Mulholland, M. J. Sutcliffe, N. S. Scrutton, D. Leys, *Science* **2006**, *312*, 237.
[8] D. J. Heyes, C. N. Hunter, *Trends Biochem. Sci.* **2005**, *30*, 642.
[9] N. Lebedev, M. P. Timko, *Photosynth. Res.* **1998**, *58*, 5.
[10] T. P. Begley, H. Young, *J. Am. Chem. Soc.* **1989**, *111*, 3095.
[11] H. M. Wilks, M. P. Timko, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 724.
[12] D. J. Heyes, A. V. Ruban, H. M. Wilks, C. N. Hunter, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 11145.
[13] D. J. Heyes, A. V. Ruban, C. N. Hunter, *Biochemistry* **2003**, *42*, 523.
[14] D. J. Heyes, C. N. Hunter, *Biochemistry* **2004**, *43*, 8265.
[15] D. J. Heyes, P. Heathcote, S. E. J. Rigby, M. A. Palacios, R. van Grondelle, C. N. Hunter, *J. Biol. Chem.* **2006**, *281*, 26847.
[16] D. J. Heyes, M. Sakuma, S. P. deVisser, N. S. Scrutton, *J. Biol. Chem.* **2009**, *284*, 3762.
[17] D. J. Heyes, M. Sakuma, N. S. Scrutton, *J. Biol. Chem.* **2007**, *282*, 32015.
[18] D. J. Heyes, C. N. Hunter, I. H. M. van Stokkum, R. van Grondelle, M. L. Groot, *Nat. Struct. Biol.* **2003**, *10*, 491.
[19] B. Dietzek, W. Kiefer, G. Hermann, J. Popp, M. Schmitt, *J. Phys. Chem. B* **2006**, *110*, 4399.
[20] O. Sytina, D. J. Heyes, C. N. Hunter, M. T. Alexandre, I. H. M. van Stokkum, R. van Grondelle, M. L. Groot, *Nature* **2009**, *456*, 1001.
[21] D. Beece, L. Eisenstein, H. Frauenfelder, D. Good, M. C. Marden, L. Reinisch, A. H. Reynolds, L. B. Sorensen, K. T. Yue, *Biochemistry* **1980**, *19*, 5147.
[22] A. Ansari, C. M. Jones, E. R. Henry, J. Hofrichter, W. A. Eaton, *Science* **1992**, *256*, 1796.

[23] P. W. Fenimore, H. Frauenfelder, B. H. McMahon, F. G. Parak, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 16047.
[24] P. W. Fenimore, H. Frauenfelder, B. H. McMahon, R. D. Young, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 14408.
[25] U. Samuni, C. J. Roche, D. Dantsker, J. M. Friedman, *J. Am. Chem. Soc.* **2007**, *129*, 12756.
[26] J. S. Zhou, N. M. Kostic, *J. Am. Chem. Soc.* **1992**, *114*, 3562.
[27] J. S. Zhou, N. M. Kostic, *J. Am. Chem. Soc.* **1993**, *115*, 10796.
[28] M. M. Ivkovic-Jensen, N. M. Kostic, *Biochemistry* **1997**, *36*, 8135.
[29] C. Feng, R. V. Kedia, J. T. Hazzard, J. K. Hurley, G. Tollin, J. H. Enemark, *Biochemistry* **2002**, *41*, 5816.
[30] L. Qin, N. M. Kostic, *Biochemistry* **1994**, *33*, 12592.
[31] A. Kohen, T. Jonsson, J. P. Klinman, *Biochemistry* **1997**, *36*, 2603.
[32] S. L. Seymour, J. P. Klinman, *Biochemistry* **2002**, *41*, 8747.
[33] S. Hay, C. R. Pudney, M. J. Sutcliffe, N. S. Scrutton, *Angew. Chem.* **2008**, *120*, 547; *Angew. Chem. Int. Ed.* **2008**, *47*, 537.
[34] S. Hay, C. R. Pudney, M. J. Sutcliffe, N. S. Scrutton, *ChemPhysChem* **2008**, *9*, 1875.
[35] E. J. Loveridge, R. M. Evans, R. K. Allemann, *Chem. Eur. J.* **2008**, *14*, 10782.
[36] A. M. Kuznetsov, J. Ulstrup, *Can. J. Chem.* **1999**, *77*, 1085.
[37] L. O. Johannissen, S. Hay, N. S. Scrutton, M. J. Sutcliffe, *J. Phys. Chem. B* **2007**, *111*, 2631.

