Nuclear Quantum Tunneling in the Light-activated Enzyme Protochlorophyllide Oxidoreductase

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In chlorophyll biosynthesis, the light-activated enzyme protochlorophyllide oxidoreductase catalyzes trans addition of hydrogen across the C-17–C-18 double bond of the chlorophyll precursor protochlorophyllide (Pchlide). This unique light-driven reaction plays a key role in the assembly of the photosynthetic apparatus, but despite its biological importance, the mechanism of light-activated catalysis is unknown. In this study, we show that Pchlide reduction occurs by dynamically coupled nuclear quantum tunneling of a hydride anion followed by a proton on the microsecond time scale in the Pchlide excited and ground states, respectively. We demonstrate the need for fast dynamic searches to form degenerate “tunneling-ready” configurations within the lifetime of the Pchlide excited state from which hydride transfer occurs. Moreover, we have found a breakpoint at −27 °C in the temperature dependence of the hydride transfer rate, which suggests that motions/vibrations that are important for promoting light-activated hydride tunneling are quenched below −27 °C. We observed no such breakpoint for the proton-tunneling reaction, indicating a reliance on different promoting modes for this reaction in the enzyme-substrate complex. Our studies indicate that the overall photoreduction of Pchlide is endothermic and that rapid dynamic searches are required to form distinct tunneling-ready configurations within the lifetime of the photoexcited state. Consequently, we have established the first important link between photochemical and nuclear quantum tunneling reactions, linked to protein dynamics, in a biologically significant system.

Hydrogen transfer reactions are fundamental chemical processes that are essential for almost all biological reactions. H-transfer by tunneling is an important feature of these reactions in enzymes (1–3), but mechanistic understanding of how protein motions (from the millisecond to sub-picosecond time domain) facilitate the H-tunneling reactions remains elusive (4–6). A major limitation has been the inability to synchronously trigger catalysis on ultrafast time scales for the majority of enzymes that require mixing strategies to initiate the reaction. However, by using the light-activated enzyme, protochlorophyllide oxidoreductase (POR; EC 1.3.1.33) (7), we have triggered two enzymatic H-transfer reactions using a single pulse of light, and we show these reactions occur sequentially by quantum tunneling in a pre-formed enzyme-substrate complex. This has provided a unique opportunity to analyze these reactions at physiological and cryogenic temperatures, on very fast time scales, that are experimentally inaccessible with other enzyme systems.

POR catalyzes the trans addition of hydrogen across the C-17–C-18 double bond of the chlorophyll precursor protochlorophyllide (Pchlide) to produce chlorophyllide (Chlide) (7), a unique light-driven reaction in the synthesis of the most abundant pigment on earth, which plays a key role in the assembly of the photosynthetic apparatus (8, 9). In addition to POR, nonflowering land plants, algae, and cyanobacteria possess a light-independent Pchlide reductase, which consists of three separate subunits and allows these organisms to produce Chlide in the dark (10). However, despite its biological significance, the detailed mechanism of this light-activated catalysis remains poorly understood. Low temperature spectroscopy has identified a number of steps in the reaction cycle, including an initial light-driven reaction (11, 12) and subsequent dark reactions (13–15). In the reaction, reduction of Pchlide by NADPH in the POR-NADPH-Pchlide complex involves hydride transfer from the pro-S face of NADPH to the C-17 atom of Pchlide (16). The valence of the C-18 atom is satisfied by the transfer of a proton from a conserved Tyr residue (Fig. 1), a finding based on previous site-directed mutagenesis studies (17) and the structural homology model of the Synechocystis enzyme (18). The remaining dark reactions involve a series of ordered product release and cofactor binding steps, which are linked to conformational changes in the enzyme (13–15).

In addition, previous ultrafast measurements on POR have revealed that spectral changes on the picosecond time scale are likely to represent conformational changes that occur before the reduction of Pchlide (19–22). Moreover, prior excitation with a laser pulse leads to a more efficient conformation of the active site and an enhancement in the catalytic efficiency of the enzyme systems.

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enzyme (22). However, a detailed description of the light-driven chemical steps is currently lacking. To access this chemistry we have now synchronized the turnover of the POR-NADPH-Pchlide complex (assembled in the dark) by using a 6-ns laser pulse tuned to the Soret region of the Pchlide absorbance spectrum for analysis in the nanosecond to microsecond time domain. We have combined kinetic analysis with studies of both the temperature and isotopic dependence of the rate of hydride and proton transfer to derive information on the mechanism of H-transfer in the photoexcited state of the ternary enzyme-substrate complex.

EXPERIMENTAL PROCEDURES

Sample Preparation—Recombinant POR from Thermosynechococcus elongatus was overexpressed in Escherichia coli and purified as described (14). Pchlide was purified as described (14). Deuterated pro-S NADPH (NADP²H) was purified and analyzed for chemical and isotopic purity as described (23). Solvent isotope effects were measured using deuterated buffer systems made with D₂O, d₆-glycerol (both Goss Scientific), and deuterated sucrose, which was produced by exchange into D₂O. For solvent isotope effect measurements, POR was deuterated by exchange into a deuterated buffer system containing 50 mM Tris, pH 7.5, 100 mM NaCl, and 1 mM dithiothreitol.

Laser Photoexcitation Measurements—Photoexcitation of POR ternary complexes (POR-NADPH-Pchlide) was by excitation at 450 nm, using an optical parametric oscillator of a Q-switched Nd-YAG laser (Brilliant B, Quantel) in a cuvette of 1-cm path length as described (15). Laser pulses (~60 ml) were between 6 and 8 ns in duration. Absorption transients were recorded at 696 and 681 nm using an LKS-60 flash photolysis instrument (Applied Photophysics Ltd.) with the detection system (comprising probe light, first monochromator, sample, second monochromator, and photomultiplier) at right angles to the incident laser beam. Rate constants were observed from the average of at least five time-dependent absorption measurements by fitting to a single exponential function. For studies of the temperature dependence of rate constants, reaction components were assembled in the dark in a volume of 1 ml and contained 100 μM POR (or deuterated enzyme), 250 μM NADPH (or NADP²H), and 30 μM Pchlide in 44% glycerol and 20% sucrose containing 50 mM Tris-HCl, pH 7.5, 0.1% Genapol, 0.1% β-mercaptoethanol. Control experiments in the absence of any cryoprotectant confirmed that the respective kinetic and solvent isotope effects were not influenced by the presence of glycerol or sucrose.

Density Functional Theory (DFT) Calculations—Calculations followed previous procedures (24) and used the hybrid density functional method B3LYP (25) in combination with a 6-311+G* basis set on all atoms (26). Full geometry optimizations were performed in Jaguar 7.0 with a dielectric constant of ε = 5.7 included using the self-consistent reaction field model (27). The theoretical model for the reduction of the C-17-C-18 bond of Pchlide contains the pigment without side chains, except for the propionate chain that is attached to C-17, which is replaced by an ethyl group to keep the overall charge of the model neutral. In addition, the nicotinamide ring of NADPH and phenol (for the Tyr-193 side chain) was added to the model, and they function as hydride and proton donors, respectively. To prevent the NADPH and phenol groups from undergoing excessive changes during the geometry optimizations, a constraint was placed on one of the atoms of each group with respect to the magnesium atom of the Pchlide. This did not cause any problems during the geometry optimization. The total model had stoichiometry of MgC₃₆N₆H₄₆O₃ and overall charge of 0. All calculations followed established procedures in the field (24) and use the hybrid density functional method B3LYP (25), as implemented in Jaguar 7.0 (27) in combination with 6-311+G* basis set on all atoms (26). Structures were fully optimized in Jaguar, followed by an analytical frequency calculation. Obtained gas-phase results predicted a stepwise reaction process via a radical intermediate between two consecutive hydrogen abstraction reactions, which is in disagreement with the experiment. We subsequently added a dielectric constant of ε = 5.7 in combination with a probe radius of 2.72 Å, as is commonly used for theoretical studies of enzyme mechanisms and has been shown to be a good representative of environmental perturbations generated by an enzyme (28, 29). Calculations, with a dielectric constant incorporated, predicted the charge distributions of intermediates and products that are consistent with sequential hydride and proton transfer. Therefore, only results in which a dielectric constant was included were used for the final calculations. Despite the large electronic differences between the gas-phase and solvent-based calculations, the differences in optimized geometries were actually quite small. The solvent optimized structures have little radical character, and hence the group spin densities are close to zero.

RESULTS AND DISCUSSION

Sequential Kinetic Mechanism for Hydride and Proton Transfer—Photoexcitation of the POR-NADPH-Pchlide complex generates an intermediate at 696 nm (Fig. 2A) with a rate constant of 2.02 ± 0.21 × 10⁶ s⁻¹ at 25 °C. Similar traces obtained between 665 and 720 nm define the “action spectrum” for this step (Fig. 2A, inset) and reveal that this species is iden-
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FIGURE 2. Kinetic absorption traces showing the hydride and proton transfer steps following photoexcitation of POR-NADPH/D-Pchlide with a 6-ns laser at 450 nm. A, typical kinetic traces measured at 696 nm over 4.5 μs for NADPH (red) and NADPD (blue). The inset shows an action spectrum for this step, created by measuring the absorbance at the respective wavelength after 75 ns and 2.5 μs. B, typical kinetic traces measured at 696 nm over 200 μs in H2O buffer (red) and D2O buffer (blue). The inset shows an action spectrum for this step, created by measuring the absorbance at the respective wavelength after 2.5 and 150 μs. C, typical kinetic transient showing increase in absorbance at 681 nm following laser photoexcitation at 450 nm. All traces were collected at 25 °C as described under “Experimental Procedures” and were fitted to a single exponential to obtain rate constants.

The rates of the hydride transfer and proton transfer steps in the presence of either pro-S NADPH or S-NADPH and in either H2O or D2O buffers

All rates were measured at 25 °C as described under “Experimental Procedures.”

| Sample | Hydride transfer | Proton transfer |
|--------|------------------|-----------------|
|        | Rate             | KIE             | Rate             | SIE             |
|        | s⁻¹ × 10⁶        | s⁻¹             | s⁻¹ × 10⁶        | s⁻¹             |
| NADPH in H2O | 2.02 ± 0.21 | 1              | 25.3 ± 1.5      | 1              |
| NADPH in H2O | 1.00 ± 0.14 | 2.03 ± 0.35     | 25.6 ± 3.1      | 0.99 ± 0.14 |
| NADPH in D2O | 1.79 ± 0.22 | 1.13 ± 0.18     | 11.5 ± 1.1      | 2.20 ± 0.24 |
| NADPH in D2O | 0.89 ± 0.12  | 2.25 ± 0.38     | 10.6 ± 0.4      | 2.39 ± 0.17 |

At longer times following laser initiation of catalysis, the 696 nm trace decays over ~100 μs (rate constant = 25.3 ± 1.5 × 10⁶ s⁻¹ at 25 °C; Fig. 2B) and is accompanied by an absorbance increase at 681 nm with a similar rate constant (Fig. 2C). Action spectra, generated from a series of kinetic traces (wavelength range 660–720 nm), confirm that the new intermediate has an absorbance peak centered at ~680 nm (Fig. 2B, inset), which has previously been shown to represent the POR-NADP⁺-Pchlide product complex (13, 14). A solvent isotope effect (SIE) of ~2.2 at 25 °C was measured for this step, implying that it represents the proton transfer reaction (Fig. 1, reaction B). Importantly, the SIE associated with reaction A is close to unity, which suggests that the SIE on the second step is a specific probe of proton transfer to substrate. Also, the lack of a KIE in reaction B indicates that hydride transfer only occurs during reaction A (Table 1) and provides conclusive proof of a sequential hydride transfer followed by proton transfer mechanism.

Both H-transfer Reactions Proceed via Quantum Mechanical Tunneling—We extended our analysis of hydride and proton transfer to cover a wide temperature range (−80 °C to +50 °C), and in particular to cryogenic temperatures, a regime that is difficult for thermally activated enzyme systems that require mixing methods for reaction initiation. Data were analyzed in the form of Eyring plots (Fig. 3), which provides thermodynamic parameters for each step (Tables 2 and 3) and reveals that both H-transfer reactions proceed by quantum mechanical tunneling. The proton transfer rate is strongly dependent on temperature (ΔH° = 74.8 kJ mol⁻¹), and the SIE is temperature-dependent with an inverse extrapolated Eyring prefactor ratio (Aₚ/Aₚ₀ = 0.041) (Fig. 3A and Table 2). This is consistent with a proton-tunneling reaction, which requires fast (sub-picosecond) promoting motions coupled to the reaction coordinate (30, 31) within the model for environmentally coupled hydrogen tunneling (32, 33). This involves motion to achieve the reactant and product well degeneracy required for tunneling, and gated motion along the H-transfer coordinate (indicated by the temperature dependence of the KIE) to enhance nuclear wave function overlap for the transferring proton (30, 31). The lack of a breakpoint in the Eyring plot suggests that the gated motion is coupled to the proton tunneling coordinate at all temperatures studied (−80 °C to +50 °C).

By contrast, the faster light-activated hydride transfer step shows a clear breakpoint at ~27 °C in the Eyring plot with both...
Temperature dependence of the rate constants for the hydride and proton transfer steps. A, Eyring plot of \( \ln(k_{\text{rate}}/T) \) versus \( 1/T \) for the proton transfer step (Fig. 1, reaction B). Data are shown fitted to the Eyring equation. Activation enthalpies, \( \Delta H^\ddagger \), and activation entropies, \( \Delta S^\ddagger \), are shown in Table 2, and the rate constants were determined at each temperature in supplemental Table S1. B, Eyring plot of \( \ln(k_{\text{rate}}/T) \) versus \( 1/T \) for the hydride transfer step (Fig. 1, reaction A) with NADPH and pro-S of NADPH. Activation enthalpies, \( \Delta H^\ddagger \), and activation entropies, \( \Delta S^\ddagger \), are shown in Table 3, and the rate constants were determined at each temperature in supplemental Table S2. For the proton and hydride transfer studies, each point is the average of at least five kinetic traces. In most cases error bars are smaller than the diameter of the data points shown and are thus not shown. C, amplitudes of the kinetic phase for hydride transfer as a function of temperature illustrating the progressive loss in signal below the breakpoint temperature of \(-27^\circ \text{C}\).

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**TABLE 2**
Thermodynamic parameters for the proton transfer step in the POR-catalyzed reaction

|            | \( \Delta H^\ddagger \) (kJ mol\(^{-1}\)) | \( \Delta H^\ddagger \) (kJ mol\(^{-1}\)) | \( \Delta S^\ddagger \) (J mol\(^{-1}\) K\(^{-1}\)) | \( \ln(A) \) | \( A'_{\text{H}}/A'_{\text{D}} \) |
|------------|------------------------------------------|------------------------------------------|------------------------------------------|------------------|------------------|
| \( \text{H}_2\text{O} \) | 74.8 \( \pm \) 0.8 | 9.8 \( \pm \) 0.8 | 65.7 \( \pm \) 0.8 | 31.7 \( \pm \) 0.4 | 0.041 \( \pm \) 0.016 |
| \( \text{D}_2\text{O} \) | 84.6 \( \pm \) 0.3 | 92.2 \( \pm \) 0.4 | 34.9 \( \pm \) 0.1 | 9.3 kJ mol\(^{-1}\) | less than unity |

The enthalpies of activation, \( \Delta H^\ddagger \), and the entropies of activation, \( \Delta S^\ddagger \), have been calculated by fitting the temperature dependence data to the Eyring equation for each step.

protiated and deuterated NADPH (Fig. 3B). Above \(-27^\circ \text{C}\) the reaction has a relatively weak temperature dependence (\( \Delta H^\ddagger = 9.3 \) kJ mol\(^{-1}\) for NADPH) but becomes considerably more temperature-dependent below the breakpoint (\( \Delta H^\ddagger = 27.2 \) kJ mol\(^{-1}\)) (Table 3). At temperatures below the breakpoint, the KIE is measurably independent of temperature, and the prefactor ratio \( (A'_{\text{H}}/A'_{\text{D}} = 3.59) \) is similar in value to the observed KIE, indicating quantum tunneling of hydride in the absence of an experimentally detectable (i.e. dominant) gating motion, coupled to the hydride transfer coordinate (3, 34). This contrasts with the situation at temperatures above \(-27^\circ \text{C}\), where the KIE is dependent on temperature and the prefactor ratio is less than unity \( (A'_{\text{H}}/A'_{\text{D}} = 0.077) \), consistent with the need for a more dominant gating motion along the hydride transfer coordinate (30).

DFT Calculations Confirm the Catalytic Mechanism—To gain further insights into the reaction mechanism, we set up a theoretical model representing the important features of the system (Fig. 4; supplemental Fig. S1). We have assumed a starting reactant complex (R) that involves Pchlide forming a long range complex with NADPH and the phenol group of the Tyr proton donor. Full geometry optimizations were performed in a dielectric constant of magnitude \( \varepsilon = 5.7 \), which has been shown to correctly predict the values of an enzyme-mimicked environment (35, 36). The overall reaction mechanism is endothermic (63.9 kJ mol\(^{-1}\)) and involves hydride transfer from NADPH to the C-17 atom of Pchlide to give an intermediate (I) via transition state TS1, followed by the subsequent proton transfer reaction to form the product complex (P) via barrier TS2. A time-dependent density functional theory calculation (37) on the reactant geometry shows that a close manifold of electronic states is accessible with the lowest Franck-Condon transition located at 210 kJ mol\(^{-1}\) (2.18 eV). The system then has sufficient energy to cross the hydride transfer barrier via quantum mechanical tunneling and relax back to the ground state potential energy surface to form the intermediate complex. The subsequent barrier \( (\Delta G^\ddagger = 67.7 \) kJ mol\(^{-1}\) at 298 K) of the proton transfer step, leading to product formation, is of much lower energy on the ground state potential energy surface and is in excellent agreement with the experimental data shown above in Fig. 3. Charge distributions (Fig. 4) confirm that the I is formed by hydride transfer, generating a positively charged NADP+ group (\( Q_{\text{NADPH}} = 1.16 \)) and a double negatively charged Pchlide (\( Q_{\text{Pchlide}} = -2.09 \)), consistent with a change in hybridization (\( sp^2 \) to \( sp^3 \)) for the C-17 atom of Pchlide. The theoretical reaction energetics agree closely with those observed from our temperature dependence studies of the hydride and proton.
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**TABLE 3**

| Thermodynamic parameters for the hydride transfer step in the POR-catalyzed reaction |
|---------------------------------------------------------------|
| The enthalpies of activation, $\Delta H^\circ$, and the entropies of activation, $\Delta S^\circ$, have been calculated by fitting the temperature dependence data to the Eyring equation for each step. |
| | Above $-27 \, ^\circ\text{C}$ | Below $-27 \, ^\circ\text{C}$ |
| | NADPH | NADPD | NADPH | NADPD |
| $\Delta H^\circ$ (kJ mol$^{-1}$) | 9.3 ± 0.4 | 17.5 ± 0.5 | 27.2 ± 0.5 | 27.7 ± 0.9 |
| $\Delta H^\circ$ (kJ mol$^{-1}$) | 8.2 ± 0.6 | -94.4 ± 1.2 | -73.2 ± 1.1 | 0.4 ± 1.0 |
| $\Delta S^\circ$ (mol$^{-1}$ K$^{-1}$) | 12.4 ± 0.2 | 15.0 ± 0.2 | 0.3 ± 0.3 | 32.4 ± 0.8 |
| $A^\#_{\text{TS}}/A^\#_{\text{P}}$ | 0.08 ± 0.02 | 15.0 ± 0.2 | 21.1 ± 0.3 | 3.6 ± 2.0 |

*FIGURE 4.* DFT calculated model of the reaction mechanism for sequential hydride and proton transfer catalyzed by POR. All energies are in kJ mol$^{-1}$ relative to the reactant complex and obtained after a full optimization in a dielectric constant of $\epsilon = 5.7$. Adding zero point, thermal, and entropic corrections to the energies gives a free energy of activation for TS2 of $\Delta G^\# = 67.7$ kJ mol$^{-1}$, which is close to the experimental data mentioned above. The calculated Franck-Condon value is in good agreement with the laser excitation used in the experiments. Also shown is the optimized geometry of the reactant complex with group charges of the reactant (R), intermediate (I) (in parentheses), and product (P) complexes (in square brackets) highlighted.

transfer kinetics (Table 2 and Table 3) and confirm that the light-activated sequential transfer of hydride (excited state) and proton (ground state) is an appropriate chemical mechanism for the POR-catalyzed reduction of Pchlide.

Our theoretical model demonstrates the need for light activation to drive an overall endothermic reaction from the Pchlide excited state. A consequence is that a dynamic search for “tunneling-ready” configurations (i.e. reactant configurations in which donor and acceptor wells become degenerate (23)) must occur within the lifetime of the photoexcited state. Significantly, there is a loss of amplitude for the hydride and proton transfer signals at lower temperatures (Fig. 3C), which suggests that dynamic searches for the tunneling-ready configuration are impeded as the temperature is reduced and that relaxation from the excited state becomes dominant. This model is supported by the presence of the $-27 \, ^\circ\text{C}$ breakpoint for the hydride transfer reaction. Below the breakpoint, the lack of a measurable temperature dependence on the KIE implies that quenching of gated motion along the hydride transfer coordinate will impede hydride tunneling as the wavefunction overlap becomes less optimal (30, 31). In addition, the observed increase in the temperature dependence of the hydride transfer rate below the transition point reflects the increased energetic cost of tunneling in the absence of an “assisting” motion coupled to the hydride transfer coordinate. By necessity, this implies that the system rarely reaches a tunneling-ready conformation, but when it does, it is at an ideal donor-acceptor distance.

**Conclusions**—In conclusion, we have established a direct link between light-activated chemistry and nuclear tunneling in a biologically important enzyme system. Furthermore, by extending our studies of light activation to cryogenic regimes, we have shown that gated motion along the H-transfer coordinate enhances the tunneling reaction. In contrast to the slower dynamic searches that generally limit H-transfer rates in thermally activated enzyme systems (1, 38, 39), we have demonstrated that fast dynamic searches within the lifetime of the excited state are required to reach a tunneling-ready configuration. Viewed globally, light-driven nuclear tunneling enables photosynthetic organisms to make chlorophyll despite the large and opposing thermodynamic gradient for this crucial reaction.

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