Laser Excitation Studies of the Product Release Steps in the Catalytic Cycle of the Light-driven Enzyme, Protochlorophyllide Oxidoreductase

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The latter stages of the catalytic cycle of the light-driven enzyme, protochlorophyllide oxidoreductase, have been investigated using novel laser photoexcitation methods. The formation of the ternary product complex was initiated with a 6-ns laser pulse, which allowed the product release steps to be kinetically accessed for the first time. Subsequent absorbance changes associated with the release of the NADP+ and chlorophyllide products from the enzyme could be followed on a millisecond timescale. This has facilitated a detailed kinetic and thermodynamic characterization for the interconversion of all the various bound and unbound product species. Initially, NADP+ is released from the enzyme in a biphasic process with rate constants of 1210 and 237 s−1. The rates of both phases show a significant dependence on the viscosity of the solvent and become considerably slower at higher glycerol concentrations. The fast phase of this process exhibits no dependence on NADP+ concentration, suggesting that conformational changes are required prior to NADP+ release. Following NADP+ release, the NADPH rebinds to the enzyme with a maximum rate constant of ∼72 s−1. At elevated temperatures (>298 K) chlorophyllide is released from the enzyme to yield the free product with a maximum rate constant of 20 s−1. The temperature dependencies of the rates of each of these steps were measured, and enthalpies and entropies of activation were calculated using the Eyring equation. A comprehensive kinetic and thermodynamic scheme for these final stages of the reaction mechanism is presented.

The light-driven enzyme protochlorophyllide oxidoreductase (POR; EC 1.3.1.33) is an excellent model system for studying the role of protein dynamics and thermodynamics in enzyme catalysis and more generally, the mechanism and timescales of enzymatic proton and hydride transfers (1). POR catalyzes the light-dependent trans addition of hydrogen across the C17=C18 double bond of the D-ring of protochlorophyllide (Pchlide) to produce chlorophyllide (Chlide), which is a key reaction within the chlorophyll biosynthesis pathway and the subsequent assembly of the photosynthetic apparatus (Fig. 1A) (1–4). Significant progress in understanding and solving the catalytic mechanism of the reaction has been made in recent years, which has consequently opened up exciting new areas in enzymology by exploring temperature ranges and very fast timescales that are generally not experimentally accessible with the majority of enzymes (5–9).

During the reaction a hydride is transferred from the pro-S face of the nicotinamide ring of NADPH to the C-17 position of the Pchlide molecule (10, 11), and a conserved Tyr residue has been proposed to donate a proton to the C-17 position (12). The close proximity of a conserved Lys residue is thought necessary to lower the apparent pKₐ of the phenolic group of the Tyr, allowing deprotonation to occur at physiological pH (12). As POR is light-driven, the ternary enzyme-substrate complex can be formed in the “dark”, prior to catalysis, thus removing the diffusive components from the kinetic analyses. Catalysis can then be triggered by illumination, using low temperatures to slow down Pchlide reduction and trap intermediates in the reaction pathway. Consequently, a number of catalytic steps, involving the initial photochemistry followed by a series of dark reactions, have been identified, and this has resulted in a detailed understanding of the entire catalytic cycle (6–8). The initial light-driven step, which can occur below 200 K, results in the formation of a non-fluorescent intermediate with a broad absorbance band at 696 nm (6). By using a combination of EPR, ENDOR, and Stark spectroscopies, in conjunction with low temperature absorbance spectroscopy, this intermediate was shown recently to be a charge-transfer complex resulting from hydride transfer from the NADPH molecule to the C17 position of Pchlide (13). This then facilitates the protonation of the C18 position of Pchlide from the protein during the first of the dark reactions (13).

The remaining dark steps only occur close to, or above, the “glass transition” temperature of proteins, implying a role for domain movements and/or reorganization of the protein for these stages of the catalytic cycle (7, 8). These latter catalytic events have been shown to represent a series of ordered product release and cofactor binding events. First, NADP+ is released from the enzyme and is replaced by the NADPH coenzyme. This is followed by release of the Chlide product and subse-
Product Release Steps of a Light-driven Enzyme

In addition to using low temperature regimes to trap and observe catalytic intermediates, POR has also been analyzed using ultrafast pulses of light (9). Catalysis was triggered with a 50-fs laser pulse, and the subsequent reaction dynamics were followed in real time by using ultrafast pump-probe absorption spectroscopy. The product state in its excited state was probed by a simultaneous large decrease in absorbance at 681 nm (8), which reveals the characteristic absorbance peak at 681 nm (8), is formed on a much slower timescale. Therefore, in this paper we are able to follow the subsequent slower product release processes on the millisecond to second timescales by monitoring the absorbance changes associated with the various bound and unbound Chlide forms. Kinetic transients were typically collected over 50 ms to 5 s. Experiments were averaged three times and repeated at various temperatures between 5–55 °C by using a circulating water bath (Fisher-brand) and at a range of glycerol and NADP concentrations. Absorbance spectra were measured before and after laser photoexcitation by transferring the cuvette to a Cary 50 UV/visible spectrophotometer (Varian) and recording data between 300–800 nm. Data from photoexcitation experiments were analyzed and fitted to appropriate exponential functions using SigmaPlot (Systat Software). In temperature dependence studies the kinetic and thermodynamic parameters were obtained by fitting the data to the Eyring equation as described previously (14, 15).

RESULTS

Kinetic Overview of the Product Release Steps—The catalytic reaction of POR has been triggered by using a 6-ns laser pulse tuned to the Soret region of the Chlide absorbance spectrum. As the POR-NADPandr Chlide ternary product complex, with a characteristic absorbance peak at 681 nm (8), is formed on a very fast timescale (9) we are able to follow the subsequent slower product release processes on the millisecond to second timescales by monitoring the absorbance changes associated with the various bound and unbound Chlide forms. Kinetic transients have been measured at wavelengths associated with POR-NADPandr Chlide (681 nm), POR-Chlide (670 nm), POR-NADPH-Chlide (685 nm), and free Chlide (670 nm), which are formed after the faster chemical steps (8). Fig. 24 shows a typical transient that is observed at 670 nm, which reveals the formation and interconversion of all of the various product states in the reaction pathway. The predominant signal appears to arise from the initial increase in absorbance at 670 nm as the NADP is released from the POR-NADPandr Chlide ternary product complex to form a POR-Chlide state. This is accompanied by a simultaneous large decrease in absorbance at 681 nm.

EXPERIMENTAL PROCEDURES

Sample Preparation—Recombinant POR from the thermo-philic cyanobacterium *Thermosynechococcus elongatus* was overexpressed in *Escherichia coli* and purified as previously described previously (8). The Pchlide and Chlide pigments were produced and purified as described previously (8). NADPH/NADP was obtained from Melford.

Laser Flash Photolysis—For laser photoexcitation experiments, 1-ml samples containing 30 μM POR, 100 μM NADPH, and 10 μM Pchlide in 50 mM Tris–HCl, pH 7.5, 0.1% Genapol, 0.1% β-mercaptoethanol were excited at 450 nm by using an optical parametric oscillator of a Q-switched Nd-YAG laser (Brilliant B, Quantel) in a cuvette of 1-cm path length. The energy output of each laser pulse was up to 200 mJ and pulses were 6–8 ns in duration. Spectral transients were recorded at 670, 681, and 685 nm using an Applied Photophysics LKS-60 flash photolysis instrument with the detection system at right angles to the incident laser beam. The probe light (150 watt xenon lamp) was passed through a monochromator before and after passage through the sample. Absorbance changes were measured using a photomultiplier tube, and kinetic transients were typically collected over 50 ms to 5 s. Experiments were averaged three times and repeated at various temperatures between 5–55 °C by using a circulating water bath (Fisher-brand) and at a range of glycerol and NADP concentrations. Absorbance spectra were measured before and after laser photoexcitation by transferring the cuvette to a Cary 50 UV/visible spectrophotometer (Varian) and recording data between 300–800 nm. Data from photoexcitation experiments were analyzed and fitted to appropriate exponential functions using SigmaPlot (Systat Software). In temperature dependence studies the kinetic and thermodynamic parameters were obtained by fitting the data to the Eyring equation as described previously (14, 15).
Characterization of NADPH Binding—Following catalysis and the formation of the ternary product complex the NADP$^+$ is released from the enzyme in a reaction that is represented by a decrease in absorbance at 681 nm and an increase in absorbance at 670 nm. Kinetic transients at each of these wavelengths reveal that this step is a biphasic process with the fast phase contributing $\sim$25% of the overall amplitude of the absorbance change. The rates of both phases have been measured over a range of temperatures from 5–50 °C by fitting to a double exponential function (Fig. 3A). Maximal rates are achieved at $\sim$50 °C with observed rate constants of 1280 ± 93.6 and 199 ± 2.8 s$^{-1}$ at 670 nm and 1210 ± 90.8 and 236.5 ± 6.2 s$^{-1}$ at 681 nm. To obtain more detailed thermodynamic information the data were refitted in the form of an Eyring plot of ln($k_{obs}/T$) versus $1/T$ (Fig. 3B). The enthalpy of activation, $\Delta H^\ddagger$, was calculated from the slope of each plot, and the entropy of activation, $\Delta S^\ddagger$, was calculated by extrapolation to the ordinate axis for each of the phases (Table 1).

We also studied the effect of increasing NADP$^+$ concentrations on the rate of both kinetic phases. There is only a minor or negligible dependence on the rate of the fast phase of the absorption decrease at 681 nm (Fig. 4A, inset). In contrast, the rate of the slower phase becomes considerably reduced at higher NADP$^+$ concentrations and therefore exhibits a significant NADP$^+$ dependence (Fig. 4A). Neither phase of the absorbance decrease at 681 nm shows any apparent dependence on NADPH nor Chlide concentrations (data not shown).

As it has previously been suggested that protein motions are required for these stages of catalysis (7, 8), it might be expected that increases in the viscosity of the solvent would affect the rate constants associated with NADP$^+$ release. Fig. 4B shows the effect of increasing glycerol concentrations on the rate of both phases of the absorption decrease at 681 nm. There is a significant reduction in both rates as the viscosity increases, resulting in an approximate 200-fold reduction at 70% glycerol.

Characterization of NADPH Binding—After the release of NADP$^+$ the NADPH coenzyme is able to rebound to the enzyme. This results in a decrease in absorbance at 670 nm and a simultaneous increase in the absorbance band at 685 nm as the POR-NADPH-Chlide complex. Finally, there is slower increase at 670 nm, together with a decrease at 685 nm, to yield the unbound or “free” Chlide product (Fig. 2C). Subsequently, to characterize these events in more detail we have carried out a thorough kinetic and thermodynamic investigation of each step and have summarized the results in Table 1.

| Product release event | Maximum $k_{max}$ (s$^{-1}$) | $\Delta H^\ddagger$ (kJ mol$^{-1}$) | $\Delta S^\ddagger$ (JK$^{-1}$ mol$^{-1}$) |
|----------------------|-----------------------------|----------------------------------|---------------------------------|
| NADP$^+$ release (fast) | 1210 ± 90.8 | 69.52 ± 6.12 | 34.30 ± 3.01 |
| NADP$^+$ release (slow) | 236.5 ± 6.2 | 70.76 ± 6.89 | 21.21 ± 2.21 |
| NADPH rebinding       | 71.6 ± 7.5 | 66.27 ± 3.05 | -0.0083 ± 0.0005 |
| Chlide release        | 20.4 ± 1.4 | 70.34 ± 6.16 | -5.30 ± 0.54 |
Chlide-state is converted to the POR-NADPH-Chlide ternary complex. Both of these absorbance changes are monophasic giving a single rate constant, which we have subsequently measured over a range of temperatures (Fig. 5A). A maximal rate is achieved at 45 °C of 71.6 s⁻¹. An Eyring plot of ln (kobs/T) vs 1/T for both phases of absorbance decreases at 681 nm. The filled circles represent the fast phase, and open circles represent the slow phase. The data were fitted to the Eyring equation, allowing the enthalpy of activation, ΔH‡, and entropy of activation, ΔS‡, to be calculated (14, 15) (Table 1). The error bars were calculated from the average of at least three traces.

Characterization of the Chlide Release Step—The final step in the catalytic cycle of POR is the release of the Chlide product. During this step the absorbance band at 681 nm (reporting on the presence of the POR-Chlide-NADPH complex) disappears, and the free form of Chlide accumulates, indicated by an absorption increase at 670 nm. Again, the decrease in absorbance at 681 nm and the absorbance increase at 670 nm are kinetically linked and can both be fitted to single rate constants. However, the temperature dependence of this step reveals that it can only proceed above 25 °C with a maximal rate achieved at approximately 55 °C of 20.4 ± 1.4 s⁻¹ (Fig. 6). The Eyring plot for this process has allowed the calculation of the enthalpy and entropy of activation to be +70.34 ± 6.16 J K⁻¹ mol⁻¹ and −5.30 ± 0.54 J K⁻¹ mol⁻¹, respectively.

DISCUSSION

The light-driven enzyme POR has previously been shown to be an important model system for studying aspects of enzyme mechanism (1) with the identification of a number of reaction intermediates allowing the entire catalytic cycle to be resolved (6–8). We have now used laser flash methods to measure the kinetics of the slower product release steps in the reaction, which have previously been suggested to require protein motions to proceed (7). Catalysis has been initiated by laser irradiation at 450 nm to form the ternary POR-NADP⁺-Chlide product complex with a characteristic absorbance band at 681 nm. Consequently, this species undergoes a series of product
release and substrate-rebinding events before a new catalytic cycle can begin. Each of these steps has a distinct spectroscopic signal and has now been studied in more detail, resulting in the complete kinetic and thermodynamic scheme depicted in Fig. 7.

Initially, NADP$^+$ is released from the ternary product complex in what appears to be a biphasic process. The rates of formation of the POR-Chlide species, absorbing at 670 nm, mirrored the disappearance of the POR-NADP$^+$-Chlide complex at 681 nm, confirming that both of these spectral events represent the same kinetic process. Maximal rates of 1210 and 237 s$^{-1}$ were measured at 50 °C with enthalpies of activation calculated to be +69.5 and +70.8 kJ mol$^{-1}$ and entropies of activation calculated to be 34.3 and 21.2 J K$^{-1}$ mol$^{-1}$. These kinetic and thermodynamic parameters are the first such values to be calculated for the product release steps of POR and will therefore prove to be invaluable in analyzing site-directed mutants and species variants in the future.

Importantly, a dependence on NADP$^+$ concentration could only be observed for the second phase of the NADP$^+$ release step, with a negligible detectable effect on the first phase. This suggests that the first phase represents a conformational change or a reorganization of the protein, which then facilitates the release of the products and substrate rebinding during the subsequent steps. This is in agreement with previous low temperature work, which revealed that these stages of the reaction could only proceed above the glass transition temperature of proteins, suggesting that protein motions or domain movements are required (7, 8).

The effects of any possible protein dynamics and conformational changes were investigated further by measuring rates of absorbance decrease at 681 nm at increasing solvent viscosities. A significant dependence on the glycerol concentration was observed for both phases of NADP$^+$ release suggesting that solvent viscosity played an important role in this process. It is widely accepted that viscosity can significantly influence and control the conformational changes of a protein, and it has been demonstrated that the dynamics of a protein are considerably slower in a high viscosity solvent (16–18). Therefore, it is likely that the slower rates observed during the first phase of NADP$^+$ release at higher solvent viscosities are caused by a reduction in the rate of the conformational changes in the enzyme that are
now proposed to be required. It is also known that the solvent viscosity will influence the rate at which a product can dissociate from the active site of an enzyme (19), and hence, at higher viscosities the NADP$^+$ product is released at much slower rates during the second phase.

Following NADP$^+$ release, NADPH coenzyme rebinds to the enzyme to form a POR-NADPH-Chlide complex in a single step with a maximal rate of $\sim 72 \text{ s}^{-1}$. Eyring analysis for this rebinding event suggests that the contribution of the entropy term is minimal in this process. No dependence on NADPH concentration was observed although we noted that in these laser photoexcitation experiments the initial concentration of NADPH was relatively high to ensure that prior to catalysis all of the Pchlide substrate was in the ternary substrate complex form. As the enzyme also has a high affinity for NADPH (18, 19) it is perhaps not surprising that a dependence on the NADPH concentration is not observed.

The final stage of the catalytic cycle is the release of the final product, Chlide. This can only occur at higher temperatures with a maximal rate of $20.4 \text{ s}^{-1}$ at $55 \, ^\circ\text{C}$. The rates of this step are faster than the overall $k_{\text{cat}}$ value for the reaction, which has been previously determined from steady-state measurements to be $\sim 0.2 \text{ s}^{-1}$ (20, 21). This suggests that the product release events are not rate-limiting in the overall catalytic cycle. We suggest, therefore, that substrate binding and any associated conformational changes or protein rearrangements to form the catalytically active ternary substrate complex are rate-limiting.

In conclusion, as a light-activated enzyme POR offers unique opportunities over the many “thermally activated” enzymes in that the product release events can be kinetically disentangled from the preceding substrate binding and chemical steps. Hence, for the first time we have been able to kinetically and thermodynamically characterize all of these latter product release steps for POR by using novel laser excitation techniques, thus advancing our general understanding of the entire catalytic cycle. This work further illustrates the importance of this light-driven enzyme as a model system for studying enzyme mechanism.

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