Plant Protochlorophyllide Oxidoreductases A and B

CATALYTIC EFFICIENCY AND INITIAL REACTION STEPS*

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Background: In plants, a key regulatory step in chlorophyll biosynthesis is catalyzed by two light-dependent isozymes.

Results: The two isozymes operate via the same reaction mechanism but differ in their catalytic efficiencies.

Conclusion: Different substrate affinities and conformational flexibilities modulate the catalytic reaction.

Significance: Detailed understanding of light-driven reactions in nature has a big impact on the development of artificial energy conversion systems.

The enzyme protochlorophyllide oxidoreductase (POR, EC 1.3.1.33) has a key role in plant development. It catalyzes one of the later steps in chlorophyll synthesis, the light-induced reduction of protochlorophyllide (PChlide) into chlorophyllide (Chlide) in the presence of NADPH. Two isozymes of plant POR, POR A and POR B from barley, which differ in their function during plant life, are compared with respect to their substrate binding affinity, catalytic efficiency, and catalytic mechanism. POR B as compared with POR A shows an 5-fold higher binding affinity for PChlide and an about 6-fold higher catalytic efficiency, and catalytic mechanism. During plant life, are compared with respect to their substrate binding affinity, catalytic efficiency, and catalytic mechanism. POR B as compared with POR A shows an 5-fold higher binding affinity for PChlide and an about 6-fold higher catalytic efficiency measured as $k_{cat}/K_m$. Based on the reaction intermediates, which can be trapped at low temperatures the same reaction mechanism operates in both POR A and POR B. In contrast to results reported for POR enzymes from cyanobacteria, the initial light-driven step, which occurs at temperatures below 180 K already involves the full chemistry of the photoreduction and yields the reaction product, Chlde, in an enzyme-bound form. The subsequent dark reactions, which include cofactor (NADP⁺) release and cofactor (NADPH) rebinding, show different temperature dependences for POR A and POR B and suggest a higher conformational flexibility of POR B in the surrounding active center. Both the higher substrate binding affinity and well adapted enzyme dynamics are held responsible for the increased catalytic activity of POR B as compared with POR A.

Chlorophyll is the main pigment of photosynthesis in plants, green algae, and cyanobacteria. The biosynthesis of chlorophyll therefore represents a fundamental metabolic process, which is strongly regulated in response to environmental cues (1, 2). The light-dependent protochlorophyllide oxidoreductase (POR) (EC 1.3.1.33) is the key regulatory enzyme of the chlorophyll synthesis pathway in all oxygen-producing photosynthetic organisms (3–7). In one of the last steps, POR catalyzes the light-driven reduction of protochlorophyllide (PChlide) into chlorophyllide (Chlide), from which chlorophyll finally derives. With reduced nicotinamide adenine dinucleotide phosphate (NADPH) as cofactor, the reduction involves the trans addition of hydrogen across the C(17)-C(18) double bond in ring D of PChlide (Fig. 1), whereas the pro-S hydride of the nicotinamide ring of NADPH is transferred to the C(17) position and, most likely, the proton of a conserved tyrosine residue to the C(18) position of the porphyrin molecule (8–9). Because of this light-driven H-transfer, which enables the exploitation of the abundant sun light as energy source for chemical synthesis, POR is also an attractive model for novel approaches in bio-inspired, green chemistry.

Based on the comparison of the amino acid sequence with other sequences in the protein database POR is a member of the large family of enzymes known as short chain dehydrogenases/reductases that catalyze a variety of NADP(H)- or NAD(H)-dependent reactions (9–11). The common structural features include a glycine-rich GXXGXXG NADPH-binding motif as part of the so-called Rossman-fold, which is located close to the N-terminal region of the enzyme. In addition, tyrosine and lysine residues are arranged at the catalytic site. They are essential for the catalytic activity of POR and belong to the YXXXX substrate-binding motif, which is highly conserved across the short chain dehydrogenases/reductases superfamily of enzymes.

In barley (Hordeum vulgare) and perhaps in other plant species two distinct isozymes of POR, termed POR A and POR B exist (12–14), whereas in Arabidopsis (Arabidopsis thaliana) apart from POR A and POR B an additional third isozyme, POR C has been identified (15, 16). POR A and POR B differ remarkably in their expression pattern during plant development (17, 18), substrate specificities (19, 20), reaction efficiencies (21) and the mechanism by which their nuclear-encoded precursor pro-

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3 The abbreviations used are: POR, protochlorophyllide oxidoreductase; PChlide, protochlorophyllide; Chlide, chlorophyllide; MST, microscale thermophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
Proteins are imported into plastids (22–24). POR A is highly abundant in etiolated plants and mainly active during seedling deetiolation. In the light, the expression of POR A is repressed resulting in its selective disappearance, whereas POR B becomes the predominant species. In contrast to POR A, POR B is not negatively regulated by light but constitutively expressed both in etiolated and light-adapted plants. Hence, in greened and adult plants, POR B is the only POR enzyme, which operates in chlorophyll synthesis (13, 14, 17, 18). With respect to their function, the two isoforms are proposed to cooperate in higher molecular weight light-harvesting complexes in the prolamellar body of etioplasts (19, 20). Based on a comparison of the amino acid sequences, POR A and POR B show a very high sequence homology (25) indicating that differences in the catalytic function can hardly be attributed to striking differences in their structure at first glance.

Thus far POR A and POR B have been mainly examined in comparative studies with respect to their function in light-harvesting complexes (19, 20, 26). Although POR B is the key enzyme in chlorophyll synthesis of light-adapted, green plants, details of the enzyme kinetics and catalytic mechanism are still unclear. In the present paper we have addressed this dearth of knowledge by comparing the two POR isozymes in parallel experiments with regard to their substrate affinities and catalytic efficiencies toward PChlide as a substrate (henceforth referred to as PChlide for reasons of simplicity). Furthermore, the catalytic mechanism is assessed by trapping transient intermediates in low temperature absorbance experiments. The results allow further conclusions on the catalytic dynamics of POR A and POR B as well as on differences in the catalytic mechanism between the two plant isoforms themselves and the enzyme from cyanobacteria, which does not exist in a multiple form. That enzyme is of interest because POR A and POR B as well as on differences in the catalytic mechanism between the two plant isoforms themselves and the enzyme from cyanobacteria, which does not exist in a multiple form. That enzyme is of interest because POR A and POR B are highly abundant in etiolated plants and mainly active during seedling deetiolation. In the light, the expression of POR A is repressed resulting in its selective disappearance, whereas POR B becomes the predominant species. In contrast to POR A, POR B is not negatively regulated by light but constitutively expressed both in etiolated and light-adapted plants. Hence, in greened and adult plants, POR B is the only POR enzyme, which operates in chlorophyll synthesis (13, 14, 17, 18). With respect to their function, the two isoforms are proposed to cooperate in higher molecular weight light-harvesting complexes in the prolamellar body of etioplasts (19, 20).

**Expression and Purification of POR from Synechocystis**—cDNA encoding the POR enzyme from *Synechocystis* sp. was also subcloned into expression vector pRSET A (Invitrogen) using BamHI and EcoRI cloning sites. The subsequent procedures including overexpression and purification of the enzyme were analogous to the experimental steps described above for the enzymes from barley (*H. vulgare*).

**Preparation of PChlide**—PChlide a was isolated from 5-day-old, dark grown oat seedlings (*Avena sativa* L.) as reported recently (28). Briefly, the coleoptiles were treated with 15 mM 5-aminolevulinic acid in 35 mM potassium phosphate buffer for 48 h. Following disruption of the coleoptiles by homogenization, PChlide was extracted into an ice-cold 10 mM Tricine
buffer, pH 7.5, 75% acetone (v/v) mixture. After centrifugation, PChlide was transferred into diethyl ether followed by extraction into a 4:1 methanol, 0.01 M ammonia mixture. Finally, PChlide was transferred into diethyl ether from the water/methanol mixture and fractionated by HPLC on a reverse phase RP-18 column in a linear 20–80% acetonitrile gradient. The fractions containing highly pure PChlide were lyophilized and stored at 250 K until use. For the experiments PChlide was dissolved in methanol and diluted with reaction buffer to concentrations as required resulting in less than 1% methanol in the sample.

Kinetic Experiments—Kinetic assays for determination of the initial rates \( (v_0) \) of PChlide, the Michaelis-Menten constant \( (K_m) \), and the turnover number \( (k_{\text{cat}}) \) were conducted in measuring buffer containing 50 mM Tris-HCl, 0.3 M NaCl, 20% glycerol, 0.1% Triton X-100, and 1 mM DTT, pH 7.6. The typical reaction mixture was composed of 10 \( \mu \)M POR, 100 \( \mu \)M NADPH, 1 mM DTT. PChlide was used at various concentrations as indicated under “Results.”

Initial reaction rates \( (v_0) \) were measured by irradiating the ternary complex POR-NADPH-PChlide, which was preformed by incubation in the dark, with a HeNe laser 30–50 (Spindler and Hoyer) at \( \lambda_{\text{exc}} = 633 \) nm and a photon flux of \( 2.5 \times 10^{-5} \) mol m\(^{-2}\) s\(^{-1}\). The reaction progress was followed by monitoring the absorbance spectra in the UV/visible region in dependence on the irradiation time using a Lambda 35 UV/visual spectrophotometer (PerkinElmer Instruments). In this setup, the reaction rate corresponds to the decrease/increase in the PChlide/Chlide concentration. The concentrations were determined in each experiment using \( \varepsilon_{630\,\text{nm}} = 48,700 \) liters/mol cm\(^{-1}\) for PChlide and \( \varepsilon_{670\,\text{nm}} = 91,000 \) liters/mol cm\(^{-1}\) for Chlide. From plots of the initial rates versus the concentration of PChlide, the \( K_m \) values were estimated according to the Michaelis-Menten equation,

\[
\frac{d[\text{Chlide}]}{dt} = v_0 \quad \text{(Eq. 1)}
\]

\[
= \frac{V_{\text{max}} \times [\text{PChlide}]}{K_m + [\text{PChlide}]} \quad \text{(Eq. 2)}
\]

by means of a nonlinear least-squares curve fitting procedure in Matlab. The turnover number, \( k_{\text{cat}} \), was derived from maximum enzyme velocity \( (V_{\text{max}}) \) according to,

\[
k_{\text{cat}} = \frac{V_{\text{max}}}{[E_0]} \quad \text{(Eq. 2)}
\]

where \( [E_0] \) is total concentration of the POR enzyme.

Microscale Thermophoresis (MST)—The affinity of PChlide to the POR enzymes was analyzed by microscale thermophoresis (29). The POR enzymes were labeled with the blue fluorescent dye NT-495-NHS (Nano Temper Technologies). The concentration of the labeled POR enzymes including the cofactor NADPH was kept constant at 83 nm for POR A and 166 nm for POR B, respectively, whereas the concentration of PChlide was varied in a 1:1 serial dilution starting from 5 \( \mu \)M. The samples, enzyme and substrate, were dissolved in measuring buffer (50 mM Tris-HCl, 0.3 M NaCl, 20% glycerol, 0.1% Triton X-100, 1 mM DTT, pH 7.6). After an incubation period of 15 min in the dark the MST measurements were carried out at 25 °C on a Monolith NT.115 instrument (Nano Temper Technologies). Thermophoresis was measured at 80% LED power and 20% MST power, respectively. From the binding curves, which yield the fraction of POR-bound PChlide as function of the titrated PChlide, the dissociation constants, \( K_{DP} \), were determined using the equation,

\[
K_D = \frac{[E] \times [S]}{[ES]} \quad \text{(Eq. 3)}
\]

with

\[
[E] = [E_0] - [ES] \quad \text{(Eq. 4)}
\]

\[
[S] = [S_0] - [ES] \quad \text{(Eq. 5)}
\]

where \([ES]\) is the concentration of the PChlide-bound POR-complex (enzyme-substrate complex) and \([S_0]\) the added PChlide concentration.

The solution of Equation 3 with substitutions of Equations 4 and 5 results in,

\[
C = \frac{1}{2[E_0]} \left( K_D + [E_0] + [S_0] - \sqrt{[K_D + [E_0] + [S_0]]^2 - 4[E_0] \times [S_0]} \right) \quad \text{(Eq. 6)}
\]

with the C− fraction of POR-bound PChlide.

From the fit of the binding curves to Equation 6 the dissociation constant, \( K_{DP} \), was derived.

The change in the free enthalpy (\( \Delta G^0 \)) upon binding of PChlide to the POR-NADPH complex was deduced from the dissociation constant, \( K_{DP} \), by Equation 7.

\[
\Delta G^0 = -RT \times \ln(K_D) \quad \text{(Eq. 7)}
\]


**Results**

**Binding of PChlide to POR A and POR B**—The affinity of PChlide to isozymes POR A and POR B was first analyzed by MST. To this end the POR enzymes were labeled with the blue fluorescent dye NT-495 and the reconstituted POR-labeled NADPH complexes, the concentration of which was kept constant, were titrated with decreasing concentrations of PChlide in serial dilution. Fig. 2 shows the binding curves for POR A and POR B with the fraction of POR-bound PChlide plotted against the concentration of titrated PChlide. The binding constants were derived from the binding curves as equilibrium dissociation constants, \( K_D \), through the curve fitting procedure given under “Experimental Procedures.” This analysis yields dissociation constants for PChlide binding in the presence of NADPH of \( K_D = 1.0 \pm 0.014 \mu M \) in the case of POR A and \( K_D = 0.19 \pm 0.009 \mu M \) in the case of POR B. From the lower \( K_D \) value for POR B it is apparent that this isozyme shows a about 5-fold higher binding affinity to PChlide as compared with POR A.

To further characterize the affinity of the two POR isozymes to the substrate, the Michaelis-Menten constants, \( K_m \), were determined in a second set of experiments. Fig. 3 shows the dependence of the initial reaction rates on the PChlide concentration when the enzyme concentration was held constant. As can be seen, both, POR A and POR B follow the Michaelis-Menten kinetics with the initial rate, \( \nu_0 \), being proportional to the PChlide concentration in the low concentration range and approaching its maximum, \( \nu_{\text{max}} \), at high PChlide concentrations thus resulting in a rectangular hyperbola. The fit of the rectangular hyperbola to the Michaelis-Menten equation (Equation 1) yielded values of \( K_m = 3.44 \pm 0.3 \mu M \) for POR A and \( K_m = 1.25 \pm 0.05 \mu M \) for POR B. The value obtained for POR A is in gross agreement with the dissociation constant, \( K_D \), whereas \( K_m \) determined for POR B differs to a slightly higher extent from \( K_D \) (cf. Table 1). In any case, the \( K_m \) values reproduce the trend observed for the \( K_D \) constants, i.e. a lower value for POR B than POR A. From the same fit, values of \( \nu_{\text{max}} = 0.05 \mu M \) for POR A and \( \nu_{\text{max}} = 0.1 \mu M \) for POR B, thus indicating that POR B reaches the maximum reaction rate at substrate saturation, \( \nu_{\text{max}} \), twice as fast as POR A.

**Catalytic Efficiency of POR A and POR B**—To assess and compare the catalytic efficiencies of the two isozymes, POR A and POR B, the turnover number, \( k_{\text{cat}} \), and the specificity constant, \( k_{\text{cat}}/K_m \), were used. \( k_{\text{cat}} \) was determined from the maximum velocity, \( \nu_{\text{max}} \), under substrate saturation (Equation 2). \( \nu_{\text{max}} \) and also \( K_m \), necessary for evaluation of the specificity constant, were derived from the plots in Fig. 3 by a nonlinear least squares fit as described in the preceding paragraphs. The efficiency constants thus obtained are summarized in Table 1. As these data reveal, \( k_{\text{cat}} \) is about two times higher for POR B than for POR A indicating a considerably enhanced rate for the conversion of PChlide when the reaction is catalyzed by POR B. This tendency is further confirmed by the specificity constant \( k_{\text{cat}}/K_m \) for the two isozymes. Compared with \( k_{\text{cat}} \), the ratio of \( k_{\text{cat}}/K_m \) does not only provide information on how fast the POR-catalyzed reaction is in the one or the other case, but also on how much of the substrate is required to reach \( \nu_{\text{max}} \). By using the respective values from Table 1 it is evident that the catalytic efficiency of POR B as compared with POR A is ~6-fold higher. Thus, POR B is clearly the more efficient enzyme in the photoconversion of PChlide into Chlide.

**Reaction Pathway of POR A and POR B Probed at Low Temperatures**—As shown by studies on POR enzymes from cyanobacteria, catalysis can be initiated by irradiation at cryogenic temperatures as low as 120 K. This allows the catalytic reaction steps to be followed by trapping reaction intermediates at low temperatures (6, 30–33). Therefore, to gain insight...
The temperature dependence of the intermediates formed at low temperatures was characterized by their absorbance spectra. To this end the catalytically active ternary POR-NADPH-PChlide complex was cooled down to 180 K. After initiation of catalysis by irradiation with red light ($\lambda_{\text{exc}} = 632$ nm) the temperature was progressively increased in the dark and the absorbance spectra were taken at definite temperatures. The spectra thus obtained and the temperature dependence of the intermediates extracted from the absorbance spectra is shown in Figs. 4 and 5.

It is striking that upon binding of PChlide to both POR isozymes in the presence of the cofactor NADPH the absorbance shifts to the red by about 10 nm to give rise to an absorbance maximum at 640 nm. This effect is clearly seen at 180 K and reflects the formation of species $S_{640}$, which represents the absorbance of enzyme-bound PChlide in the POR-NADPH-PChlide ternary complexes. In the room temperature spectra the red shift in absorbance is manifested solely by a broadening of the absorbance band between 630 and 650 nm (data not shown).

After photoexcitation of $S_{640}$ at 180 K, the initial light-induced step involves the formation of a first intermediate with an absorbance maximum at 677 nm ($A_{677}$). No intermediate form that precedes $A_{677}$ can be observed even at temperatures below 180 K. The temperature dependence of the $A_{677}$ intermediate reveals that it mainly occurs between 180 and 240 K in both POR A and POR B. At temperatures above 240 K the $A_{677}$ absorbance band disappears accompanied by the simultaneous appearance of a new intermediate ($A_{672}$) absorbing with a maximum at 672 nm. This first dark reaction, which requires temperatures close to or above the phase transition of proteins, is maximum at 672 nm. This effect is clearly seen at 180 K and reflects the formation of species $S_{640}$, which represents the absorbance of free, unbound Chlide. Hence, this last reaction step indicates the release of Chlide, the final product of the enzymatic reaction, from the enzyme complex.

The dissociation constants, $K_D$, are derived from MST measurements and the values of the kinetic parameters are obtained from plots of the initial rates, $v_0$, against the PChlide concentration by using a nonlinear regression analysis for the Michaelis-Menten Equation 1. The estimates are the average of four independent experiments.

### TABLE 1
Comparison of the binding parameters and kinetic constants between POR A and POR B

| Parameter | POR A (ternary complex) | POR B (ternary complex) |
|-----------|-------------------------|-------------------------|
| $K_D$ ($\mu$M) | 1.0 ± 0.014 | 2.0 ± 0.009 |
| $K_M$ ($\mu$M) | 3.44 ± 0.3 | 2.0 ± 0.007 |
| $v_{max}$ (M s$^{-1}$) | 0.05 ± 0.005 | 0.1 ± 0.015 |
| $k_{cat}$ (s$^{-1}$) | 5.5 × 10$^{-3}$ ± 5.5 × 10$^{-4}$ | 1.16 × 10$^{-2}$ ± 1.5 × 10$^{-3}$ |
| $k_{cat}/K_M$ (s$^{-1}$ M$^{-1}$) | 1.6 × 10$^{-2}$ ± 1.6 × 10$^{-3}$ | 9.3 × 10$^{-3}$ ± 9.3 × 10$^{-4}$ |

In POR A, the further increase in temperature above 290 K leads to the disappearance of the 681-nm absorbance band and simultaneous formation of a blue-shifted band at 670 nm, which represents the absorbance of free, unbound Chlide. Hence, this last reaction step indicates the release of Chlide, the final product of the enzymatic reaction, from the enzyme complex.

The corresponding reaction is missing in POR B. There are no further spectral changes after formation of the $A_{681}$ intermediate in the time period of the measurement.

Another difference between POR A and POR B refers to the yield of the first photoprodut ($A_{677}$) formed after irradiation at 180 K (Fig. 4). Under the conditions of the low temperature
experiments, which allow only a single catalytic turnover, it is
evident that POR A generates a significantly larger amount of
the first intermediate than POR B.

Activation Energy for the First Light-induced Reaction Step—
To gain further insight into the reaction mechanism the activation
energies for the initial light-driven step were determined.
For this purpose the rate constants for the formation of the first
photoproduct, \( A_{677} \), were estimated over a range of tempera-
tures from 160–200 K both for POR A and POR B. The data
were plotted in an Arrhenius graph (\( \ln(k) \) versus \( 1/T \)) and fitted
to the Arrhenius equation (Fig. 6). Thus, activation energies
(\( \Delta G^\circ \)) of 24.9 ± 2.1 kJ mol\(^{-1}\) for POR A and 35.9 ± 1.8 kJ mol\(^{-1}\)
for POR B could be calculated implying a higher activation bar-
rier that has to be crossed in POR B as compared with POR A to
produce \( A_{677} \).

Comparison with the Reaction Intermediates in the Catalytic
Cycle of POR from Cyanobacteria—To compare the reaction
intermediates in the catalytic pathway of POR A and POR B
with those already identified in the reaction cycle for POR from
cyanobacteria (6, 30–33), the low temperature absorbance
spectra were also monitored for the enzyme from Synechocystis
under identical experimental conditions. The spectra of the
intermediates resolved are summarized in Fig. 7. In the follow-
ing, they are briefly discussed in respect of the results reported
for Synechocystis POR (6, 30, 31). In full agreement with the
Synechocystis studies, the first light-induced step yields a pho-
toproduct, which is characterized by a broad absorbance band
with a maximum at 698 nm (\( A_{698} \)). In the subsequent dark reaction
\( A_{698} \) is converted into another intermediate form, \( A_{677} \), with
an absorbance band centered at 677 nm. In the last reaction
step occurring above the glass transition temperature, \( A_{677} \)
undergoes a blue-shift in its absorbance and forms the final
product, \( A_{670} \), the absorbance features of which mirrors those

FIGURE 5. Spectroscopic characteristics of the intermediates in the reaction cycle of POR A and POR B as obtained from exploratory factor analysis. A
and B, absorbance spectra of the intermediates approached for the reaction pathway of POR A and POR B, respectively. C, temperature dependence of the
intermediates in the reaction path of POR A (■) and POR B (□).

FIGURE 6. Arrhenius plots displaying the logarithm of the rate constant
(\( \ln(k) \)) for the initial photoreaction of the ternary POR complex to the
first photoproduct, \( A_{677} \), as a function of the inverse temperature (1/T).
The plots are given for the reactions catalyzed by POR A (■) and POR B (□)
in the temperature range from 160 –200 K. From a fit of the data to the Arrhenius
equation (Equation 8) the activation energies (\( \Delta G^\circ \)) are calculated to be
24.9 ± 2.1 kJ mol\(^{-1}\) for POR A and 35.9 ± 1.8 kJ mol\(^{-1}\) for POR B.
Catalytic Activities of POR A and POR B

![Graphs](images)

**FIGURE 7. Spectroscopic characterization of the reaction intermediates in the catalytic pathway of the POR enzyme from Synechocystis as detected by low temperature absorbance spectroscopy.** The ternary complexes of Synechocystis POR (60 μM POR, 3.5 μM PChlide, 400 μM NADPH, 1 mM DTT in measuring buffer with 60% glycerol) were frozen at 180 K, irradiated by actinic light at λ_{act} = 632 nm, and then the temperature was raised in the range between 180 and 305 K step by step. The absorbance spectra were monitored at the temperatures indicated in the *insets*. The figure reproduces the results reported in Refs. 6 and 30–32. It is shown to demonstrate that identical results are obtained under the experimental conditions used with the enzymes from barley.

Discussion

In the present work the two isozymes of POR, POR A and POR B, are compared with respect to their substrate-binding parameters, catalytic efficiency, and photochemical reaction cycle assessed at low temperatures. In this direct comparison, heterologously expressed plant enzymes from barley (*H. vulgare* L.) are used. Those enzymes allow a detailed spectroscopic and kinetic analysis of the enzyme-catalyzed reaction because disturbances, as for instance caused by aggregation or limited amounts of available enzymes, can be excluded.

The PChlide-binding affinities of the two POR isozymes are analyzed by a direct comparison of the dissociation constant, \( K_D \), and the Michaelis-Menten constant, \( K_m \). By using the MST technique, \( K_D \) values of 1.0 ± 0.014 μM for POR A and 0.19 ± 0.009 μM for POR B have been calculated. The value of \( K_m \) for POR A is in gross agreement with the \( K_m \) value, which is derived from kinetic measurements and determined to be 3.44 ± 0.3 μM. POR B the \( K_D \) constant differs to a slightly higher extent from the \( K_m \) value, which is estimated as 1.25 ± 0.07 μM (Table 1). This discrepancy shows that the Michaelis-Menten constant does not represent a measure of the substrate affinity at least in case of POR B. The difference between \( K_m \) and \( K_D \) is not uncommon and applies for many enzymes. This can be explained by the fact that the rate constant for breakdown of the ES complex is not rate-limiting, *i.e.* not negligible relative to that for the formation of the ES complex or that other reaction steps such as the product release are rate-determining. Consequently, \( K_m \) remains a complex function of various rate constants and cannot be considered as a measure of the substrate affinity even though the Michaelis-Menten equation and the characteristic saturation behavior of the enzyme still apply.

However, irrespective of the situation for \( K_m \), the considerably smaller \( K_D \) constant of POR B indicates a significantly higher (~5-fold) affinity of this isozyme for PChlide than POR A. This result is in line with findings from *in vitro* reconstitution experiments of light-harvesting POR-PChlide-NADPH complexes. As shown in these studies by quantification of the acetone-extractable pigments, POR B as compared with POR A is more specific to PChlide by binding and photoconverting a higher amount of PChlide into Chlide (20). Moreover, the \( K_m \) value of ~3 μM obtained for POR A is of the same order as the values of 6.8 and 1.8 μM reported for *Synechocystis* POR and the POR enzyme from *Thermosynechococcus*, respectively (35, 36).

On the other hand, there is a difference to the \( K_m \) values of 0.27 and 0.47 μM estimated for plant PORs from pea and oat (37, 38). The reason for this discrepancy is most likely due to the use of “inhomogeneous” enzyme preparations in the assays. In the case of the enzyme from pea, dimers of POR fused with the high molecular affinity tag maltose-binding protein were assessed, whereas in the case of the oat enzyme, POR was directly extracted from etiolated seedlings and therefore most probably contains a mixture of POR A and POR B. With respect to the change in the free enthalpy \( (\Delta G^0) \) the binding of PChlide to the POR-NADPH complexes does not greatly differ between POR A and POR B. It is estimated from the \( K_D \) as ~34 and ~38 kJ mol⁻¹ for POR A and POR B, respectively.

The higher binding affinity of POR B to PChlide and therefore the higher concentration of the enzyme-substrate complex in the steady-state correlates with a considerably increased catalytic efficiency as compared with POR A. Both the turnover number \( k_{cat} \) and the specificity constant \( k_{cat}/K_m \) commonly used for comparing enzyme activity are 2- and about 6-fold, respectively, larger for POR B than POR A. The 2-fold higher turnover number corresponds well with the previously reported 2-fold higher quantum efficiency of POR B versus POR A (21). One can speculate that the higher catalytic efficiency of POR B is related to its biological function. Although POR A appears mainly transiently in dark-grown seedlings and is the main enzyme during seedling de-etiolation, POR B becomes the predominant one in green plants, in which it is responsible for maximal PChlide photoreduction (13, 14, 18). Accordingly, it can be assumed that the high enzymatic activity of POR B guarantees the delivery of sufficient amounts of chlorophyll needed to meet the demands of plant growth. This role of POR B is of special importance for angiosperms, which only contain the light-dependent enzyme and lack the light-independent enzyme. Furthermore, it is known that free PChlide generates reactive oxygen species, primarily singlet oxygen, after light absorption and provokes photooxidative damage (39–42). Hence, the high binding constant and enhanced activity of POR B most likely prevents the accumulation of free PChlide and lowers the risk of photosensitized side reactions.

Very recently, reconstituted enzyme complexes of POR A, POR B, and POR C from *A. thaliana* were studied with respect to their low temperature fluorescence spectra and catalytic activity (26). Whereas the fluorescence spectra indicate formation of highly aggregated POR complexes under the experimental conditions used, POR A and POR B did not differ in their
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charge-transfer intermediate similar to \( A_{696} \) is not to be observed in the two *Hordeum* PORs. The comparative analysis of the low temperature intermediates trapped in *Synechocystis* POR and the two barley isozymes confirms this conclusion. The results show that a charge-transfer intermediate (\( A_{699} \)) can be identified in the case of *Synechocystis* POR, whereas it is definitely missing in the low temperature spectra of POR A and POR B (Figs. 4 and 7). That means that either the activation barrier separating \( A_{699} \) from \( A_{681} \) is too small in the two isozymes studied so that an intermediate similar to \( A_{699} \) cannot be trapped at low temperatures, or that in general the \( H^+ / H^+ \) transfer reaction occurs in a concerted process that directly produces Chlide as reaction product. In any case it seems that the active site has optimally evolved for the reaction chemistry in the two barley isozymes compared with the *Synechocystis* enzyme.

As is further evident from Fig. 4, a higher amount of the first photoprodut, \( A_{677} \), is generated in POR A as compared with POR B. This finding agrees with the lower activation energy estimated with 24.9 ± 2.1 kJ mol\(^{-1}\) for POR A as opposed to 35.9 ± 1.8 kJ mol\(^{-1}\) for POR B. However, on second glance it seems to be in contrast with the higher reaction quantum yield reported for POR B as compared with POR A (21). The reason for this is that in the low temperature experiments the reaction pathway is followed for only one reaction cycle, whereas the results on the quantum yields were achieved in steady-state measurements, i.e. in multiple turnover assays at room temperature. Under those conditions substrate binding and enzyme flexibility also affect the catalytic efficiency (see below).

In both POR A and POR B the initial light-induced reaction is followed by three and two, respectively, dark reactions, which take place above the glass transition temperature. Therefore, they most likely require the rearrangement of protein domains/sites near or at the catalytic center to modulate the product binding interactions and to release the cofactor and product. The absorbance shifts, characteristic of the different dark reaction stages, are very similar to those identified in low temperature binding studies of Chlide to POR in the presence of NADPH and NADP\(^+\) (32). Taking the assignment that has been made there, the dark reactions can be attributed to the release of NADP\(^+\) (\( A_{677} \rightarrow A_{672} \)), its replacement by NADPH (\( A_{672} \rightarrow A_{681} \)), and the expulsion of Chlide from the active site (\( A_{681} \rightarrow A_{670} \)). However, in case of POR B it appears that Chlide remains trapped in the binding pocket under the experimental conditions used. It is most likely that this effect reflects a higher binding affinity of Chlide to POR B than to POR A, similar to the distinct binding affinities of PChlide.

Furthermore, a difference in the temperature dependence of the second dark step, which involves the conversion \( A_{672} \rightarrow A_{681} \), and goes along with the liberation of NADP\(^+\) and rebinding of NADPH, was observed. This reaction is shifted to lower temperatures in POR B as compared with POR A (Fig. 5). Because the reaction only occurs above the glass-transition temperature where protein dynamics plays a prominent role, the temperature shift suggests higher protein flexibility in the POR B enzyme, which finally facilitates the replacement of NADP\(^+\) by NADPH within the active site.
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Apart from possible differences in protein dynamics, the low temperature experiments clearly indicate that both the initial photoactive POR-NADPH-PChlide complex and the reaction intermediates, which are formed in the course of PChlide reduction, are spectroscopically identical in POR A and POR B. Beyond that, the reaction sequence in the appearance of the different intermediate species is also the same in the two isozymes. These findings imply that the same mechanism of catalysis is likely to operate in POR A and POR B. Thus, the higher catalytic efficiency of POR B as compared with POR A is not the result of differences in the catalytic mechanism. It is first of all due to the higher binding affinity of PChlide to POR B and most probably to a higher conformational flexibility of POR B, as a result of which the cofactor release and cofactor rebinding steps are modulated. In addition, enzyme dynamics may also play a role for PChlide binding in the ternary enzyme-substrate complex as it was recently observed for POR enzymes from cyanobacteria (45). Thus, even if protein dynamics might not directly affect the catalytic rate itself it seems to be an integral part in the catalytic function of POR B that could explain the higher catalytic activity when compared with POR A.

In conclusion, the results of the present study reveal significant differences between the two isozymes of barley POR, POR A and POR B. In accordance with its prominent role for chlorophyll biosynthesis in light-adapted plants, POR B shows an about 6-fold higher catalytic efficiency as compared with POR A. As is apparent from the intermediates trapped at low temperatures, the two isozymes do not differ in their reaction mechanism. Therefore, the higher catalytic efficiency of POR B obviously results from the higher binding affinity of PChlide to the enzyme and, most likely, from dynamic effects, which control conformational fluctuations of protein sites around the catalytic center.

Moreover, with respect to the initial photochemistry the two studied isozymes differ from the enzymes of cyanobacteria. The initial photoreaction, which occurs below 180 K, yields already fully reduced Chlide, even if Chlide remains at first still bound to the enzyme and is only released in the following dark reaction steps at increasing temperatures. The photoreduction in two separate reaction steps including the (i) light-induced hydride transfer from NADPH to PChlide followed by (ii) the proton transfer reaction in the subsequent dark reaction as it is reported for the enzymes from cyanobacteria (31, 32, 45) is not realized in the two barley isozymes.

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