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Protochlorophyllide Oxidoreductase: A Homology Model Examined by Site-Directed Mutagenesis

Helen E. Townley, Richard B. Sessions, Anthony R. Clarke, Timothy R. Dafforn, and W. Trevor Griffiths*
Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol, United Kingdom

ABSTRACT **An homology model of protochlorophyllide reductase (POR) from *Synechocystis* sp. was constructed on a template from the tyrosine-dependent oxidoreductase family. The model showed characteristics appropriate to a globular, soluble protein and was used to generate a structure of the ternary complex of POR, nicotinamide adenine dinucleotide phosphate (NADPH), and protochlorophyllide. The POR ternary model was validated by mutagenesis experiments involving predicted coenzyme-binding residues and by chemical modification experiments. A core tryptophan residue was shown to be responsible for much of the protein's fluorescence. Both quenching of this residue by coenzyme and fluorescence resonance energy transfer (FRET) from the protein to the coenzyme allowed the binding constant of NADPH to be determined. Replacement of this residue by Tyr gave an active mutant with approximately halved fluorescence and a negligible FRET signal, consistent with the role of this residue in energy transfer to the NADPH at the active site and with the model. The mechanism of the enzyme is discussed in the context of the model and semiempirical molecular orbital calculations. Proteins 2001;44:329–335.**

Key words: synechocystis; NADP; AM1; fluorescence; photoenzyme; chlorophyll; protochlorophyll; dehydrogenases

INTRODUCTION

The bulk of the chlorophyll in O_2 evolving plants is produced via a biosynthetic pathway that includes the light-dependent enzyme protochlorophyllide reductase (POR) (EC 1.3.1.33). The reaction catalyzed by this enzyme has been extensively studied in vitro and involves light-induced hydride transfer from the *pro*-S face of nicotinamide adenine dinucleotide phosphate (NADPH), trans to the C_{17} position of protochlorophyllide (pchlide), together with the addition of H^+ to C_{18}, forming chlorophyllide,[1–3] Figure 1. Detailing the mechanism of this catalytic process represents a considerable challenge, currently attracting the interest of scientists from a wide range of disciplines.[3] Progress in this direction has been facilitated by the heterologous expression of the maltose-binding protein (pmal) fusions of POR from various plant sources, and these have been shown to generate high yields of the active enzyme.[4–6] pMal-POR from the *Synechocystis* gene is highly active in producing chlorophyllide under the appropriate conditions in vitro.[5] The heterologous fusion product has many advantages over preparations of the native protein. It is easy to prepare in large quantities, and such preparations are free from interfering contaminants such as pigments and other proteins, which invariably accompany the enzyme isolated from higher plant tissues. These plasmid constructs also allow the structure-function relationship in POR to be probed by site-directed mutagenesis.

Progress in evaluating the mechanism of POR has been hampered by the current lack of a crystal structure of the enzyme. Here, we present a detailed homology model of the protein from *Synechocystis* based on the assignment of POR to the "RED" superfamily[7] (Reductases, Epimerases, and Dehydrogenases) and an, albeit low, sequence similarity with the tyrosine-dependent oxidoreductase family. The validity of this model has been tested by site-directed mutagenesis and chemical modifications of certain residues predicted to have key roles in substrate/cofactor recognition.

MATERIALS AND METHODS

Homology Modeling of *Synechocystis* POR

Comparison of the *Synechocystis* sequence with the sequences of known structures from the SCOP database highlighted the most suitable structural templates for modeling. These were the tyrosine-dependent oxidoreductases of the NAD(P)-binding Rossmann-fold superfamily, namely, the short-chain alcohol dehydrogenase from *Mus musculus* (1cyd), 3α,20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* (2hsd) and 7α-hydroxysteroid dehydrogenase from *E. coli* (1ahi). A multiple sequence alignment between POR sequences and the three structural templates (1cyd, 2hsd, and 1ahi) shows complete conservation of the glycine-rich motif of the Rossmann-fold. This motif corresponds structurally to part of the surface of the adenine-binding cleft and varies in sequence detail between classes of nucleotide-binding pro-

Abbreviations: POR, protochlorophyllide oxidoreductase; pchlide, protochlorophyllide; chlide, chlorophyllide; MBP, maltose-binding protein; PGO, phenylglyoxal; NPM, N-phenylmaleimide.

H.E. Townley's present address is Department of Plant Sciences, University of Oxford, Oxford, UK.

T.R. Dafforn's present address is Department of Haematology, University of Cambridge, Cambridge, UK.

*Correspondence to: W. Trevor Griffiths, Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, UK. E-mail: T.Griffiths@bris.ac.uk

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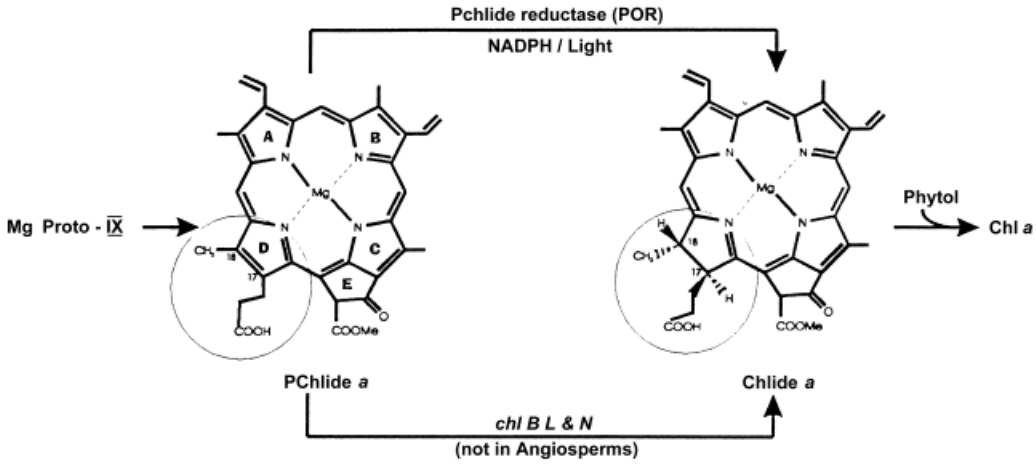

Fig. 1. The reduction of pchlide to chlide during chlorophyll synthesis in plants. The scheme shows addition of hydrogen atoms to the C_{17}-C_{18} double bond of pchlide.

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     1        10        20        30        40        50        60        70        80
por  MKPTVIITGASSGVGLYGRKALIDKGWHVIMACRNLDKTQKVADELGFPKDSYTIIKLDLGYLDSVRRFVAQFRELGRPL
          β-1       α-A       β-2       α-B       β-3       α-C
ahi  DGKCAIITGAGAGIGKEIAITFATAGASVVVSDINADAANHVVDEIQQLGGQAFACRCDITSEQELSALADFAISKLGKV

     81        90        100       110       120       130       140       150       160
por  KALVCNAAVYFPLLDEPLWSADDYELSVATNHLGHFLLCNLLLEDLKACPDADKRLIILGTVTANSKELGGKIPIPAPPD
     β-4                           α-D                           β-5
ahi  DILVNNAGGGGPKPFD..MPMADFRRAYELNVFSFFHLSQLVAPEM....EKNGGGVILTITSMAAENK..........

     161       170       180       190       200       210       220       230       240
por  LGNFEGFEAGFKKPIAMINNKKFKSGKAYKDSKLCNMLTTRELHRRFHQETGIVFNSLYPGCVADTPLFRNHYSLFRTIF
               α-E                 α-F                 β-6       α-G
ahi  .....................NINMTSYASSKAAASHLVRNMAFDL.GEKNIRVNGIAPGAIL.TDALKSVIT.PEIEQ

     241       250       260       270       280       288
por  PWFQKNVTKGYVSQELAGERVAMVVADDKFKDSGVHWSWGNRQQAGREafvqelseqgsdaqkaqrmwdlseklvglv
     α-H       α-I                 β-7
ahi  KMLQHTPIRRLGQPQDIANAALFLCSPAASWVSGQILTVSGGGVQELN.............................
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Fig. 2. The sequence alignment of *Synechocystis* POR and the 7α-hydroxysteroid dehydrogenase from *E. coli* used to generate the homology model. The residues shown in lower case were not modeled because of the lack of a template for this C-terminal portion. Secondary structural elements of POR are labeled. The GxxxGxG and YxxxK motifs are underlined.

teins, being GxxxGxG in POR. The sequence motif YxxxK corresponds to the catalytic dyad, which gives the tyrosine-dependent oxidoreductase family its name. The multiple sequence alignment software (Pileup; GCG) fails to match this motif between the two classes of protein, principally because there is a 33-residue insertion in POR with respect to the known tyrosine-dependent oxidoreductases between the GxxxGxG and YxxxK motifs. Multiple sequence alignments of the regions between the two motifs suggest that the insertion should be placed just before the second motif. These considerations led to the homology model being built according to the alignment shown in Figure 2. The 7α-hydroxysteroid dehydrogenase (1ahi) was chosen as the structural template largely because the substrate of this enzyme is most similar in shape to pchlide. The model was built according to standard methods.[8] The 33-residue insertion was built as a strand-turn-helix, both on the basis of secondary-structure prediction and a FPS (v2.0) search of the protein structure database that found a sequence match to this motif in the structure of UDP-N-acetylmuramoyl-L-alanine:D-glutamate ligase (1uag, residues 107–140). After docking the cofactor NADPH and substrate Mg-pchlide, the ternary complex was soaked with a 5 Å layer of water and relaxed by energy minimization. The programs InsightII v97 and Discover v2.97 (MSI) were used to perform the modeling.

Overlap Extension Polymerase Chain Reaction Mutagenesis

Site-directed mutagenesis was performed on the wild-type cyanobacterial POR gene by using the overlap extension PCR method of Ho et al.[9] The oligonucleotides used were as follows, with the mutation sites in upper case letters:

R34V:	g att atg gcc tgc GTG aat ttg gat
C33D:	g att atg gcc GAT cga aat ttg gat
C33DR34V:	g att atg gcc GAT GTG aat ttg gat
W27Y:	ta att gac aaa ggt TAC cac gtg att atg gcc
5′ terminus primer:	tcag GAA TTC atg aaa ccc acg gtg atc atc
(restriction site)	Eco RI
3′ terminus primer:	taag GGA TCC cta aac cag acc cac taa ctt
(restriction site)	Bam HI

Constructs were sequenced to ensure that cloned inserts were correct.

Expression and Purification of the *Synechocystis* Por-MBP Fusion Protein

Fusion protein was overexpressed in XL-1Blue *E. coli* cells and purified by a combination of anion exchange and affinity chromatography as previously described.[5]

Enzyme Activity Assay

MBP-POR activity was measured spectroscopically as flash-induced formation of products as previously described.[10]

Chemical Modification

Reactive Cys and Arg groups in POR were modified by reaction with N-phenylmaleimide and phenylglyoxal, respectively, as previously described.[11,12]

Titration by Fluorescence Spectroscopy

Spectra were recorded by using a Perkin Elmer Luminescence Spectrometer LS 50B. The excitation wavelength was fixed at 290 nm (5-nm bandwidth). The fusion protein MBP-POR (5 mM) was incubated in 10 mM Tris buffer, pH 7.5, in a final volume of 3 mL in quartz cuvettes. Emission spectra were scanned between 300 and 500 nm after each addition of NADPH (5-mM aliquots). The data were fitted to the tight ligand-binding equation with slope (see below) to correct for the inner-filter effect of NADPH. Similar binding constants were obtained when fitting either the quench at 340 nm or the fluorescence resonance energy transfer (FRET) signal at 460 nm.

$$\text{Signal} = (((L + E_0 + K_d) - ((L + E_0 + K_d)^2 - 4E_0L)^{0.5})/2E_0)(F_{mzx} - F_{min}) + Lm$$

where E_0 is the enzyme concentration, L is the ligand concentration, F_{min} and F_{max} are the minimum and maximum values of fluorescence, and m is the slope correction for the inner-filter effect of L.

Semiempirical Molecular Orbital Calculation

A single Mg-pchlide molecule was calculated by using MOPAC 7.0 and the AM1 Hamiltonian. The acid group was protonated, and the metal ion was replaced by a +2 sparkle, such that the overall charge of the system is +2. The geometry optimization converged (using the "precise" option) to a structure with a heat-of-formation of 126.2 kcal/mol. The calculation was conducted in cartesian space such that the ring system lay in the X-Y plane. Consequently, components of the eigenvectors in the Z direction approximately correspond to coefficients of the π-system. A single node of a 32x(twin PIII 750 MHz) Beowulf was used to perform the calculation.

RESULTS AND DISCUSSION

Homology Model

Protochlorophyllide oxidoreductases characterized from a wide range of O_2 evolving photosynthetic organisms display a remarkably conserved sequence of amino acids but, in contrast, show a very low homology with other functionally related enzymes. Despite this finding, regions of similarities have been recognized between POR and sequences found in members of the family of tyrosine-dependent oxidoreductases (also known as short-chain dehydrogenases), especially when secondary structural features of established crystal structures are considered. Figure 2 shows such a sequence comparison between the POR of *Synechocystis* and a typical structurally characterized member of the tyrosine-dependent oxidoreductases, that is, 7α-hydroxysteroid dehydrogenase from *E. coli*. Although the overall homology of the amino acid sequences is quite low, motifs already established as necessary for structural and functional reasons are conserved between POR and the rest of the family. These include a glycine-rich motif GxxxGxG[7,13] characteristic of the NAD(P)-binding Rossmann-fold superfamily and the YxxxK catalytic motif characteristic of the tyrosine-dependent oxidoreductase family.[7]

The homology model of *Synechocystis* POR, built on the 7α-hydroxysteroid dehydrogenase structure of *E. coli* as described in Materials and Methods, is shown in Figure 3. The structure has a typical Rossmann-fold, consisting of a central parallel β-sheet comprised of 7 β-strands (β-1 to β-7, Fig. 2) surrounded by 9 α-helices (α-A to α-I, Fig. 2), providing a cofactor-binding pocket. Extensions of some of the central helices provide a cleft to accommodate the substrate, pchlide. The highly conserved YxxxK catalytic motif is on helix α-F, which forms a side of this cleft where the side chains of Y and K can interact with the substrate. The sequence alignments (Fig. 2) show a 33-residue insertion in POR just before the YxxxK motif. The sequence predicts as an α-helix for the final three quarters of its length and was modeled between β-4 and α-F, forming an extension to the face of the substrate-binding cleft containing the YxxxK motif (Fig. 3). Such insertions in this fold are not unknown; for example, human carbonyl reductase (HCR) has a 41-residue insertion at this position. Although it has been suggested this loop may modulate intermolecular interactions,[14] this may not be the case, because both HCR and POR have been shown recently to be functionally active as monomers.[6,14]

The POR model has a hydrophobic core with no buried charged residues, whereas hydrophilic and charged residues adorn the surface. The stereochemistry and geometry of the structure was satisfactory when analyzed with Procheck.

Chemical Modification of POR

The substrate and cofactor specificity of the expressed and purified MBP-POR fusion has been shown[15,16] to be identical with those already reported for the purified higher plant enzyme.[17,18] The expressed enzyme also shows similar responses as the higher plant enzyme to various inhibitors, for example, sensitivity to quinacrine (I_{50} = 40 μM) and diphenyleneiodinium (I_{50} = 5 μM) in addition to inhibition by thiol reagents such as the maleimides and the arginine specific reagent, phenylglyoxal

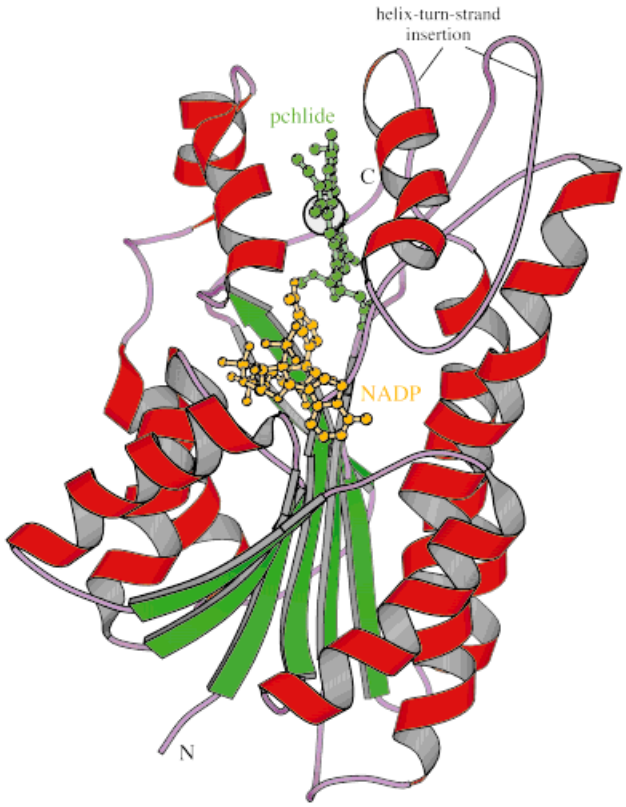

Fig. 3. A ribbon representation of the *Synechocystis* homology model. α-Helices are shown as red tapes and β-strands as blue arrows. The substrate, pchlide, is shown as green ball and sticks with the Mg ion as a gray sphere. The cofactor NADPH is shown as orange ball and sticks. The N and C termini of the protein chain are labeled. The position of the 33-residue insertion is indicated.

(PGO).[15] As already well documented for the isolated plant enzyme,[11] incubation of the *Synechocystis* fusion protein alone at 22° in darkness for 15 min results in a slight (8%) loss in activity (control level). Addition of 0.5 μM of the thiol reagent, N-phenylmaleimide (NPM) over 15 min attenuates the activity to 50% of the unsupplemented control. Although preincubation with pchlide offered no protection against the inhibitor, preincubation with NADPH does protect the enzyme, resulting in retention of 71% of the activity of the unsupplemented control incubation. This protection is enhanced in the ternary complex such that preincubation with both NADPH and pchlide allows 85% retention of the control activity. Addition of the arginine modifying reagent, phenylglyoxal (PGO), at 8 mM to the enzyme also results in loss of activity over 15 min, resulting in recovery of only 10% of the control activity. The protection against PGO inhibition afforded by formation of the ternary complex with added pchlide and NADPH is even more striking in this case, resulting in 95% retention of wild-type activity.[15] These data strongly suggest that cysteine and arginine residue(s) may be involved with substrate and/or NADPH binding in POR. Site-directed mutagenesis of recombinant POR offers the prospect of identifying these potential substrate-binding residues and validating the predicted model.

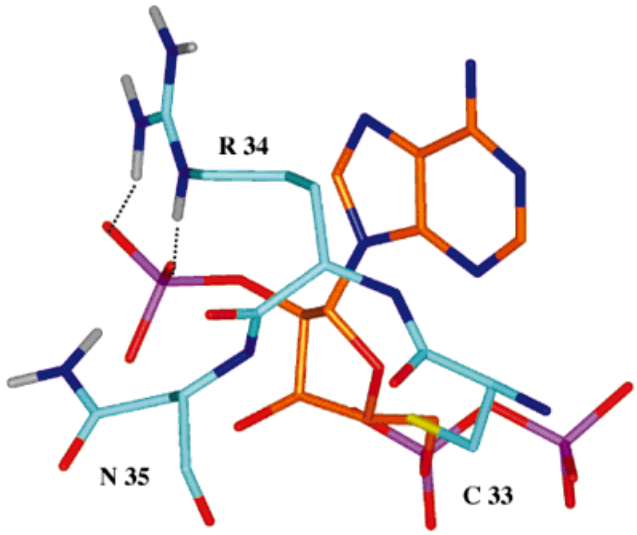

Fig. 4. Detail around the adenine-ribose binding pocket of NADPH in the *Synechocystis* POR model. This shows the proposed interaction of Arg 34 with the 2′-ribose-phosphate group, and the position of the neighboring Cys 33. Carbon atoms of the protein are colored pale blue, and those of NADPH are orange. Polar hydrogen atoms on the side chains of residues 34 and 35 are shown in gray. Hydrogen bonds between Arg 34 and the 2′-phosphate are shown as dotted lines.

NADPH-Binding Site and Site-Directed Mutagenesis

Docking the coenzyme into the POR model is straightforward because the template structure itself contains NADPH. The coenzyme cleft is well conserved between the template and modeled structures such that no inappropriate residues appear in the modeled NADPH pocket. POR shows absolute specificity for NADPH, with NADH, and a range of other biochemical reductants, being completely inactive as hydrogen donors.[10] As discussed in the preceding section, inhibition of the POR fusion protein by chemical modification, and the protection against this afforded by substrate and cofactor, strongly indicate roles for Arg and Cys in the substrate/cofactor binding sites. A pair of such residues, Cys 33 and Arg 34, occur in the model in a loop adjacent to the 2′-hydroxyl group of the adenosine ribose of the cofactor. This site has been identified as a key position in determining the NAD/NADP cofactor selectivity of a number of NAD(P) linked dehydrogenases.[19–21] The model shows (Fig. 4) how Arg 34, a structural feature normally associated with NADP-linked dehydrogenases, can form a salt bridge with the 2′-phosphate group of NADP. This residue is absent and usually preceded by an aspartic acid in NAD-dependent dehydrogenase sequences, where the side chain can hydrogen bond to both adenosine ribose hydroxyl groups. Site-directed mutagenesis was used to change Cys 33 to Asp and Arg 34 to Val, generating both single and double-mutant enzymes. Both of the single mutations reduce the activity of the protein with NADPH by more than 10-fold. R34V and C33D show 3% and 4% of wild-type activity, respectively. The corresponding double mutant abolishes any detectable activity. Consequently, the results of chemical modification and the mutagenesis

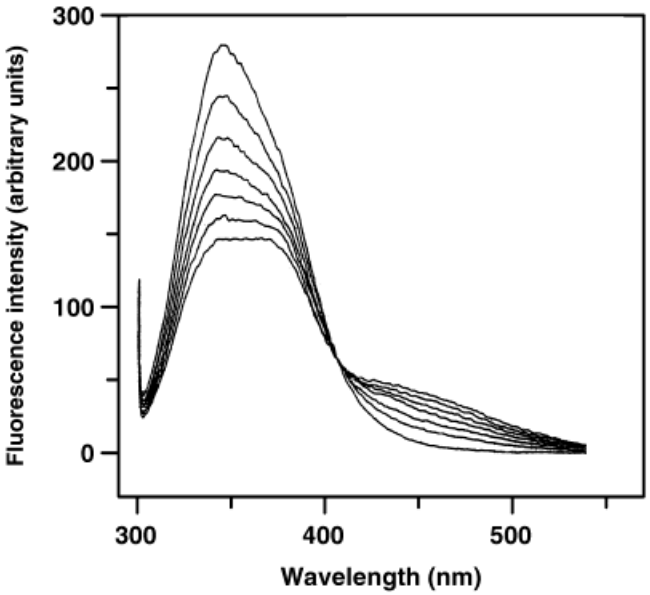

Fig. 5. Successive fluorescence spectra of *Synechocystis* POR as NADPH is added. During addition, the peak at 340 nm is quenched, whereas the FRET peak at 460 nm is enhanced. A clear isosbestic point is seen at 412 nm. The protein concentration is 5 μM and NADPH is added in aliquots to give 5-μM increments in the cell.

experiments conducted at positions 33 and 34 are consistent with the known cofactor specificity and our model of the POR ternary complex.

Cofactor Binding by Fluorescence Titration

Figure 5 shows the tryptophan fluorescence emission from POR and the effect of added NADPH. The presence of pchlide had no effect on the system, suggesting that the binding of NADPH is independent of the presence of the pigment substrate. Addition of NADH has no effect on the protein fluorescence (data not shown), suggesting that this coenzyme does not bind to the enzyme, a result consistent with the observation that POR shows absolute specificity for NADPH as a hydride source.

Successive additions of NADPH results in progressive specific quenching of the 340-nm emission accompanied by simultaneous increase in emission centered at 460 nm, consistent with FRET from the excited tryptophan to the bound NADPH. The quenching/FRET spectra in Figure 5 feature a sharp isosbestic point at 412 nm, which may indicate that a single tryptophan residue in the protein is ideally located for efficient FRET to the enzyme-bound NADPH. This behavior is observed despite the fact that 5 tryptophan residues are present in *Synechocystis* POR together with a further 8 in the maltose-binding protein fusion. However, the model of POR shows that only one of the POR tryptophan residues, Trp 27, is buried in the hydrophobic core of the protein (albeit within hydrogen-bonding distance of the carbonyl group of L 22), whereas the remainder are surface exposed and would be expected to be quenched by solvent (Fig. 6). Furthermore, this residue is proximal to the nucleotide-binding residues Cys 33 and Arg 34 in the linear sequence and is around 18 Å from the nicotinamide group of NADPH, well within the limit of the Förster radius required for efficient FRET. The W27Y mutant of *Synechocystis* POR was constructed to ascertain the contribution of Trp 27 to the intrinsic fluorescence of the enzyme and to the quenching by NADPH. This mutant protein has a catalytic rate of 0.32 nmoles chlide formed $min^{-1} \cdot$ mg protein, comparable with rates routinely measured for the wild-type enzyme under similar conditions. The W27Y mutant fusion protein has a low fluorescence yield at 340–350 nm, some 55% of the wild-type fusion protein, and shows little quenching of this emission on titration with NADPH. Furthermore, the W27Y mutant shows a negligible FRET signal at 460 nm in saturating amounts of NADPH. This indicates that Trp 27 is the principal source of the fluorescence signal change that occurs on binding of NADPH to the wild-type enzyme.

The titration curve of wild-type POR with NADPH is shown in Figure 7. The data have been fitted to the tight binding equation with slope to correct for the inner-filter effect of NADPH, as described in Materials and Methods. This plot yields a value of $K_d = 7.4 \pm 0.5$ μM for the dissociation constant of the enzyme with NADPH. This may be compared with a value of 10 μM measured for the K_M for NADPH of the enzyme.[15]

Protochlorophyllide-Binding Model

Pchlide was modeled into the binding cleft in POR corresponding to the hydroxysteroid-binding site in the template structure. Docking was performed manually, guided both by visual inspection and by limitations imposed by the following experimental observations:

1. Rings C, D, and E of the pigment (Fig. 8) must be embedded in and in intimate specific contact with protein at the active site because any modification to this part of the molecule leads to loss of activity as substrate.[17,18] Specificity for this part of the molecule is so precise that even the minimal alteration of the C_{17} propionate group to an acrylate chain, as present in chlorophyll c_1, renders it inactive as a substrate for POR.[1]
2. Within the pigment-binding cleft formed by the two longer helices of the inner core (helices α-D and α-F in POR), the pigment has to be orientated in such a way that C_{17} and C_{18} in ring D lie adjacent to the catalytic YxxxK motif on helix α-F. This motif has already been shown to be directly involved with provision of H^+ for the catalysis by pea POR using site-directed mutagenesis.[22] Furthermore, the C_4 hydrogens of the nicotinamide ring of NADPH must also be close to this site to provide the H^- used in the reduction of the C_{17}-C_{18} double bond.
3. In contrast to the strict requirement for chemically unaltered rings D and E in pchlide, rings A and B can be subjected to chemical modification without any impairment of their activity as substrates for POR.[17,18] This finding suggests that this part of the pigment makes no essential contacts with the protein and in the model (Figs. 3 and 8), rings A and B are seen as freely suspended in the neck of the substrate cleft.

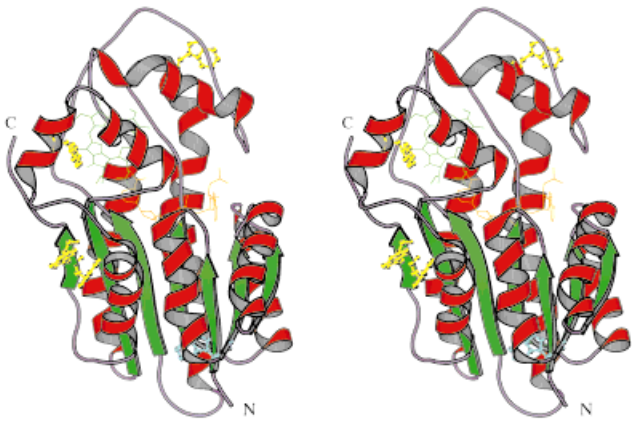

Fig. 6. A stereo view of the *Synechocystis* POR homology model. α-Helices are shown as red tapes and β-strands as blue arrows. The substrate, pchlide, is shown as green sticks with the Mg ion as a gray sphere. The cofactor NADPH is shown as orange sticks. The N and C termini of the protein chain are labeled. The solvent exposed tryptophan residues in the structure are shown as yellow ball and sticks, whereas the buried tryptophan 27 (the subject of mutagenesis) is shown as cyan ball and sticks.

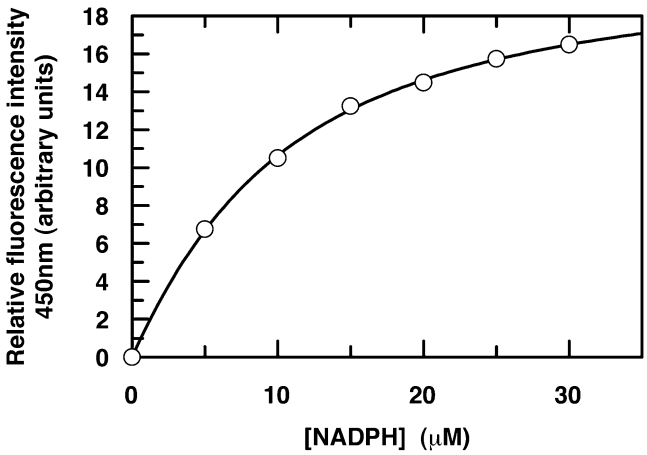

Fig. 7. The FRET signal at 460 nm observed when POR is titrated with aliquots of NADPH. The data are corrected for the inner-filter effect of NADPH as described in Materials and Methods.

4. Pchlide analogues, effective as POR substrates, must have either a Mg or Zn atom as the chelating metal.[17] It is unclear whether this requirement is to satisfy some essential photochemical role during the photocatalysis or else in the binding of the pigment at the active site. We had considered the possibility that the two unusual Histidine motifs H(112)xxH(115) and H(204)xxxH(208) (Fig. 2) might provide the ligands for chelating the central Mg or Zn in the pigment. Identical motifs are found in the ChlB subunit of the light-independent pchlide reducing system,[23,24] which might indicate a pigment-binding role for the ChlB subunit. Despite this interesting possibility, both Histidine motifs in our model are located distal from the pigment. However, we do find a Histidine residue H 232 located on helix α-G that is perfectly placed to form an axial ligand to the pchlide metal ion. Although such a histidine coordination is likely to be stronger in the product Mg-chlide rather than the substrate Mg-pchlide, we may speculate that dissociation of the product is facilitated by the relative movement of the propionic acid side chain with respect to the tetrapyrrole ring of the substrate. Reduction of C_{17} from sp^2 (trigonal) to sp^3 (tetrahedral) geometry will displace the acid group some 3–4 Å further out of the plane of the tetrapyrrole ring and so disrupt the binding interaction of this group to K 193 in the protein.

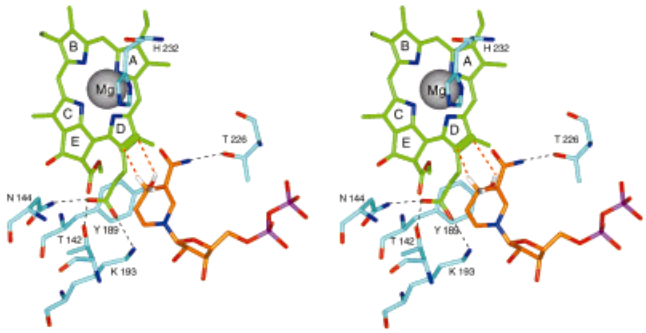

Fig. 8. Stereo view of the active site of the POR ternary complex. Protein residues are shown with pale blue, pchlide with green, and NADPH with orange, carbon atoms. Black dotted lines represent possible intermolecular hydrogen bonds. The C_{17}-C_{18} double bond is represented with a thick line. The only hydrogen atoms shown are those on C_4 of the nicotinamide ring, and the OH group of Tyr 189. Red dotted lines highlight the distance between the *pro*-S nicotinamide hydrogen and C_{17}, and the hydroxyl proton of Tyr 189 and C_{18}.

Mechanism of POR

Previous spectroscopic experiments have shown that POR-catalyzed reduction of pchlide to chlide undergoes reaction in two distinct steps. A stable ternary complex of POR, pchlide, and NADPH is formed in the dark.[1] Illumination of this ternary complex causes an initial fast (<1 ns) reaction to an unstable intermediate that decays via a slower (≈1 μs) reaction, to the product, chlide.[25] It is generally agreed that the initial, fast reaction involves transfer of hydride from NADPH to the excited state of pchlide. However, the precise oxidation state or spin state of the ensuing intermediate is open to question, because some experiments have identified paramagnetic species after irradiation of the POR ternary complex at low temperature,[6] whereas others have not.[5] It is of interest to consider how the electron-rich C_{17}-C_{18} double bond could become electrophilic enough to accept a hydride anion on irradiation with light. Some clues are afforded by a semiempirical molecular orbital calculation performed on pchlide itself (see Materials and Methods). Inspection of the eigenvectors from this calculation shows that the P_z atomic orbital of C_{17} comprises 22% of the HOMO. Hence, absorption of a photon of light, which is most likely to promote an electron out of the HOMO, will create an electron-deficient site at C_{17}. This site can satisfy its electron deficiency by abstracting hydride from NADPH.

The second step of the reaction appears to be the transfer of a proton from the protein to the intermediate to generate the product, chlide. Supporting evidence is pro-

vided by the effects of NADPD, D_2O, and pH on the kinetics of the postillumination decay of the intermediate species[12] (Oliver, Matthis, and Griffiths, unpublished data) and also by site-directed mutagenesis of the putative proton donor residue, Tyr 275 in Pea POR. None of the mutant proteins Y275F, Y275C, K279R, and K279I showed POR-type activity.[22]

The POR ternary model developed here is consistent with the above mechanism, or one involving radical intermediates, because the hydride of the nicotinamide ring of NADPH is in close (3.8 Å) contact with C_{17} of pchlide. Likewise, Y 189 and K 193 (equivalent to Y275 and K279 in Pea POR) are juxtaposed on the opposing face of the pchlide ring, ideally placed to donate a proton to C_{18} in the second step of the reaction. Additions of H^- and H^+ in this manner yield the correct stereochemistry[26] of (17 S, 18 S) for the product chlide.

CONCLUSIONS

The premise that POR is a member of the tyrosine-dependent oxidoreductase family allowed us to build a model of *Synechocystis* protochlorophyllide reductase. This model shows all the characteristics of a globular protein, namely, a hydrophobic core with no buried charged residues, and a surface decorated with hydrophilic and charged amino acids. Modeling of the ternary complex of POR, NADPH, and protochlorophyllide identified certain key residues involved in the binding of NADPH. Replacement of these by site-directed mutagenesis gave results consistent with this prediction, as did chemical modification of the generic residue types (Arg and Cys). A single buried tryptophan in the model was considered to be responsible for most of the fluorescence shown by the protein, and this signal could be used to measure cofactor-binding affinity. Replacement of this residue by Tyr gave an active mutant but with drastically reduced fluorescence, again in agreement with prediction. Hence, the homology model is supported by all available experimental evidence and provides a rational basis for the design of further experiments to explore the mechanism of this intriguing enzyme.

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