Purification and kinetic analysis of pea (*Pisum sativum* L.) NADPH:protochlorophyllide oxidoreductase expressed as a fusion with maltose-binding protein in *Escherichia coli*

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NADPH:protochlorophyllide oxidoreductase (POR) catalyses the light-dependent reduction of protochlorophyllide to chlorophyllide, a key reaction in the chlorophyll biosynthetic pathway. To facilitate structure–function studies, POR from pea (*Pisum sativum* L.) has been overexpressed in *Escherichia coli* as a fusion with maltose-binding protein (MBP) at 5–10% of the total soluble cell protein. The fusion protein (MBP–POR) has been purified to greater than 90% homogeneity by a two-step affinity-purification procedure. This represents the first successful overexpression and purification of a plant POR. MBP–POR was found to be active, and the kinetic properties were determined using a continuous assay in which the rate of chlorophyllide formation was measured. The V_{max} was 20.6 ± 0.9 nmol·min^{-1}·mg^{-1} and the K_m values for NADPH and protochlorophyllide were 8.7 ± 1.9 μM and 0.27 ± 0.04 μM respectively. These results represent the first determination of the kinetic properties of a pure POR and the first report on the kinetics of POR from a dicotyledenous plant. The experiments described here demonstrate that the enzyme is not a 'suicide' enzyme, and the only components required for catalysis are NADPH, protochlorophyllide and light. Size-exclusion chromatography on a Superose 6 HR column indicated that MBP–POR has a molecular mass of 155 kDa (compared with the molecular mass of 80 kDa estimated by SDS/PAGE), indicating that it behaves as a dimer in solution. This is the first direct determination of the oligomerization state of POR.

INTRODUCTION

NADPH:protochlorophyllide oxidoreductase (POR; EC 1.3.1.33) catalyses the light-dependent reduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) [1]. This reaction is an important regulatory step in chlorophyll biosynthesis and chloroplast development of angiosperms as a result of this unique requirement for light. In contrast, purple non-sulphur photosynthetic bacteria are able to synthesize bacteriochlorophyll in the dark as a result of the fact that they have a light-independent protochlorophyllide reductase [2]. Cyanobacteria, algae and non-flowering land plants possess both the light-dependent and light-independent protochlorophyllide reductases [3–5]. In these cases the activity of the light-dependent enzyme POR seems to be important for maximum chlorophyll accumulation.

POR from pea (*Pisum sativum* L.) is a nuclear-encoded protein which is synthesized in the cytoplasm as a 400-amino-acid precursor [6]. On import into the plastids, a 64-amino-acid transit peptide is removed by proteolysis and the mature protein is localized to the inner plastid membranes. The enzyme accumulates in the dark, but is rapidly degraded upon illumination. This is important for the regulation of the chlorophyll biosynthetic pathway and the development of the photosynthetic apparatus. However, this instability and the association with membranes creates problems for isolation and purification of the enzyme for kinetic and structural studies. In addition, it is currently not possible to analyse the activities of mutant enzymes in transgenic plants.

One way of overcoming these problems is to use a heterologous expression system to overproduce the enzyme. We recently reported the expression of POR from pea in *Rhodobacter capsulatus* strains which have mutations in one of the three subunits of the light-independent protochlorophyllide reductase [7]. The plant enzyme was active and complemented the mutations, restoring the synthesis of bacteriochlorophyll, but only in the light. This has allowed manipulation of the protein-coding sequence and subsequent analysis of the effects of the changes on enzyme activity. However, one drawback of this expression system is that the enzyme is produced at very low levels, which are only detectable by Western-blot analysis. Therefore it has not been possible to isolate sufficient quantities of the enzyme for detailed kinetic and structural studies.

POR is one of only two enzymes known to require light for catalysis; the other is DNA photolyase [8]. To date, little is known about the relationships between structure and function of POR and the role of light in the catalytic reaction mechanism. In the present paper we report on the high-level expression of a catalytically active fusion of pea POR with maltose-binding protein (MBP) in *Escherichia coli*. A two-step affinity purification procedure yielded sufficient recombinant protein for detailed kinetic analysis. In addition, size-exclusion chromatography has been used to determine the molecular size of the native fusion in order to elucidate the oligomerization state of the protein.

MATERIALS AND METHODS

Materials

Unless otherwise stated, chemicals were obtained from Sigma Chemical Co. and were of analytical grade. Chemicals for SDS/PAGE and nitrocellulose membranes were purchased from

Abbreviations used: Chlide, chlorophyllide; DTT, dithiothreitol; IPTG, isopropyl β-D-thiogalactoside; Pchlide, protochlorophyllide; POR, NADPH:protochlorophyllide oxidoreductase; MBP, maltose-binding protein; RED, reductases/epimerases/dehydrogenases.

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Bio-Rad. The pMAL-c2 expression vector, *E. coli* strain PR745 [RR1 *lon*::miniTn*10*(Tet^r) Δ(malB)Δ(argF-lac)U169 Pro$^+$ *zjc*::Tn*5*(Kan^r)], amylose resin, rabbit anti-MBP antiserum and *Xmn*I DNA restriction endonuclease were from New England Biolabs. Other enzymes for DNA restriction and modification were from Northumbria Biologicals Ltd. Red Sepharose CL-6B resin and the Superose 6 gel-filtration column were from Pharmacia. Rabbit anti-POR antiserum was a gift from Dr. W. T. Griffiths (Department of Biochemistry, University of Bristol, U.K.).

Construction of pMAL-POR

The DNA encoding the mature form of POR (lacking the codons for the first 64 amino acids of the full-length pre-protein) was amplified from plasmid pBluescript KS (Stratagene) containing the pea POR cDNA [6] in a Perkin–Elmer Cetus DNA thermal cycler as follows: 1 ng of plasmid in a total volume of 50 μl was denatured for 10 min at 94 °C in the presence of 0.25 μM each oligonucleotide primer (forward primer: 5′-GAGACAGCGGC-TCCGGC-3′; reverse primer: 5′-GGAAACAGCTATGACC-ATG-3′), 200 μM dNTPs and 2.5 units of *Taq* DNA polymerase in 50 mM KCl/15 mM $MgCl_2$/10 mM Tris/HCl, pH 8.3. This was followed by 40 cycles (94 °C, 45 s; 50 °C, 45 s; 72 °C, 90 s) and a final extension for 10 min at 72 °C. The resulting PCR product was treated with the Klenow fragment of DNA polymerase I to create blunt ends and digested with *Xba*I prior to ligation into *Xmn*I/*Xba*I-cut pMAL-c2. *E. coli* JM109 cells were transformed with the recombinant plasmid (pMAL-POR) and a clone over-expressing MBP–POR on induction with 0.3 mM isopropyl β-D-thiogalactoside (IPTG) was isolated. The entire insert of pMAL-POR was sequenced using the ABI PRISM Dye Terminator Sequencing Kit with AmpliTaq DNA polymerase, FS (Perkin-Elmer) and found to contain the expected nucleotide sequence of mature pea POR. Plasmid pMAL-POR was subsequently transformed into *E. coli* PR745, which lacks a protease responsible for degrading aberrant proteins and also contains a deletion of the *malB* region eliminating expression of MBP.

Expression and purification of the MBP–POR fusion protein

E. coli PR745(pMAL-POR) was grown in 1 litre of rich medium (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl and 2 g of glucose per litre) containing 100 μg/ml ampicillin and 15 μg/ml tetracycline to an A_{600} of 0.5. The expression of MBP–POR was induced by the addition of IPTG (0.3 mM final concentration) and the cells were grown for a further 2 h before harvesting by centrifugation. The cell pellet was resuspended in chilled lysis buffer [50 mM Tris/HCl (pH 7.5)/500 mM NaCl/5 mM EDTA/0.1 %Triton X-100/1 mM DTT (dithiothreitol)] and frozen at −20 °C. The cells were thawed, sonicated for 3 min and the cell debris removed by centrifugation (9000 ***g***, 30 min). The resulting crude cell extract was diluted with buffer to give a protein concentration of 2–3 mg/ml in 50 mM Tris/HCl (pH 7.5)/200 mM NaCl/2 mM EDTA/0.1 % Triton X-100/1 mM DTT and loaded at 0.37 ml/min on to a 1.5 cm × 20 cm column packed with amylose resin equilibrated with column buffer [50 mM Tris/HCl (pH 7.5)/1 mM EDTA/0.1 % Triton X-100/1 mM DTT], containing 200 mM NaCl. The resin was washed with 10–20 column vol. of this buffer and the fusion protein was eluted with 10 mM maltose in the same buffer. The eluant was diluted to decrease the concentration of NaCl to 100 mM and then applied to a second column (1.5 cm × 30 cm, 0.37 ml/min) containing Red Sepharose CL-6B equilibrated with column buffer containing 100 mM NaCl. The resin was washed with 10–20 column vol. of this buffer and the fusion protein was eluted with column buffer containing 1 M NaCl.

Protein determination, SDS/PAGE and Western-blot analysis

Protein concentrations were determined by the method of Schaffner and Weismann [9]. The expression level and purification of MBP–POR was examined by SDS/PAGE, the separated proteins being stained with Coomassie Brilliant Blue [10]. For Western analysis, the separated proteins were electrophoretically transferred on to supported nitrocellulose, which was then blocked with 5 % powdered milk in 150 mM NaCl/10 mM Tris, pH 7.5, and allowed to react with rabbit polyclonal antiserum to either *E. coli* MBP or wheat POR. Immobilized primary antibody was detected with alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antiserum. The immunoreactive bands were subsequently revealed with 5-bromo-4-chloroindol-3-yl phosphate and NitroBlue Tetrazolium [11].

Pigment preparation

Pchlide was purified either from *R. capsulatus* ZY5 [12] or *R. sphaeroides* V3 [13] cultures grown in the dark at 32 °C. Both strains have mutations in subunits of the light-independent protochlorophyllide reductase and accumulate Pchlide. *R. capsulatus* ZY5 was cultured in RCV$^+$ [14] and *R. sphaeroides* V3 was grown in M22$^+$ supplemented with 0.1 % casamino acids [15]. During growth of the cultures, Pchlide released into the media was adsorbed on to polyurethane foam bungs. The pigment was extracted from the bungs with 100 % methanol, diluted 10-fold with 0.1 % triethylamine in water and then loaded on to a Sep-Pak C18 cartridge (Waters chromatography). The cartridge was washed with 50 % methanol/0.1 % triethylamine and the Pchlide was eluted with 100 % methanol. The eluant was diluted with water and the Pchlide extracted into diethyl ether. This was then concentrated by evaporation and stored in the dark at −20 °C until required. Before use, the pigment was dried under nitrogen and redissolved in 100 % methanol.

Activity measurements

The concentrations of the different pigments were determined using the following absorption coefficients in aqueous solution: NADPH, 6.22 $mM^{-1} \cdot cm^{-1}$ at 340 nm; Pchlide, 30.4 $mM^{-1} \cdot cm^{-1}$ at 630 nm [16]; and Chlide, 91.2 $mM^{-1} \cdot cm^{-1}$ at 670 nm [17]. For the initial determination of enzyme activity, MBP–POR was mixed with Pchlide and NADPH in assay buffer [50 mM Tris/HCl (pH 7.5)/0.1 % Triton X-100] at 25 °C. Spectra were recorded using a Shimadzu 2101 double-beam UV–visible spectrophotometer equipped with a thermostatically controlled cell holder. Measurements were recorded pre- and post-illumination by a 60 W tungsten bulb held at a distance of approx. 10 cm from the reaction cuvette.

The kinetic parameters for catalysis by MBP–POR were determined by monitoring the initial rate of Chlide production (change in absorbance at 670 nm) over a range of substrate concentrations during continuous illumination of the sample in assay buffer at 25 °C. A Schott KL1500 electronic cold light source with a blue insert filter and a short-pass interference filter (Ealing 35-5362) provided illumination (approx. 120 $\mu mol \cdot s^{-1} \cdot m^{-2}$) in the Soret region of the Pchlide absorption spectrum. A red cut-on filter (Schott RG 610) which blocks transmission of light below 600 nm was used to protect the photomultiplier detector from the actinic light. The apparent K_m and V_{max} values (under these light conditions) were obtained by

fitting the initial reaction rate against the concentrations of each substrate to the following equation:

$$\frac{v}{[E_t]} = \frac{V_{max}[A][B]}{[A][B] + K_{mA}[B] + K_{mB}[A] + K_{AB}} \quad (1)$$

where $v/[E_t]$ is the specific initial rate (the rate/mg of protein), [A] and [B] are the substrate concentrations, V_{max} is the initial rate achieved as both [A] and [B] approach ∞, K_{mA} is the value of [A] giving $V_{max}/2$ when [B] approaches ∞ and K_{mB} is the value of [B] giving $V_{max}/2$ when [A] approaches ∞. Data were fitted using the Sigma Plot program (Jandel Scientific).

Gel-filtration chromatography

Size-exclusion gel-filtration chromatography was carried out at 20 °C on a pre-packed Superose 6 HR column (1 cm × 30 cm) equilibrated with column buffer containing 100 mM NaCl. The column was calibrated with the following molecular-mass markers (Sigma): 2 mg/ml horse heart cytochrome *c* (12.4 kDa), 3 mg/ml bovine erythrocyte carbonic anhydrase (29 kDa), 10 mg/ml BSA (66 kDa), 5 mg/ml yeast alcohol dehydrogenase (150 kDa) and 3 mg/ml sweet-potato β-amylase (200 kDa). To determine the molecular mass of MBP–POR in solution, 200 μl aliquots of purified fusion protein (1.2 mg/ml) were loaded on to the column in the presence or absence of 100 μM NADPH and 10 μM Pchlide (before or after illumination for 2 min). Protein and enzyme assays were performed on the peaks which eluted from the column. In addition, spectra were recorded between 600 and 700 nm to determine where Pchlide and Chlide were eluted.

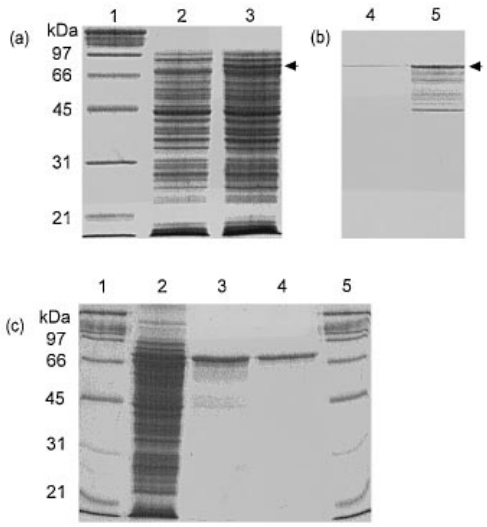

Figure 1 SDS/PAGE and Western-blot analysis of MBP–POR expression and purification

(**a**) Coomassie Blue-stained gel; (**b**) Western immunoblot using anti-MBP antiserum. Lane 1, molecular-mass standards (myosin, 200 kDa; β-galactosidase, 116 kDa; phosphorylase *b*, 97.4 kDa; BSA, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa and aprotinin, 6.5 kDa); lanes 2 and 4, *E. coli* lysate before induction; and lanes 3 and 5, lysate from cells harvested 2 h after induction with IPTG; (**c**) Coomassie Blue-stained gel. Lanes 1 and 5, molecular-mass standards (as above); lane 2, crude cell extract (60 μg of protein); lane 3, protein eluted from the amylose column (15 μg of protein); and lane 4, protein eluted from the Red Sepharose column (5 μg of protein).

RESULTS

Expression and purification of MBP–POR

The pMAL-c2 expression vector was used to express mature pea POR as a fusion with MBP in *E. coli*. The level of expression of MBP–POR in *E. coli* was monitored by examining a Coomassie Blue-stained SDS/polyacrylamide gel of soluble cell extracts taken before and after induction with 0.3 mM IPTG (Figure 1a). After IPTG induction, a band with a molecular mass of approx. 80 kDa appeared. This corresponded to the MBP–POR fusion, which has a predicted molecular mass of 78.8 kDa. The identity of this band was confirmed by Western-blot analysis using both the anti-MBP antiserum (Figure 1b) and the anti-(wheat POR) antiserum (results not shown). The Western immunoblot also revealed that MBP–POR is partially degraded. The use of a protease-deficient (lon^-) host strain *E. coli* PR745, and limiting the time for expression to 2 h reduced the level of proteolysis. MBP–POR was routinely expressed at high levels, and the full-length fusion was estimated to constitute 5–10 % of the total soluble cell protein.

The recombinant MBP–POR was purified from soluble *E. coli* extracts by a two-step affinity-chromatographic procedure. The first step involved the use of an amylose resin column which purified the protein of the basis of affinity of the MBP portion of the fusion for the resin [18]. MBP–POR was eluted from this column with 10 mM maltose. The second chromatographic step involved the use of a Red Sepharose CL-6B column, which has been shown to bind NADP(H)-utilizing enzymes [19]. The yield and level of purification at each step for a typical MBP–POR preparation is shown in Table 1. From 2 litres of induced culture, the total yield of MBP–POR after the two chromatography steps was 8 mg, representing a 22-fold purification. The fusion was estimated to be at least 90 % pure by SDS/PAGE (Figure 1c). Routinely 4–5 mg of purified fusion protein were obtained from a 1-litre culture. Purified MBP–POR was stored in elution buffer at 4 °C and retained full activity for many weeks.

MBP–POR catalyses the light-dependent reduction of Pchlide

Crude extracts from induced cells were assayed for POR activity. The extract was shown to catalyse the reduction of Pchlide to Chlide, but only upon illumination. Figure 2(a) shows the absorption spectra of solutions of MBP–POR in the presence of Pchlide and NADPH before and after illumination. In the dark there is an absorbance peak at 630 nm that corresponds to Pchlide. Upon illumination this peak diminishes and a peak appears which has a maximum at 670 nm and corresponds to Chlide. This result indicates that heterologously expressed MBP–POR is active.

To determine the kinetic parameters for the MBP–POR fusion protein it was necessary to measure the initial rate of Chlide production for a range of NADPH and Pchlide concentrations. This was accomplished by continuously illuminating the enzyme reaction mix within the spectrophotometer using a light source fitted with filters to provide light at 390–460 nm coinciding with the Soret region of the Pchlide absorption spectrum. Figure 2(b) shows a typical time course for Chlide production. The rate of Chlide production was calculated from the increase in absorbance at 670 nm with time. Under conditions of continuous product measurement the enzyme was shown to perform multiple turnovers and the dependence of the initial rate on substrate concentration followed Michaelis–Menten kinetics. These results are shown graphically in Figure 3. The V_{max} was calculated to be 20.6 ± 0.9 nmol·min^{-1}·mg^{-1} (which is equivalent to a catalytic rate constant of 0.027 s^{-1} assuming the enzyme preparation to be 100 % pure and active), $K_m^{Pchlide}$ was 0.27 ± 0.04 μM, K_m^{NADPH} was 8.67 ± 1.86 μM, and K_{AB} was 5.14 ± 1.38.

Table 1 Purification of MBP–POR from *E. coli* PR745 (pMAL–POR)

POR activity was determined spectrophotometrically at each of the purification stages. The specific activity is given in arbitrary units based on ΔA_{670}/min. The data shown are for the purification of MBP–POR from a 2-litre culture.

Purification step	Total protein (mg)	Total activity (units/min)	Specific activity (units/min per mg)	Yield (%)	Purification factor (fold)
Crude extract	331	24408	74	100	1
Amylose chromatography	45	10710	234	44	3
Red Sepharose chromatography	8	13041	1630	53	22

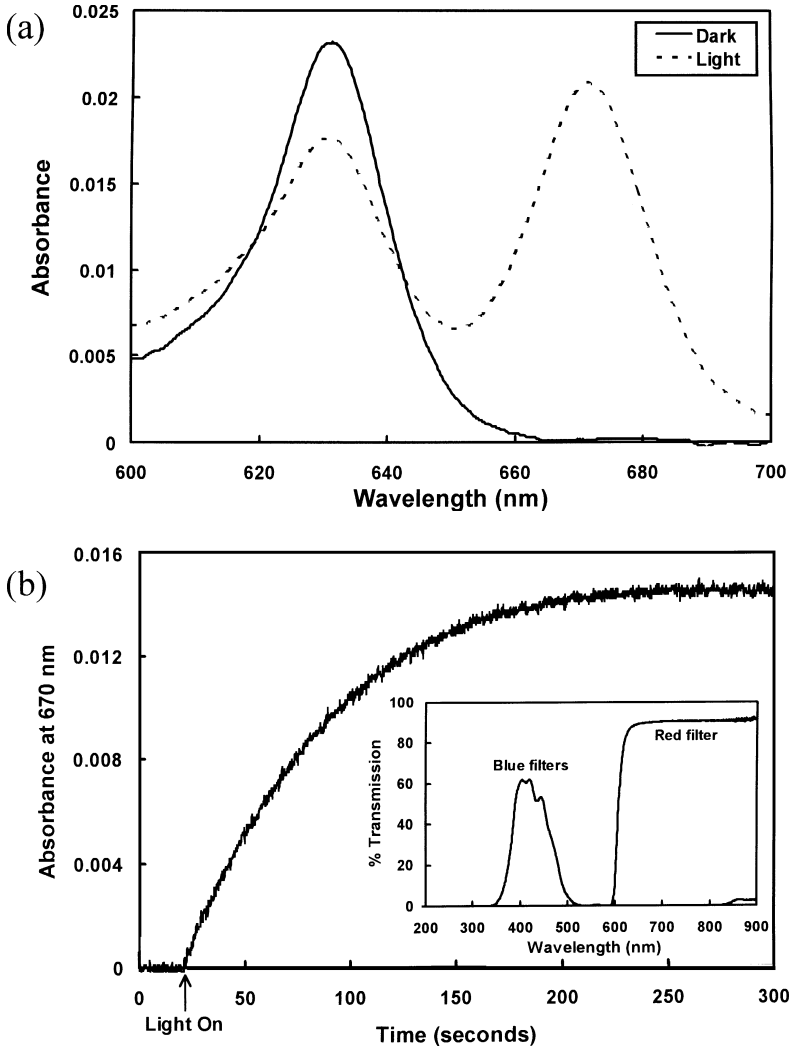

Figure 2 MBP–POR catalyses the light-dependent reduction of Pchlide

(**a**) A sample of crude extract was incubated in the dark with Pchlide (3.7 μM) and NADPH (160 μM) in assay buffer at 25 °C. Absorption spectra shown were taken before (——, dark) and after (– – – –, light) illumination by a 60 W tungsten bulb held at a distance of approx. 10 cm from the reaction cuvette for 2 min. The absorbance peak at 630 nm corresponds to Pchlide and that at 670 nm to Chlide. (**b**) A typical time course for light-dependent production of Chlide by MBP–POR. This time course was obtained with purified MBP–POR eluted from the Superose 6 gel-filtration column in column buffer containing 100 mM NaCl. An aliquot of the eluant (25 μl) was equilibrated with Pchlide (10 μM) and NADPH (100 μM) in a total volume of 1 ml prior to illumination. Inset are the transmission spectra of the blue filters used with the cold light source to provide illumination in the Soret region of the absorption spectrum of Pchlide and the red filter used to protect the photomultiplier detector of the spectrophotometer from actinic light.

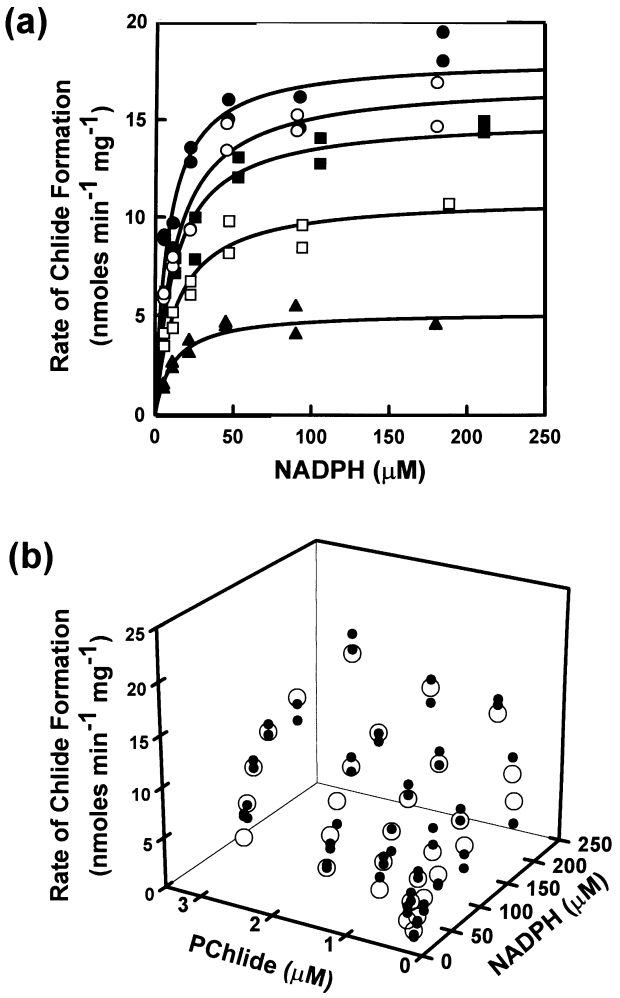

Figure 3 Graphical representation of the data used to determine the kinetic parameters

(**a**) Shows the dependence of the initial rate of Chlide production on the concentration of NADPH at a series of fixed concentrations of Pchlide: 0.15 μM (▲), 0.24 μM (□), 0.61 μM (■), 1.3 μM (○) and 2.42 μM (●). (**b**) Shows a three-dimensional plot of the same data (denoted by ●). All the points were fitted to eqn. (1) (see the Materials and methods section) by non-linear regression analysis. ○ Represent the best-fit values for each pair of substrate concentrations.

Determination of the oligomerization state of MBP–POR

The molecular mass of the native recombinant MBP–POR was evaluated by size-exclusion chromatography on a Superose 6 gel-filtration column. Figure 4 shows the elution profile of the fusion protein which had been illuminated in the presence of NADPH and Pchlide (to produce $NADP^+$ and Chlide) prior to loading on to the column. The protein (and POR activity) eluted from the column with a peak at 14.55 ± 0.1 ml (average of three results) in the presence or absence of substrates or products. Using this value, the molecular mass was calculated to be 155 ± 11 kDa. As the predicted molecular mass of the fusion is 78.8 kDa (confirmed by SDS/PAGE) these results suggest that MBP–POR behaves as a dimer in solution. The MBP–POR peak was slightly asymmetric, which may be due to the presence of a small amount of degraded fusion protein or possibly a small population of monomer. In the presence of enzyme, the coenzyme was eluted with a peak at 18.65 ml and the other pigments (Pchlide and Chlide) were eluted between 24 and 29 ml. Pchlide and Chlide were absent from the protein and coenzyme peaks, indicating that the column succesfully separated the substrates and products from the enzyme.

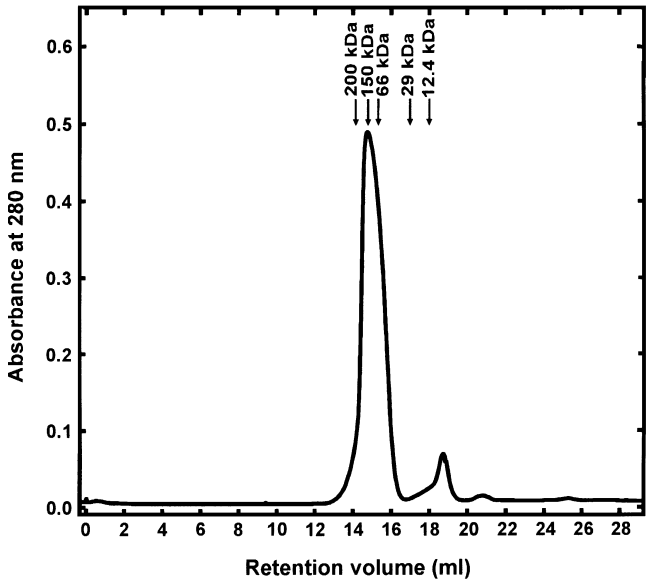

Figure 4 Gel-filtration chromatography of purified MBP–POR

MBP–POR (1.2 mg/ml) was loaded on to a Superose 6 gel-filtration column and eluted at a rate of 0.4 ml/min. Prior to loading on the column NADPH and Pchlide were added to final concentrations of 100 μM and 10 μM respectively, and the sample was illuminated. Arrows indicate the elution positions of proteins used to calibrate the column (see the Materials and methods section).

DISCUSSION

In order to begin to analyse the kinetic properties of POR, it was necessary to obtain large quantities of the enzyme from a heterologous expression system. Initial attempts to overproduce the enzyme in *E. coli* using the expression plasmid pKK233-2 (Pharmacia) were unsuccessful due to low expression levels and rapid degradation of the protein (H. M. Wilks and M. P. Timko, unpublished work). We therefore decided to express the enzyme as a fusion, in order to try to stabilize the enzyme. Using the pMAL-c2 expression vector, a fusion of MBP and POR was produced at up to 10% of the total soluble *E. coli* cell protein. The fusion was soluble, active and could be purified by affinity chromatography.

The MBP–POR fusion was purified to near homogeneity by a two-step affinity purification procedure. The first step involved affinity chromatography on amylose resin, to which the MBP portion of the fusion binds [18]. After purification on this column, the major band observed on a Coomassie Blue-stained gel is the approx. 80 kDa band corresponding to the fusion protein. In addition there are also a number of smaller bands which are immunoreactive with anti-MBP antiserum, suggesting that these are degradation products. Since the protein retains the ability to bind amylose resin, these smaller proteins are likely to be degraded at the C-terminal end of the protein within the POR portion. Previously POR has been shown to utilize NADPH (and not NADH) as coenzyme for the reduction of Pchlide [17]. Chromatography matrices carrying triazine dyes have been shown to have affinity for dinucleotide-binding proteins, and

investigation of a number of resins revealed that MBP–POR bound strongly to Red Sepharose CL-6B, a resin to which several NADP(H)-utilizing enzymes have been shown to bind [19]. After elution from this column, the fusion was essentially pure, with less than 10% contamination with degradation products (which were immunoreactive with both anti-MBP antiserum and anti-POR antiserum).

One of the benefits of expressing POR as a fusion with MBP is that the enzyme is soluble, whereas in plants it is normally associated with the plastid membranes. This has facilitated the purification and determination of the kinetic parameters for the reaction. Previously, the $K_m^{Pchlide}$ was determined to be 0.103 and 0.108 μM for dark and illuminated barley etioplast preparations respectively [20]. Using barley etioplast membrane preparations, Griffiths determined the $K_M^{Pchlide}$ as 0.46 μM and the K_m^{NADPH} as 35 μM [1]. The $K_m^{Pchlide}$ obtained for MBP–POR (0.27 μM) is comparable with these values. The K_m^{NADPH} obtained for the POR from etioplast membranes is considerably higher than the value obtained for MBP–POR (8.67 μM), and this may be accounted for by the presence of other NADPH-utilizing enzymes in the crude membrane preparations. In addition, differences in the kinetic parameters may be due to the differences between the pea and barley enzymes or differences in the conditions under which the kinetic parameters were determined. Likewise the kinetic properties of MBP–POR are similar to those recently determined for the enzyme from the alga *Scenedesmus obliquus* [21]. The K_m values were 4.2 μM for NADPH and 0.19 μM for Pchlide. However, it should be noted that these values were obtained at sub-saturating concentrations of the other substrate in both cases, and that the kinetic measurements were determined for a cell-free crude homogenate and not a purified enzyme preparation as in the present study. These comparisons reveal that the properties of MBP–POR are similar to those of native plant and algal enzymes and are likely to be comparable with native pea POR, although the possibility that the presence of MBP may affect the kinetic properties cannot be excluded. Therefore the MBP–POR fusion protein is an excellent model system for analysing the kinetics of POR and has allowed the kinetic properties of the enzyme from a dicotyledenous plant to be determined for the first time. More detailed kinetic studies are now necessary to determine the mechanism of catalysis.

On the bais of sequence comparisons, POR has previously been shown to be a member of the family of short-chain dehydrogenase or RED (reductases/epimerases/dehydrogenases) enzymes [7,22]. The enzymes in this family are all single-domain dinucleotide-binding oxidoreductases. They are generally dimers or tetramers, with the tetrameric forms being essentially dimers of dimers [23]. The results of the size-exclusion chromatography on Superose 6 HR indicate that native MBP–POR exists predominantly as a dimer. MBP exists naturally as a monomer [24], and so the fact that the fusion is a dimer is due to the properties of POR. This direct determination of the oligomerization state is supported by previous cross-linking studies, which have shown that POR from wheat is present in prolamellar bodies as aggregates and the fundamental aggregated unit is a dimer [25]. From X-ray structural analysis of several RED proteins, the dimer interface has been identified as two long conserved parallel helices (α4 and α5) [23] that correspond to residues 190–208 and 271–292 in pea POR. The N-terminus of POR is likely to be exposed on the surface of the protein, away from the dimerization interface, in order that proteolytic processing of the enzyme on import into plastids may occur. Therefore attachment of the C-terminus of the MBP to the N-terminus of POR via a flexible linker region does not prevent dimerization, despite the fact that MBP is a large globular protein (40 kDa). The fact that MBP–POR is a dimer, in addition to being catalytically active, suggests that it is a good model for analysing structure–function relationships in POR.

In whole leaves [26] and isolated etioplast membranes [17] several different spectroscopic forms of Pchlide have been observed. Forms which absorb maximally at approx. 637 and 650 nm and have a fluorescence peak at 655 nm have been shown to be immediately phototransformable and have been designated as 'photoactive'. These forms of Pchlide were believed to be in ternary complexes with POR and NADPH, whereas 'non-active' Pchlide, which absorbs and fluoresces maximally at 630 nm, was thought to be free pigment. In contrast, the fluorescence spectra of Pchlide in the presence of NADPH and *in vitro*-translated barley POR [27] or isolated, solubilized wheat POR [28] lack the longer-wavelength peak associated with 'photoactive' Pchlide. In addition, a pea mutant (Lip 1) which shows light-independent photomorphogenesis due to the lack of phytochrome I [29] also lacks the longer-wavelength forms of Pchlide, but contains the 630 nm form [30]. However, in all these cases, the short-wavelength form of Pchlide can be phototransformed. Similarly, the absorption maximum of Pchlide occurs at 630 nm in the presence of MBP–POR and NADPH, and the pigment is readily phototransformable. The etioplasts of Lip 1 plants are characterized by their small number and size of prolamellar bodies. Taken together, these results suggest that the spectral properties of the long-wavelength forms of Pchlide observed in plant preparations may be due to the association and aggregation of pigment-POR complexes on the prolamellar bodies, and where these are not present, such as in our assays or in the Lip 1 mutant plants, the form absorbing at 630 nm predominates.

Previously it has been reported that Chlide remains tightly bound to the enzyme after catalysis [31] and it has been proposed that the enzyme is a 'suicide' enzyme [32]. The results of the size-exclusion-chromatography experiments reported here demonstrate that neither Pchlide nor Chlide is tightly bound to the fusion protein and both are able to diffuse from it freely. In addition, it does not appear that the enzyme is inactivated during catalysis, as it is capable of carrying out multiple turnovers and also remains active once substrates and products have been removed by size-exclusion chromatography. The experiments also show that the enzyme is active in the presence of NADPH, protochlorophyllide and light and that neither a flavin nor ATP is required for the catalytic reaction, contrary to previous reports of their involvement [33–35].

In conclusion, we have succeeded in overexpressing pea POR as a fusion with MBP in *E. coli*. The fusion protein is soluble and can be purified by a simple two-step purification procedure using affinity chromatography. The purified protein catalyses the light-dependent reduction of Pchlide to Chlide in a similar manner to the native plant enzyme, and the kinetics of the reaction can now be analysed in detail. As a result of these properties the MBP–POR fusion will be invaluable for detailed mechanistic and structural studies of this unique and very important enzyme.

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