A reappraisal of the mechanism of the photoenzyme protochlorophyllide reductase based on studies with the heterologously expressed protein

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Abstract It is widely believed that protochlorophyllide reductase is a flavoenzyme effecting catalysis by a radical mechanism. Here the cyanobacterial reductase has been isolated from *Escherichia coli* overexpressing the Synechocystis gene. The purified enzyme, while retaining full activity, has no detectable flavine. No radical derived ESR signal was observed during catalysis or on photoexcitation under non-catalytic conditions. Mechanistic implications of the findings are discussed.

Key words: Protochlorophyllide reductase; Photoenzyme mechanism; Radical intermediate; Flavoenzyme

1. Introduction

The role of chlorophyll *a* in harvesting and storage of the energy of sunlight during oxygenic photosynthesis in plants is fundamental to life on earth. Chlorophyll *a* formation in flowering plants is obligatorily dependent on light to satisfy the key enzyme in its biosynthesis, viz. protochlorophyllide reductase (EC 1.3.1.33) (*por*). This catalyses the NADPH and light dependent reduction of protochlorophyllide to chlorophyllide. In chlorophyll *a* containing non-flowering plants the role of light in biosynthesis of this pigment is less clear due to the co-presence in such organisms of a second light independent mechanism of protochlorophyllide reduction catalysed by the products of at least three genes, *Chl*, *BL* and *N*. Whereas the dark system is completely uncharacterised the light enzyme has been extensively studied for many years. This is undoubtedly due to the fact that very high levels of this enzyme occur in dark grown plants. The biochemical properties of the enzyme have been studied in such material and also in enzyme enriched fractions derived from etiolated leaf tissue [1]. These have been useful in devising schemes for the overall macromolecular events associated with photoconversion but have not led to any detailed molecular mechanism of the actual photoreduction. A spectral entity, X690, has been identified as an established intermediate in the photoreduction but its chemical structure has still to be established [2]. Separate from this the involvement of flavine as hydrogen carrier in the process was suggested from the co-purification of FAD and the enzyme from higher plant extracts and the demonstration of an inhibition of the enzyme in vitro by quinacrine, a flavine inhibitor [3]. A chemical model system for the enzyme has been synthesised based upon a flavine-protochlorophyllide complex [4,5]. Illumination of this results in reductant transfer from the photoexcited flavine to the protochlorophyllide in a radical mediated process. Furthermore a photoinduced ESR signal ($g = -2.0021$), stable at low temperature, has been detected in etiolated wheat leaves and putatively ascribed to the photoreduction process [6,7]. Such data, despite their preliminary nature and the possibility of artefacts inherent in the analysis of heterogeneous biological material, have nevertheless been widely accepted as implying a flavine involvement and a radical mechanism for *por* catalysis. In the present paper such conclusions have been re-examined using purified samples of the heterologously expressed protein. The findings have forced a complete reappraisal of earlier ideas about the mechanism of *por*. While this work was in progress an account appeared of expression of the pea reductase as an enzymically active mbp fusion [8].

2. Materials and methods

2.1. Chemicals

Standard sources were used for routine chemicals which were of the highest purity grade available. Protochlorophyllide for assay of *mbp-por* was extracted from the *Rb. sphaeroides* V3 mutant as described previously [9].

2.2. Expression and purification of the Synechocystis-mbp-por-maltose binding protein fusion

The DNA sequence encoding the Synechocystis *por* [10] was amplified by PCR from a plasmid harbouring the gene kindly provided by Dr. Carl Bauer (Indiana University, Bloomington, IN, USA). The amplifying primers introduced a 5′ *Eco*RI site and a 3′ stop codon and *Bam*HI site to facilitate subsequent cloning. The resulting purified fragment was ligated into the 'TA cloning vector' *p*CR2.1 (Invitrogen) and transformed into INV&F[1] 'One Shot' Competent cells (Invitrogen) from which the amplified plasmid was recovered by standard techniques [11]. The gene was excised by restriction digestion and recloned into the expression vector *p*MAL-c2 (New England Biolabs) and transformed into *Escherichia coli* Blue. Expression of the enzyme as a mbp fusion was induced at 37°C by added IPTG (0.3 μM) and after 3 h the cells, from typically 1 l of culture, were harvested by centrifugation. After washing once these were resuspended in 20 mM Tris pH 8.6, immediately frozen as aliquots in liquid N_2 and stored at −80°C.

For enzyme isolation the stored cells were thawed in cold running water and sonicated on ice (Vetrasonics Ltd., 2.0 cm diameter probe) for 5×30 s on power setting 10. The soluble fraction containing >90% of the cell *mbp-por* fusion, was recovered by centrifugation of the sonicate at 10 000×*g* for 15 min. Anion exchange chromatography was performed on DEAE Sepharose Fast Flow (Pharmacia Biotech) at pH 8.6. The column (2.5×10 cm) was eluted with a linear salt gradient from 0–0.5 M NaCl. Fractions containing the enzyme fusion, identified by SDS-PAGE and activity assays (see below), eluted between 0.23 and 0.26 M NaCl were bulked. Subsequent purification was carried out by affinity chromatography on immobilised amylase resin (New England Biolabs). The enzyme was eluted with buffer containing 10 mM maltose as described in the manufacturer's protocol. Fractions containing the enzyme were finally bulked and stored as aliquots at −80°C.

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Abbreviations: *mbp-por*, maltose binding protein fusion of protochlorophyllide oxidoreductase; IPTG, isopropyl-β-D-galactoside

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2.3. Analytical methods

2.3.1. SDS-PAGE. Analysis was carried out on 10% polyacrylamide gels and proteins detected by staining with Coomassie brilliant blue [12].

2.3.2. Enzyme assay. *mbp-por* activity was measured spectroscopically as flash induced chlorophyllide formation in the presence of substrates as previously described [13].

2.3.3. Protein assay. Protein assay was carried out by the method of Bradford [14] using BSA as standard.

2.3.4. UV-VIS spectroscopy. Spectra were recorded on a Unicam UV-2 spectrophotometer.

2.3.5. ESR spectroscopy. Spectra were recorded at room temperature in Bristol (Bruker ESP300E) and at reduced temperatures in liquid helium at UCL, London (Joel RE1X EPR spectrometer equipped with an Oxford Instruments liquid He cryostat). The sample could be illuminated in situ during the recording of spectra.

3. Results

Progress in the purification of *mbp-por* fusion from the soluble fraction of *E. coli* cells overexpressing the Synechocystis gene are shown in Fig. 1. The enzyme fusion is seen as a major component, migrating slightly slower than the 66 kDa marker amongst the soluble proteins of the cells (lane 2). During anion exchange chromatography on DEAE-Sepharose the enzyme appears in fractions eluted between 0.23 and 0.26 M NaCl. On bulking and SDS-PAGE analysis this fraction is seen to be highly enriched in the enzyme (Fig. 1, lane 3) but accompanied by traces of other proteins notably at 47 and 53 kDa. Affinity purification on maltose resin and elution with 10 mM maltose yields a sample consisting of $>95\%$ of the fusion protein with only a trace of a faster running contaminant (Fig. 1, lane 4). The identity of this peptide as the reductase fusion in the various fractions has been confirmed by western blotting using antibodies against both the maltose binding protein (New England Biolabs) and Synechocystis *mbp-por* (H. Townley, unpublished data).

mbp-por activity assays on the various fractions representing stages in the purification confirm this conclusion. In the original sonicate the specific activity of the enzyme was 0.06 units/mg protein (1 unit representing the formation of 1 nmol of product/min under our standard assay condition) [13]. This increased to 11.2 and 8.90 units/mg protein for the anion exchange and affinity purified fractions respectively confirming enrichment of the enzyme. The slightly lowered specific activity value of the highly purified final sample (Fig, 1, lane 4) is difficult to explain but may reflect some slight inactivation/denaturation of the enzyme during the final purification step.

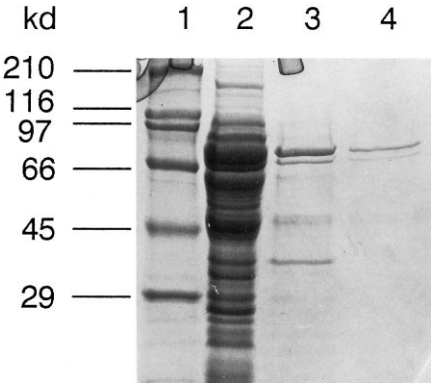

Fig. 1. Stages in the purification of the Synechocystis protochlorophyllide reductase maltose binding protein fusion (*mbp-por*). Fractions were separated on 10% (w/v) polyacrylamide gels and proteins detected by Coomassie blue staining. Lanes: 1, molecular weight markers; 2, total soluble proteins (17.9 μg); 3, bulked reductase containing fractions from DEAE column (3 μg); 4, bulked affinity chromatography fractions (2.28 μg).

The absorption spectrum of the purified material between 250 and 500 mm is shown in Fig. 2. Recording at lower wavelength is difficult due to interference by buffer and salt absorbance. The spectrum shows characteristic protein aromatic residues absorption at approx. 280 nm (Fig. 2) but more significantly shows no absorption in the visible part of the spectrum which might indicate the presence of any redox cofactors. This is further confirmed by re-recording of the spectrum after addition of solid sodium dithionite as reductant (Fig. 2). This shows a non-specific increase in absorbance below 250 nm due to the reagent. Apart form this chemical reduction results in no discernible specific change in absorption in the visible even when the spectra are recorded at a fivefold increase in sensitivity (Fig. 2).

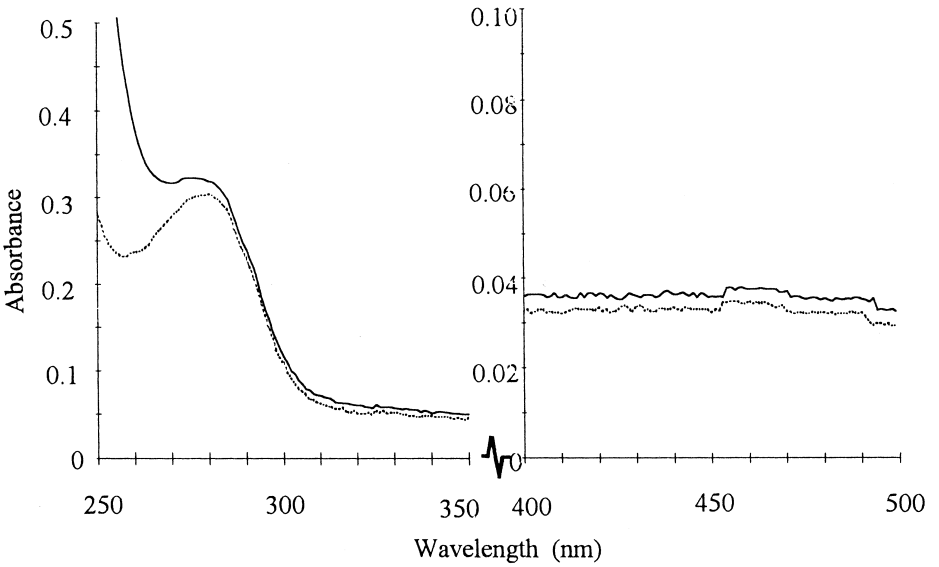

Fig. 2. UV-VIS absorption spectra of purified *mbp-por*. Spectra were recorded before (dotted line) and after (solid line) addition of sodium dithionite. Note change in absorbance scale for recording of the visible part of the spectrum.

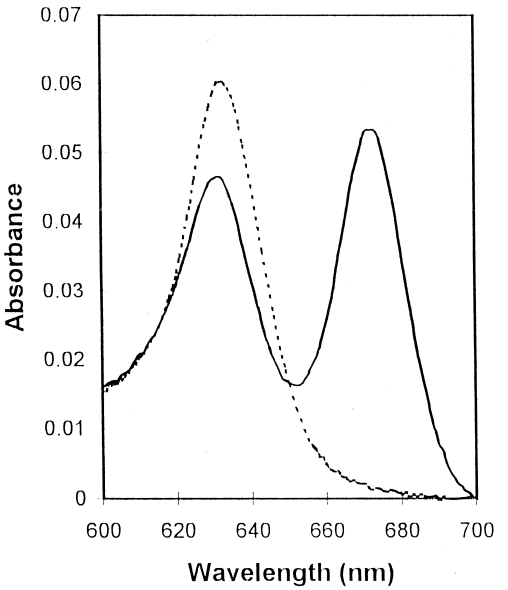

Fig. 3. Catalytic activity of the purified reductase. The purified fusion protein (50 μg/3 ml buffer) was supplemented with protochlorophyllide and NADPH and spectra recorded before (dotted line) and after (solid line) illumination as described in the text.

Fig. 3 illustrates qualitatively the enzymatic activity of the protein as determined by routine spectroscopy. For these spectra the sample (50 μg of protein in 3.0 ml) was supplemented with 0.5 mM NADPH and 2 μM protochlorophyllide and the mixture incubated in darkness for 20 min. The spectrum was then recorded followed by exposure to a 60 W lamp for approx. 2 min at a distance of 10 cm and finally re-recording of the spectrum. Light dependent turnover of the enzyme is obvious from the observed increase in absorbance at 672 nm on illumination indicating the formation of chlorophyllide (Fig. 3). Aliquots of the dark incubated material were also transferred, in darkness, into 4 mm diameter ESR sample tubes and spectra recorded at room temperature (298 K) and at reduced temperatures of 11, 66 and 100 K. ESR spectra were recorded both before and during illumination under these conditions. The purity of the material allowed spectra to be recorded at maximum gain. Despite this no signal indicating radical formation was evident in any of the spectra.

4. Discussion

Several aspects of the properties of the enzyme *por* have been profitably studied over the years but as yet very little progress has been made in elucidating the enzymes' unique mechanism of catalysis. The main reason for this has undoubtedly been the lack of an effective purification procedure for the membrane associated enzyme. Mechanistic studies to date have therefore been carried out on incompletely purified material from etiolated plants such as fractionated detergent extracts or membrane preparations enriched in the enzyme. From such studies the enzyme has emerged as a flavoprotein catalysing the reaction via free radical intermediate(s) [3,6,7]. Furthermore, a flavine-porphyrin adduct has been synthesised as a model for the reductase [5]. Illumination of this results in hydrogen transfer form the reduced flavine to the porphyrin via a sequential electron transfer proton transfer mechanism involving radical intermediates supporting the generally held view about the enzyme. Work described here on a highly purified and active soluble recombinant form of the enzyme suggest that such a view is no longer tenable.

The cyanobacterial *por* [10] has been expressed as the mbp fusion in *E. coli* (H. Townley, C.E. Bauer and W.T. Griffiths, in preparation). Sonication of the cells followed by SDS-PAGE reveals the fusion as constituting approx. 15% of the total soluble cellular protein (Fig. 1) and the extract on assay is seen to be enzymatically very active. By a combination of anion exchange and affinity chromatography of this extract a highly purified and active form of the reductase has been recovered (Fig. 1). In fact the mutant enzyme from the anion exchange purification step with a specific activity of 11.2 units/mg protein represents the most active preparation of the reductase ever recorded. This figure compares with a value of 3.53 for the enzyme in wheat membrane preparations and only 0.5 in homogenates of cyanobacterial cells [15]. The results of studies on this material form the basis of this report.

What distinguishes this product from earlier samples of the native enzyme purified form etiolated plants is its soluble nature and the complete absence of any contaminating natural pigments such as carotenoids, etc. The purified material in buffered solution appears completely colourless and spectroscopically shows a single absorption band at approx. 280 nm typical of tyrosine and tryptophan chromophores (Fig. 2). Assuming an average value [16] of 1.0 for the A_{280} 1 mg/ml for the protein (mol. wt 78 000 – containing 21 tyrosines and 12 tryptophans) the A_{280} of the sample (0.3) equates with a protein concentration of approx 3.8 μM. The absence of any specific absorbance in the 400–500 nm region of the spectrum, even when recorded at a fivefold greater sensitivity (Fig. 2), suggests the absence of flavine from the material as oxidised flavine absorbs in this spectral region. Furthermore, addition of dithionite (Fig. 2) produced no change in absorbance in this region. Rather, a slight non-specific absorbance increase is observed with this reagent attributable to an increase in scattering. An equimolar ratio of FAD/protein should under such treatment have resulted in a bleaching at approximately 450 nm of 0.03. Clearly no such change is observed (Fig. 2) indicating the absence of FAD. On pretreatment of the original sample with the oxidant, $K_3Fe(CN)_6$, followed by a removal of excess reagent by gel filtration, the recovered material again showed no absorbance decrease in the 400–500 nm region on subsequent reduction with dithionite (data not presented). This qualitatively indicates the unlikelihood of the presence of any reduced flavine in the original sample.

Despite the obvious absence of flavin this material is very active in photoreduction of added protochlorophyllide in the presence of NADPH (Fig. 3). Illumination at room temperature results in a decrease of absorbance at 630 nm due to substrate disappearance. This is accompanied by an absorbance increase at 672 nm indicating that production of chlorophyllide. This catalysis by the Synechocystis reductase, like that of the higher plant enzyme [3], is however sensitive to inhibition by the flavine inhibitor quinacrine (H. Townley and W.T. Griffiths, unpublished). The basis for such inhibition is currently unexplained. The ESR spectra of the substrate supplemented material were recorded in darkness and during illumination in the spectrometer over a range of temperatures but in no case was there any evidence of a free radical signal observed. While the enzyme is obviously turning over at room temperature (Fig. 3) the failure to detect radicals at this tem-

perature may perhaps be not unexpected in view of their transient nature under these conditions. Attempts at stabilisation of such transients and increasing the likelihood of their detection, by illumination at low temperatures – when chlorophyllide formation is blocked – again, repeatedly failed to generate any ESR signal. The failure to detect a radical signal makes it unlikely that the triplet state is involved in the process. This would be in keeping with the well established ultra-fast rates of the early events in this process (see [1]).

In summary, the data presented here suggest that *por* is not a flavoprotein and that catalysis by the enzyme is unlikely to involve radical intermediates.

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