Cloning, characterization and import studies on protochlorophyllide reductase from wheat (*Triticum aestivum*)

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A full-length protochlorophyllide reductase clone (pWR5) has been isolated from a dark-grown wheat (*Triticum aestivum*) cDNA library generated in the phage vector λgt10. Comparison of the sequence of pWR5 with published sequences indicates a high degree of conservation of the structure of the mature protein amongst species but with the structure of the transit peptide less highly conserved. Within the cereals, the structure of the complete preprotein shows a remarkable degree of sequence homology (98% between barley and wheat). *In vitro* expression of pWR5 generates a preprotein of the expected molecular mass, approx. 41 kDa. Isolated pea chloroplasts can import, process and locate the mature reductase to the thylakoid membranes. From analysis of the CNBr-cleavage fragments of the *N*-[^{3}H]phenylmaleimide-treated enzyme, the substrate-protected cysteine group in the enzyme is tentatively identified as Cys-296.

INTRODUCTION

The penultimate reaction of chlorophyll biosynthesis in plants is the reduction of protochlorophyllide to chlorophyllide catalysed by the enzyme protochlorophyllide reductase (Pchlide reductase) (Griffiths, 1978). This is an abundant protein in higher plant etioplasts and functionally is abolutely dependent on light and NADPH and with reactive cysteine residue(s) associated with its active site (Oliver and Griffiths, 1980). Besides utilizing light as a 'substrate' during catalysis, formation and accumulation of the enzyme in plants is also markedly influenced by light in an unusual manner. In the absence of light, high levels of the reductase are found in tissues. On illumination, however, a dramatic reduction in the amount of enzyme is seen (Mapleston and Griffiths, 1980; Santel and Apel, 1981). The reductase is a nuclear gene product synthesized as a precursor protein in the cytoplasm to be finally located as the processed form on the internal membranes of the plastics (Batschauer et al., 1982).

In order to characterize the mechanism of catalysis by the reductase and to understand other events that occur during its import and maturation, it is necessary to determine its structure and to assign roles to domains or individual residues in the molecule. Primary sequence information for the barley and oat enzymes has been deduced from the sequence of corresponding cDNA clones (Schulz et al., 1989; Darrah et al., 1990). Despite this, however, there have as yet been no unambiguous functional assignments to any specific residue. Neither have there to date been any studies reported on the processing and import of the enzyme nor its final targetting to the inner membrane system of the plastids. This contrasts with the extensive data available on the targetting of other chloroplast proteins [reviewed in von Heijne et al. (1989)].

In the present report we describe the isolation of a full-length Pchlide reductase cDNA clone from a wheat λgt10 cDNA library. Expression of the clone *in vitro* generates the precursor of the reductase which is used to investigate the import and processing of the enzyme by isolated pea chloroplasts. Preliminary evidence is also presented on the identity of the substrate-protected cysteine in the reductase.

MATERIALS AND METHODS

Plant material

Wheat (*Triticum aestivum* var. Avalon) seeds were purchased locally (British Seed Houses, Avonmouth, Bristol, U.K.). Seeds were grown on dampened Levington potting compost (Fison, Harston, Cambridge, U.K.) at 25 °C for 6 days in the dark as previously described (Mapleston and Griffiths, 1978). Peas (*Pisum sativum* var. Feltam first) were grown for 10 days under a 12 h photoperiod as described by Highfield and Ellis (1978).

Enzymes and reagents

Oligo(dT)–cellulose was from Collaborative Research Inc. (Lexington, MA, U.S.A.), and restriction enzymes were purchased from Pharmacia LKB Biotechnology Ltd, as were the cDNA synthesis, nested deletion and sequencing kits. The random-primed DNA-labelling kit was from Boehringer Corporation (London) Ltd. Hybond-N filters (0.45 μm), the λgt10 cloning kit, Amplify and all radionucleotides were from Amersham International. CNBr was purchased from Sigma Chemical Company, Poole, Dorset, U.K., and the poly(vinylidene difluoride) blotting membrane, Immobilon-P (0.45 μm), was from Millipore. *N*-[^{3}H]Phenylmaleimide (NPM) was prepared as described by Cava et al. (1961). Stromal-processing protease (SPP), thylakoid-processing protease (TPP) and *Escherichia coli* leader peptidase (LEP) were gifts from Dr. C. Robinson (University of Warwick).

mRNA isolation

Total RNA and poly(A)$^+$ mRNA were prepared from frozen (77 K) etiolated wheat leaves (approx. 40 g) by grinding under N_2 in a sterile mortar, followed by extraction with proteinase

Abbreviations used: NPM, *N*-phenylmaleimide; Pchlide reductase, NADPH: protochlorophyllide reductase; poly(A)$^+$, polyadenylated; OEC, O_2-evolving complex of photosystem II; LEP, *E. coli* leader peptidase; SPP, stromal-processing protease; TPP, thylakoid-processing protease.

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K/phenol and purification by oligo(dT) chromatography using the method described by Apel and Kloppstech (1978).

Construction and screening of a λgt10 cDNA library

cDNA was synthesized from the isolated mRNA template using a Pharmacia LKB cDNA synthesis kit. This was cloned, with *Eco*RI linkers, into λgt10 using an Amersham cloning kit. The library was screened with two fragments (see below) of the p127 oat reductase cDNA clone (Darrah, 1988; Darrah et al., 1990), radiolabelled using the Boehringer random-primed labelling kit. Clone pWR5 was selected on the basis of its strong hybridization to both fragments of p127, and its size of 1.5 kb, and was subcloned into the *Eco*RI site of pBluescript(+). The methods used were as recommended by the kit manufacturers or were as described by Sambrook et al. (1989).

DNA sequence analysis

The pWR5 clone was sequenced from the pBluescript vector primers using Pharmacia LKB T7 sequencing and nested deletion kits and completed using two synthetic oligonucleotide primers (synthesized within this department) complementary to sequences within the clone.

Protein sequence analysis

Sucrose-density-gradient-purified etioplast membranes were labelled with [^{3}H]NPM as described by Oliver and Griffiths (1980). The radiolabelled reductase was purified from these membranes, cleaved with CNBr (Darrah et al., 1990) and analysed by SDS/PAGE (Laemmli, 1970) and fluorography. CNBr-cleavage fragments were purified for sequence analysis by separation on Tris/Tricine SDS/polyacrylamide gels (Schagger and von Jagow, 1987), blotted on to Immobilon-P in 3-cyclohexylamino-1-propane buffer and subsequently stained with Coomassie Blue (Ploug et al., 1989). Pulsed-liquid sequencing was performed on bands excised from the stained blot by Dr. W. Mawby of this department.

In vitro synthesis and chloroplast import analysis

The methods used here were basically as described by Robinson and Ellis (1985), and were carried out at Warwick in collaboration with Dr. Colin Robinson and members of his group. pWR5 was transcribed from the T7 promoter and translated in the presence of [^{35}S]methionine to give radiolabelled reductase precursor. This was tested for its susceptibility to cleavage by crude chloroplast stroma and by purified preparations of SPP, TPP and LEP. The precursor was also incubated with Percoll gradient-purified intact pea chloroplasts for 15 min at 25 °C. These were analysed intact or were fractionated into the stroma and thylakoids and treated with protease as required (see below). All samples were analysed by SDS/PAGE (Laemmli, 1970) and fluorography in Amplify.

RESULTS

The poly(A)$^+$ mRNA (typically 60 μg) isolated from 40 g of etiolated wheat seedlings was used to construct a cDNA library of approx. 120000 independent recombinants in the *Eco*RI site of the phage, λgt10. For selective screening of this library in favour of full-length reductase clones, two purified restriction fragments representing the 5′ and 3′ ends of our original oat reductase clone p127 (Darrah et al., 1990) (Figure 1a) were radiolabelled by random-priming with ^{32}P and used as screening probes. In total, approx. 200 plaques were found to hybridize to these, of which 10 were selected as being at least 750 bp long, on the basis of their very strong hybridization to both probes. This screening strategy therefore resulted in selecting against 90% of the initial positives as representing clones shorter in length than p127.

The selected clones were purified and the inserts subcloned into pBluescript(+) and grown in *E. coli* TG-2. Finally the plasmids were reisolated, cut with *Eco*RI and analysed electrophoretically on agarose gels, and those showing inserts in the 1–1.8 kb size range were selected for further study. After preliminary sequence analysis of the termini of the inserts within these clones, one, designated pWR5, was estimated on the basis of its overlap with the oat clone, p127, to encode the complete amino acid sequence of the precursor of the wheat Pchlide reductase (Schulz et al., 1989; Darrah et al., 1990).

After initial restriction mapping of pWR5, a series of nested deletions was generated by using a combination of exonuclease III (deleting into the 3′ end of the clone) or by enzymic incision between restriction sites common to the clone and the pBluescript multiple cloning site (Figure 1). Cloned overlapping deletions of pWR5 were sequenced by the dideoxy method from priming sites within the pBluescript vector. Sequencing in both directions was completed using two synthetic oligonucleotide primers (Figure 1aii) complementary to sequences in the clone. The generated sequence (Figure 1b) reveals an open reading frame of 1484 bp which entails the complete oat p127 sequence and extends it in the 5′ direction by a further 233 residues to the first in-frame ATG codon, deemed to be the initiation codon of the gene. The sequence translates into a protein of molecular mass 41171 Da with 71 bases of 5′ untranslated sequence and a longer 3′ untranslated segment terminating in a poly(A) tail.

Figure 2 shows the results obtained on specific labelling with [^{3}H]NPM of the substrate-protected cysteine group(s) of Pchlide reductase. Etioplast membranes were used as a source of the enzyme. Of the large number of Coomassie-stained bands in the membrane sample (Figure 2a), the reductase was specifically labelled with [^{3}H]NPM (Figure 2b) as expected from earlier data (Oliver and Griffiths, 1980). The labelled enzyme, after elution from a preparative gel, was cleaved with CNBr, re-electrophoresed and subjected to fluorography (Figure 2c).

As might have been expected from the methionine content of the enzyme (Figure 1), CNBr treatment generated a complex range of Coomassie-staining cleavage fragments with a prominent band at approx. 14 kDa but with several less intense bands covering the complete size spectrum (results not presented). Autoradiography also reveals that the prominent 14 kDa Coomassie-staining fragment is also the most highly radiolabelled (Figure 2c). Radioactivity is also detectable in bands of higher molecular mass, although, interestingly, no labelling of specific bands smaller than 14 kDa is seen.

The CNBr-cleaved enzyme was separated on a Tris/Tricine SDS/polyacrylamide gel, which allowed for greater resolution of the low-molecular-mass fragments, followed by blotting on to an Immobilon-P membrane and Coomassie staining. Finally, the 14 kDa band was excised and sequenced directly on a pulsed-liquid sequencer. Despite bulking the excised strips from five separate tracks representing a total loading of 250 μg of cleaved enzyme, only small quantities of peptide were recovered, rendering sequencing difficult. However, by comparison of the data with those expected from sequencing downstream of the various methionines in the predicted sequence (Figure 1), it has been possible to rationalize the results obtained in terms of two superimposed series of sequencing cycles (Table 1). The most obvious of these generates 22 cycles of sequence compatible with a starting point at Met-271, with a second less-apparent series starting at the adjacent Met-274 (Table 1). The calculated

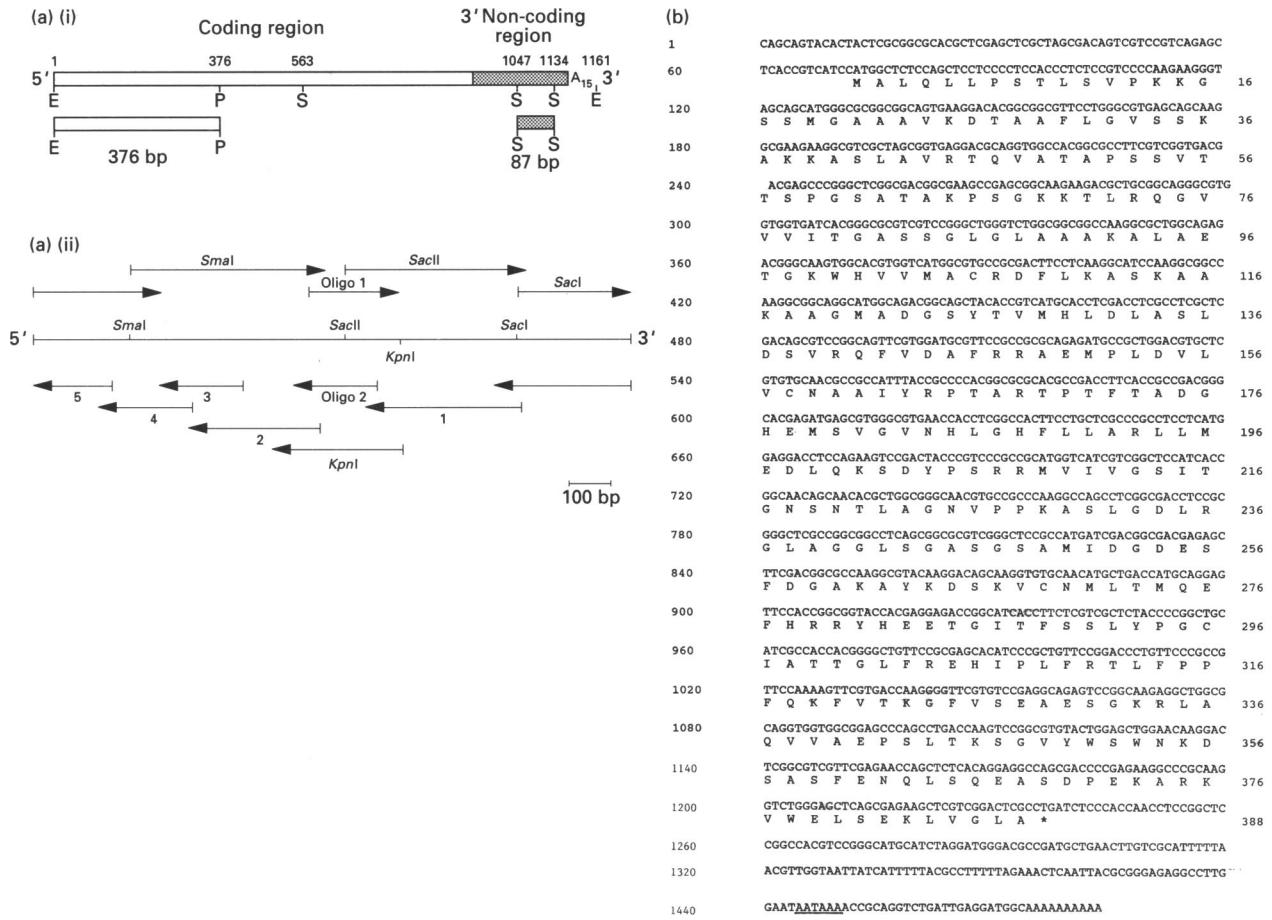

Figure 1 (a) Screening and sequencing strategy for the wheat reductase clone pWR5 and (b) nucleotide and derived amino acid sequence of the pWR5 cDNA clone

(**a**) (i) The 376 bp *Eco*RI–*Pst*I and the 87 bp *Stu*I fragments of p127 that were purified, radiolabelled with ^{32}P and used to screen the cDNA library. E, P and S are *Eco*RI, *Pst*I and *Stu*I restriction sites respectively. (**a**) (ii) The subclones used in sequencing pWR5. The nested deletions were created by either using endonuclease III deleting into the 3′ end of pWR5 (clones 1–5) or excision restriction sites common to pWR5 and the pBluescript multiple cloning site, deleting into both ends of pWR5. The deleted clones were sequenced using the dideoxy method in the direction marked by the arrows, using priming sites within the pBluescript vector. The sequencing was completed using two synthetic oligonucleotide primers (oligos 1 and 2) complementary to sequences in the clone. Oligo 1, 5′-CGCCCGCCTCCTCATGG-3′; oligo 2, 5′-ATGTTGCACACCTTGCT-3′. (**b**) The sequence in the 5′ → 3′ direction and the polyadenylation site is underlined. Numbering of the predicted amino acid sequence is given to the right, beginning with the putative initiation Met at position 1.

molecular mass of each of these fragments is 13287 and 13842 Da respectively and, as such, would have been unresolved by the original SDS/PAGE and account for the complex sequencing data observed.

Having identified pWR5 as coding for the Pchlide reductase precursor (see below), the clone was used in studies to investigate processing and import of the product. pWR5 was transcribed from the pBluescript T7 promoter and translated in the presence of [^{35}S]methionine. Portions of the resulting labelled Pchlide reductase precursor were treated with various preparations of purified processing proteases and finally the mixtures were analysed by SDS/PAGE and autoradiography (Figure 3). Lane 1 shows that, on translation of pWR5 in the absence of any additions, a major labelled band at a position compatible with a molecular mass of approx. 41 kDa is observed corresponding to the *in vitro* synthesized reductase precursor. On subsequent incubation of the labelled precursor with purified LEP or TPP, migration of the labelled band remains unchanged (lanes 5 and 6), suggesting that the precursor is not processed in the thylakoid lumen. Surprisingly, addition of SPP either during (lane 2) or subsequent to (lane 4) the translation also did not result in processing of the precursor. Under identical conditions, incubation of the radiolabelled pre-23 kDa O_2-evolving complex (OEC) protein (28 kDa) (lane 7) with SPP (lane 9) and LEP (lane 10) resulted in generation of intermediates (26 kDa) and mature (23 kDa) forms respectively of this protein, confirming the expected activity of the proteases used, as demonstrated previously (James et al., 1989). Incubation of the reductase precursor with crude stromal extract, however, (lane 3) generated a radiolabelled band of molecular mass approx. 35 kDa, corresponding to the mature reductase, implying that the processing events are carried out by stroma-located enzymes. The same stroma extract also, as expected, processed the pre-23 kDa OEC protein to its intermediate (26 kDa) form (lane 8).

Figure 4 shows the consequence, in terms of processing and import, of incubating the labelled precursor of the reductase with isolated intact pea chloroplasts under different experimental conditions. In the presence of chloroplasts, the radiolabelled band (lane 1) is largely replaced by a band (lane 2) corresponding to the mature reductase, indicating active processing. That this had occurred within the stroma (see Figure 3) after import of the precursor into the chloroplast is confirmed by subsequent treat-

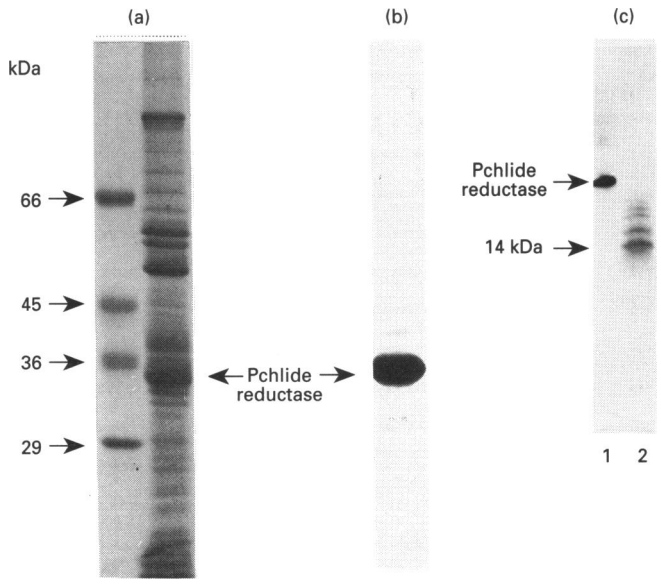

Figure 2 [^{3}H]NPM labelling of Pchlide reductase in membranes and CNBr cleavage of the labelled enzyme

Etioplast inner membranes were isolated on a 20/40% (w/v) sucrose density gradient. The reductase was specifically labelled in the membranes as described by Oliver and Griffiths (1980). Proteins were separated on an 11% polyacrylamide/SDS gel and detected by Coomassie Blue staining (**a**) and fluorography (**b**). In (**c**) a sample of the labelled reductase was purified by elution from a preparative gel and portions were re-electrophoresed on a 16% polyacrylamide/SDS gel before and after cleavage with CNBr. Finally, the gel was impregnated with Amplify and subjected to fluorography. Lane 1, original labelled enzyme; lane 2, CNBr-cleaved enzyme.

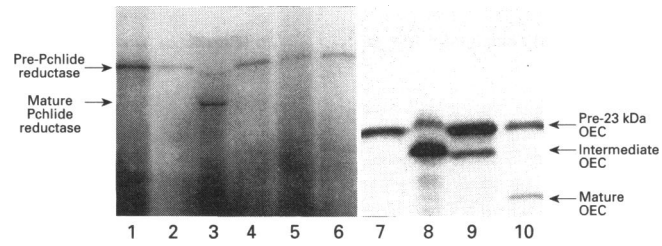

Figure 3 Processing peptidase cleavage susceptibility of pre-Pchlide reductase synthesized *in vitro* from pWR5

pWR5 was transcribed and translated *in vitro* using T7 RNA polymerase and a wheat germ translation system in the presence of [^{35}S]methionine (lane 1). A second translation was also carried out in the presence of SPP (lane 2). The synthetic precursor was incubated with a crude stromal extract (lane 3), SPP (lane 4), TPP (lane 5) and LEP (lane 6) and analysed by SDS/PAGE and fluorography. As a positive control, processing of the precursor to the 23 kDa protein of the OEC by the various fractions was also analysed as previously described (James et al., 1989). Lanes 7–10 represent the radiolabelled pre-23 kDa OEC translation product (lane 7) after incubation with crude stroma (lane 8), purified SPP (lane 9) and LEP (lane 10).

ment of the chloroplasts with trypsin. This only removed a faint precursor-sized band (compare lanes 2 and 3 of Figure 4) which probably represents precursor bound to the outside of the chloroplast, while having no effect on the major band, which corresponds to the mature protein. Subsequent fractionation of the chloroplasts into stroma (lane 4) and thylakoid fractions (lane 5) shows the mature protein to be localized exclusively to the thylakoids. Furthermore, at such a location it is susceptible to digestion by trypsin, as incubation with this enzyme (lane 6) results in substantial loss of labelling from the thylakoids.

Table 1 Protein sequence of the 14 kDa Pchlide reductase CNBr-cleavage fragment

The 14 kDa Coomassie Blue-stained band on the Immobilon-P membrane was excised and sequenced on a pulsed-liquid sequencer. The amino acids from each sequence cycle were analysed by h.p.l.c. A sequenced amino acid corresponding to the predicted amino acid sequence was identified as an increase in its level in the appropriate cycle followed by its decrease in the next. The amino acid sequenced at each cycle compared with the relevant sequence of pWR5 is shown. The amino acids in upper case in the observed sequence represent definite peaks on the chromatograms that correspond to the predicted sequence. Those in lower case represent possible peaks, while a question mark indicates that the amino acid predicted from the cDNA sequence could not be identified as a peak. (a) represents the order for the main series of sequencing cycles with (b) corresponding to the secondary superimposed series.

Sequencing cycle	Observed sequence (a)	Predicted sequence	Observed sequence (b)	Predicted sequence
—	—	Met-271	—	Met-274
1	LEU	Leu	GLN	Gln
2	THR	Thr	glu	Glu
3	MET	Met	PHE	Phe
4	GLN	Gln	his	His
5	GLU	Glu	?	Arg
6	PHE	Phe	?	Arg
7	HIS	His	TYR	Tyr
8	ARG	Arg	?	His
9	ARG	Arg	glu	Glu
10	TYR	Tyr	?	Asp
11	HIS	His	THR	Thr
12	GLU	Glu	gly	Gly
13	glu	Glu	ile	Ile
14	THR	Thr	THR	Thr
15	GLY	Gly	?	Phe
16	ILE	Ile	SER	Ser
17	THR	Thr	SER	Ser
18	phe	Phe	leu	Leu
19	SER	Ser	TYR	Tyr
20	ser	Ser	?	Pro
21	LEU	Leu	GLY	Gly
22	TYR	Tyr	?	Cys

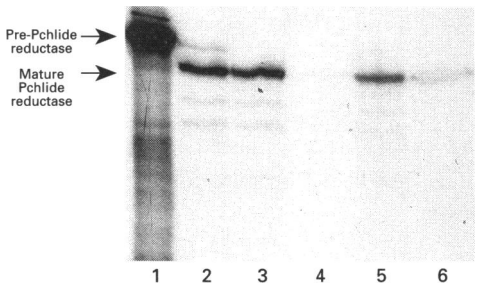

Figure 4 Import of *in vitro*-synthesized pre-Pchlide reductase into isolated intact pea chloroplasts

In vitro-synthesized pre-Pchlide reductase (lane 1) was incubated with intact pea chloroplasts (lane 2) followed by trypsin treatment of the chloroplasts (lane 3). The chloroplasts were then fractionated into stromal (lane 4) and thylakoid (lane 5) fractions and the thylakoids were also trypsin-treated (lane 6). The samples were separated by SDS/PAGE and visualized by fluorography.

DISCUSSION

Pchlide reductase is a novel enzyme catalysing a unique light- and NADPH-dependent reductive reaction during the

biosynthesis of chlorophyll by higher plants. This system offers many advantages over traditional dehydrogenases in studies on reaction mechanisms because of the ease and rapidity with which Pchlide reduction can be initiated by light. Also several highly coloured, but as yet uncharacterized, transients in the process have also been described (Iwai et al., 1984). Furthermore, the enzyme may well be involved as a photoreceptor mediating some of the photomorphogenetic processes in plants (Thompson and Weier, 1991; Harpster and Apel, 1985). Such detailed information on the structure and mechanism of the enzyme is of considerable interest.

The first primary structural information on the reductase appeared with the characterization of cDNA clones for the enzyme from barley (Schulz et al., 1989) and oat (Darrah, 1988; Darrah et al., 1990). Our oat clone, however, was shown to be incomplete when compared with that from barley, e.g. it completely lacked the presequence, and as such would be of only limited use in future experiments. In the present paper, our original oat reductase clone p127 has been used to provide probes to enable us to isolate a full-length clone (pWR5) from an etiolated wheat cDNA library constructed in the phage vector, λgt10. Comparison of the sequence of pWR5 with the earlier data for the oat and barley clones shows a remarkable degree of homology in overlapping sequences between the three monocot species with, for example, the homology between oat and wheat being 92.8% at the gene and as high as 97.8% at the amino acid level. Interestingly, the extra 5′ sequence, which encodes the transit peptides in the wheat and barley clones, also displays the same high degree of homology between these two monocot species, but see below.

Since completion of this work, the characterization of Pchlide reductase from other species, notably arabidopsis (Benli et al., 1991), pea (Spano et al., 1992a) and two species of pine (Spano et al., 1992b), has been reported. The high degree of conservation of reductase structure amongst these species, especially at the amino acid level and particularly so for the mature protein, has already been alluded to (Spano et al., 1992a, b). For the dicots, this homology does not extend to the structure of the transit peptide, in marked contrast with the situation in cereals mentioned above. Despite these reports, some uncertainty still persists as to the identity of the *N*-terminal residue of the reductase. Our attempts at sequencing and radiosequencing of the mature wheat and oat proteins have been unsuccessful, and different residues have been claimed as the *N*-terminus of the reductase from barley, arabidopsis and pea (Schulz et al., 1989; Benli et al., 1991; Spano et al., 1992a). To avoid confusion, in the present discussion the initiating methionine of the preprotein will be designated as the first residue and serve as a reference for numbering of the amino acids in the sequence.

The pWR5 sequence (Figure 1b), in common with all the others published, shows a highly conserved hydrophobic segment (residues 74–107) generating a local ($\beta\,\alpha\,\beta$) unit of secondary structure and with two essential glycine residues (81 and 98). This segment, highlighted initially in the oat reductase by Darrah et al. (1990), constitutes part of the cofactor pyrophosphate-binding loop found in virtually all nicotinamide nucleotide-dependent enzymes (Rice et al., 1984). Furthermore, Arg-107, again conserved in all the sequenced Pchlide reductases, could provide the positive charge for electrostatic interaction with the 2′-phosphate group in $NADP^+$. Of interest here is the observation that the reductase has already been shown to be sensitive to the arginine reagent, phenylglyoxal, and that, furthermore, inhibition can be prevented by prior binding of NADPH (Oliver, 1982).

Earlier experiments have also highlighted the importance of cysteine residues in substrate binding in the reductase (Oliver and Griffiths, 1981). Of the four cysteine residues present in pWR5, only three, residues 106, 267 and 296, are conserved amongst the characterized reductases, with the equivalent of the pWR5 Cys-157 replaced in the pea by asparagine (Asn-171). This makes it unlikely that Cys-157 fulfils an essential role in the reductase. Of the remaining three cysteines in pWR5, we have demonstrated that Cys-296 at least becomes labelled during our [^{3}H]NPM-labelling/substrate-protection experiments (Table 1). This was concluded because the major radiolabelled fragment isolated from CNBr cleavage of [^{3}H]NPM-labelled protein was identified, by sequencing, as originating from cleavage at Met-273, generating a fragment containing a single cysteine residue (Cys-296) (Figure 1). The absence of labelling in CNBr fragments smaller than 14 kDa (Figure 2c) implies that the remaining three cysteines are not significantly labelled. We therefore tentatively propose Cys-296 as the substrate-protected cysteine in the reductase. This particular cysteine is adjacent to a glycine residue (Figure 1), and it is interesting that the Cys-Gly dipeptide has already been identified in a widespread group of reductases such as cytochrome *P*-450 reductase, ferrodoxin reductase and cytochrome b_5 reductase (Porter, 1991), although its significance remains obscure. It is somewhat surprising in our labelling experiments that no labelling of Cys-106 was observed, despite its location within the assumed nucleotide-binding domain.

Two aspects of the cell biology of Pchlide reductase have yet to be adequately described. These concern the related problems of plastic import/processing of the enzyme and its eventual location. If it is assumed that the functional reductase is associated with the plastic inner membrane [Shaw et al. (1985) and Dehesh et al. (1986a) but see, however, Joyard et al. (1990) and Dehesh et al. (1986b)], the cytoplasmically synthesized precursor would be expected to be transported across the plastid envelope. Within the stroma, an SPP would generate the mature peptide, which, in view of its hydrophobicity (approx. 30% hydrophobic residues in wheat) would locate to the plastid inner membrane.

Against this background, the features of the N-terminal segment (transit peptide) of the wheat preprotein (Figure 1b) conform with the general properties expected of chloroplast transit peptides (von Heijne and Nishikawa, 1991). Residues 1–60 in pWR5 contain a high percentage of hydroxylated amino acids with 30% serine and threonine, a single acidic amino acid residue, Asp-26, and no obvious features of secondary structure. *In vitro* expression of pWR5 in the presence of [^{35}S]methionine generates the expected labelled preprotein of approximate molecular mass 41 kDa (Figure 3). Incubation of this with intact pea chloroplasts results in its import and stromal processing to the mature form and its location to the exposed surface of the thylakoid membrane (Figure 4).

There are several important implications in the results of this import/processing experiment. The data suggest that functional chloroplasts retain the ability to import and process the reductase. This endorses the importance of the enzyme in chlorophyll synthesis, not only in etiolated tissues but also in chloroplasts of green tissues (Griffiths et al., 1985). The lack of well-defined structural features amongst chloroplast transit peptides has already been mentioned. In fact, the same protein in different species can have transit peptides that are completely unrelated, as is the case in Pchlide reductase. Despite this, the wheat reductase transit peptide is effectively recognized by the pea chloroplast import machinery (Figure 4), implying that the import receptor is similar in all plastids, irrespective of origin.

No clear-cut conclusions can be drawn regarding the processing of the imported precursor. As expected from the location of the mature reductase on the external (stromal) side of the

thylakoid (Figure 4), it is not surprising that neither LEP nor TPP had any effect on the precursor (Figure 3). In contrast, in the presence of a crude stromal extract, effective processing of the precursor to the mature form is observed, supporting the role of stroma-located enzyme(s) in the process. The lack of processing of the prereductase by the purified stromal protease (Figure 3) is surprising, especially in view of the efficient processing of the pre-23 kDa OEC protein to its intermediate form by this preparation (Mold et al., 1992). It is possible that other factors besides the protease, e.g. chaperonins in the crude stroma, are required for processing of the reductase precursor.

Finally, how the mature reductase locates to the membrane is not clear. Its interaction with the membrane is, at least in part, electrostatic, as low-salt (50 mM KCl) washing can readily displace it (G. R. Teakle and W. T. Griffiths, unpublished work). One important element in the membrane contributing to such an interaction could be the enzyme's substrate, i.e. the pigment protochlorophyllide, the C_{17}-propionate group of which is known to interact electrostatically with the enzyme's active site (Griffiths, 1980). Also, despite the absence of any obvious transmembrane-spanning domains in the reductase, it does possess hydrophobic patches (Figure 1b) which may contribute to anchoring of the enzyme by hydrophobic interaction with the membrane structure.

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REFERENCES

Apel, K. and Kloppstech, K. (1978) Eur. J. Biochem. **85**, 581–588
Batschauer, A., Santel, H. J. and Apel, K. (1982) Planta **154**, 459–464
Benli, M., Schulz, R. and Apel, K. (1991) Plant Mol. Biol. **16**, 615–625
Cava, M. P., Deanna, A. A., Muth, K. and Mitchel, M. J. (1961) Org. Synth. **41**, 93–95
Darrah, P. M. (1988) Ph.D. Thesis, University of Bristol
Darrah, P. M., Kay, S. A., Teakle, G. R. and Griffiths, W. T. (1990) Biochem. J. **265**, 789–798
Dehesh, K., Klaas, M., Hauser, I. and Apel, K. (1986a) Planta **169**, 162–171
Dehesh, K., von Kleeve, B., Ryberg, M. and Apel, K. (1986b) Planta **169**, 172–183
Griffiths, W. T. (1978) Biochem. J. **174**, 681–692
Griffiths, W. T. (1980) Biochem. J. **186**, 267–278
Griffiths, W. T., Kay, S. A. and Oliver, R. P. (1985) Plant Mol. Biol. **4**, 13–22
Harpster, M. and Apel, K. (1985) Physiol. Plant **64**, 147–153
Highfield, P. E. and Ellis, R. J. (1978) Nature (London) **271**, 420–424
Iwai, J., Ikenhi, M., Inoue, Y. and Kobayashi, J. (1984) in Protochlorophyllide Reductase and Greening (Sironval, C. and Brouers, M., eds.), pp. 99–112, Martinus Nijhoff/Dr. W. Junk Publishers, The Hague, Netherlands
James, H. E., Bartling, D., Musgrove, J. E., Kirwin, P. M., Hermann, R. G. and Robinson, C. (1989) J. Biol. Chem. **264**, 19573–19576
Joyard, J., Block, M., Pineau, B., Albrieux, C. and Douce, R. (1990) J. Biol. Chem. **265**, 21820–21827
Laemmli, U. K. (1970) Nature (London) **277**, 680–685
Mapleston, R. E. and Griffiths, W. T. (1978) FEBS Lett. **92**, 168–172
Mapleston, R. E. and Griffiths, W. T. (1980) Biochem. J. **185**, 125–133
Mold, R. M., Meadows, J. W., Shackelton, J. B. and Robinson, C. (1992) in Transport and Receptor Proteins of Plant Membranes (Cooke, D. T. and Clarkson, D. T., eds.), pp. 201–207, Plenum Press, New York
Oliver, R. P. (1982) Ph.D. Thesis, University of Bristol
Oliver, R. P. and Griffiths, W. T. (1980) Biochem. J. **191**, 277–280
Oliver, R. P. and Griffiths, W. T. (1981) Biochem. J. **195**, 93–101
Ploug, M., Jensen, A. L. and Barkholt, V. (1989) Anal. Biochem. **181**, 33–39
Porter, T. D. (1991) Trends Biochem. Sci. **16**, 154–158
Rice, W., Schultz, G. E. and Guest, J. R. (1984) J. Mol. Biol. **174**, 483–496
Robinson, C. and Ellis, R. J. (1985) Eur. J. Biochem. **152**, 67–73
Sambrook, J., Fritsch, E. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
Santel, H. J. and Apel, K. (1981) Eur. J. Biochem. **120**, 95–103
Schagger, H. and von Jagow, G. (1987) Anal. Biochem. **166**, 368–379
Schulz, R., Steinmuller, K., Klaas, M., Forreiter, C., Rasmussen, S., Hiller, C. and Apel, K. (1989) Mol. Gen. Genet. **217**, 355–361
Shaw, P. J., Henwood, J. A., Oliver, R. P. and Griffiths, W. T. (1985) Eur. J. Cell Biol. **39**, 50–55
Spano, A. J., Zhenqui, H. and Timko, M. P. (1992a) Mol. Gen. Genet. **236**, 86–95
Spano, A. J., Zhenqui, H., Hanspeter, M., Hunt, D. F. and Timko, M. P. (1992b) Plant Mol. Biol. **18**, 967–962
Thompson, J. and Weier (1991) Annu. Rev. Plant Physiol. Plant Mol. Biol. **42**, 423–466
von Heijne, G. and Nishikawa, K. (1991) FEBS Lett. **278**, 1–3
von Heijne, G., Steppuhn, J. and Hermann, R. (1989) Eur. J. Biochem. **180**, 535–545

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