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Magnesium chelatase subunit D from pea: characterization of the cDNA, heterologous expression of an enzymatically active protein and immunoassay of the native protein

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Abstract

Mg-chelatase catalyzes the insertion of Mg into protoporphyrin and lies at the branchpoint of heme and (bacterio)chlorophyll synthesis. In prokaryotes, three genes – *BchI*, *D* and *H* – encode subunits for Mg-chelatase. In higher plants, homologous cDNAs for the I, D and H subunits have been characterized. Since the N-terminal half of the D subunit is homologous to the I subunit, the C-terminal portion of the pea D was used for antigen production. The antibody recognized the chloroplast D subunit and was used to demonstrate that this subunit associated with the membranes in the presence of $MgCl_2$. The antibody immunoprecipitated the native protein and inhibited Mg-chelatase activity. Expression in *Escherichia coli* with a construct for the full-length protein (minus the putative transit peptide) resulted in induction of 24.5 kDa (major) and 89 kDa (minor) proteins which could only be solubilized in 6 M urea. However, when host cells were co-transformed with expression vectors for the full-length D subunit and for the 70 kDa HSP chaperonin protein, a substantial portion of the 89 kDa protein was expressed in a soluble form which was active in a Mg-chelatase reconstitution assay.

Introduction

In photosynthetic organisms, heme and (bacterio)chlorophyll synthesis share a common metabolic pathway up to the level of protoporphyrin IX (Proto), after which the pathways diverge into what are known as the 'iron branch' and the 'magnesium branch' (Beale and Weinstein, 1990). In the iron branch, an Fe^{2+} ion is chelated into Proto to form heme which can then be further metabolized to modified hemes or broken down to form linear tetrapyrrole pigments such as the phytochrome chromophore. In the magnesium branch, a Mg^{2+} ion is chelated into Proto to form Mg-protoporphyrin (Mg-Proto) which is then converted in a series of enzymatic steps to the (bacterio)chlorophylls. Iron chelation and Mg chelation are catalyzed by the enzymes ferrochelatase (protoheme ferro-lyase, EC 4.99.1.1) and Mg-chelatase, respectively, and it is the relative activity of these two enzymes which will dictate the relative flux of biosynthetic intermediates into heme or (bacterio)chlorphyll synthesis (Beale and Weinstein, 1990).

The key, potentially regulatory, positions of ferrochelatase and Mg-chelatase in the biosynthetic pathway have made these enzymes a target for research. Although ferrochelatase has been well characterized (Ferreira *et al.*, 1995) it is only recently that progress on describing the mechanism of Mg-chelatase has been made through identification of genes for the enzymes and refinement of biochemical activity studies (Walker and Willows, 1997).

In photosynthetic bacteria the genes for bacteriochlorophyll synthesis, carotenoid synthesis and other photosynthetic proteins are all present in a 45 kb

cluster, designated the photosynthesis gene cluster (reviewed in Alberti *et al.*, 1995). The cluster has been completely sequenced and transposon mutagenesis of selected ORFs was used to show that three genes (*BchI*, *D* and *H*) appeared to be involved with magnesium chelation (Bollivar *et al.*, 1994). Subsequently, these genes were cloned and expressed in *Escherichia coli*, and Mg-chelatase activity was demonstrated when the cell extracts were combined, confirming that Mg-chelatase was indeed made up of three protein subunits (Gibson *et al.*, 1995). In prokaryotes, *bchI*, *D* and *H* homologues have now been isolated in *Synechocystis* (Jensen *et al.*, 1996a) and *Chlorobium* (Petersen *et al.*, 1996), and Mg-chelatase activity has again been reconstituted from the expressed gene products.

In higher plants, Mg-chelatase has been demonstrated to be composed of three subunits by protein fractionation techniques (Guo *et al.*, 1998). The pea enzyme could be completely solubilized by lysing chloroplasts in a buffer lacking $MgCl_2$. The soluble extract (SP) could be separated into three fractions: chromatography on blue agarose yielded a blue-bound (BB) fraction; the flow through the blue agarose column was then separated by size-selective (100 kDa) centrifugal separation into a FT-hi fraction and a FT-lo fraction. Since antibodies to the I and H subunits were available, western blotting was used to demonstrate that the BB fraction contained the H subunit and the FT-lo fraction contained the I subunit. The FT-hi fraction was suggested to contain the D subunit by a process of elimination.

Genetic studies on mutant barley lines have also shown that when either one of three loci, *Xantha-f*, *-g* and *-h*, are mutated, Mg-chelatase activity is destroyed (Kannangara *et al.*, 1997). Biochemically it was shown that the reconstitution of Mg-chelatase activity was possible in the mutants by the pairwise combination of extracts derived from the mutant chloroplasts, indicating again that higher plants have three Mg-chelatase subunits.

The multiple subunit composition of Mg-chelatase was initially surprising since ferrochelatase, which catalyzes an almost identical reaction, is composed of a single protein (Ferreira *et al.*, 1995). Another notable difference between the two chelatases is that Mg-chelatase requires ATP for activity whereas ferrochelatase does not (Pardo *et al.*, 1980). The cause of these differences may lie in regulatory features of the enzyme. Biochemical work has shown that the Mg-chelatase catalyzes a two-step reaction, ATP-dependent activation followed by ATP-dependent metal-ion insertion (Walker and Weinstein, 1994). Work with the *E. coli*-expressed *Rhodobacter* proteins demonstrated that only the I and D subunits were involved in the activation step, H being required in addition to I and D for catalysis (Willows *et al.*, 1996). The role of the I and D subunits in activation was subsequently confirmed biochemically with the fractionated higher-plant system (Guo *et al.*, 1998). However, further elucidation of the roles of the individual subunits in catalysis or regulation has not been established.

Counterparts for the I and H subunit genes in higher plants have also been identified and cloned (Koncz *et al.*, 1990; Hudson *et al.*, 1993; Nakayana *et al.*, 1995; Gibson *et al.*, 1996; Jensen *et al.*, 1996b; Kruse *et al.*, 1997; Nakayama *et al.*, 1998). While activity was not demonstrated in the heterologously expressed proteins, they typically shared over 40% amino acid identity with the *Rhodobacter* sequences and so are most likely the higher-plant counterparts to the Mg-chelatase genes.

Based on the obvious similarities between these higher-plant and the prokaryotic Mg-chelatase genes and correspondence of mutations in these genes with a lack of Mg-chelatase activity (Kannangara *et al.*, 1997), it was reasonable to assume that the higher-plant D gene (*chlD*) would also have strong sequence similarities with its prokaryotic counterparts. This information could then be used to take an 'evolutionary walk' to isolate the higher-plant gene. This strategy has now been successful in pea and tobacco and has resulted in the determination of the higher-plant D cDNA sequences (Luo and Weinstein, 1997; Papenbrock *et al.*, 1997). Most recently, all three tobacco Mg-chelatase subunits have now been co-expressed in yeast and Mg-chelatase activity has been demonstrated in the extracts (Papenbrock *et al.*, 1997). However, the authors only demonstrated activity when all three subunits were co-expressed and did not address whether expression of individual subunits gave correctly folded subunits. For biochemical studies, it is most convenient to have subunits expressed separately from each other in order to avoid subsequent purification steps.

In this paper, we describe work with the cloning and expression of the pea Mg-chelatase *chlD* cDNA in *E. coli*. We have demonstrated that the full-length protein was expressed in an active form in *E. coli* if the host cells were co-expressing the 70 kDa HSP chaperonin protein. Therefore we have a ready source of correctly folded Mg-chelatase D subunit, which is

not contaminated by other subunits and can be rapidly purified by virtue of a 6× His tag. In addition, using expressed truncated protein as an antigen, we have raised an antibody against the D subunit and have been able to demonstrate the partition of the D subunit between the chloroplast membranes and soluble fractions according to the buffer composition to complement the previous biochemical fractionation work from our laboratory.

Materials and methods

Biological materials

Pea seeds (*Pisum sativum* L. cv. Spring) were purchased from Asgrow. Restriction enzymes were obtained from either Promega or New England Biolabs. Vectors pGEM-4G and pGEM-T were purchased from Promega, and pProEXHT from Life Technologies. Primers were synthesized by Integrated DNA Technologies.

Messenger RNA isolation

Messenger RNA was purified by oligo(dT) chromatography (Bartels *et al.*, 1990). from leaves of 6-day old pea seedlings which were grown in trays of moist vermiculite at room temperature under a 12 h light/12 h dark cycle (Walker and Weinstein, 1991). The last light/dark/light cycle before harvesting was 4 h/12 h/4 h.

PCR reactions, DNA purification and bacterial transformation

Unless otherwise stated, PCR reactions were performed under conditions recommended by the *Taq* polymerase supplier (Perkin Elmer Cetus). A Perkin Elmer Cetus DNA Thermocycler 480 was used for all reactions. Plasmid DNA, DNA fragments from digestions or PCR reactions were purified (from agarose gels for the fragments) using appropriate kits from Qiagen. Bacterial transformation was accomplished by electroporation (BioRad).

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR using GeneAmp RNA PCR kit (Perkin Elmer Cetus) was performed according to the manufacturer's instructions with minor modifications. The reverse transcription reaction contained about 0.5 μg of messenger RNA and oligo$(dT)_{16}$ was used as the primer. In the PCR reaction, 1 μM of each primer D6, 5′-NA(AG)NGG(AGT)AT(AG)TTNCCNC(GT)NCC-3′ and PolyP, 5′-CAA(CG)A(AG)G(AC)(AG)CCNCCN CCNCC-3′ were used. Cycling conditions were: 2 min at 95 °C followed by 5 cycles of 50 s at 95 °C, 50 s at 55 °C, 2 min at 72 °C; 5 cycles of 50 s at 95 °C, 50 s at 50 °C, 2 min at 72 °C; 30 cycles of 50 s at 95 °C, 50 s at 45 °C, 2 min at 72 °C; and 10 min extension at 72 °C.

To increase the specificity, a nested PCR was performed in a 50 μl cocktail containing 1 μM of each internal primer, 2.5 units of *Taq* polymerase and 5 μl of 1:20 dilutions of the first PCR products. The internal primers used were D1, 5′-AC(TGA)(TC)T(TGC)(CA)GN(TAG)CNGC(TA)(TA G)CNCC-3′; D2, 5′-TT(TC)(TCG)(TC)NGTNGA(T C)GCNA(CG)NGG; D3, 5′-(AGT)ATNA(AG)N(GC) (AT) (TAG)ACNTG(AG)TC-3′ and D5, 5′-AT(AG)TT NCCNC(GT)NCC(AG)TCNGT-3′. Cycling conditions were the same as above except that extension at 72 °C in each cycle was 1 min instead of 2 min.

Probe preparation and library screening

The DNA probe was non-radioactively labelled with digoxigenin according to the manufacturer's instructions (Boehringer) and was used to screen a λgt11 pea cDNA library (Clontech). Phage (about 5×10^5) were transferred onto Hybond N^+ membranes (Amersham) according to standard protocols (Sambrook *et al.*, 1989). Hybridization was performed at 64 °C overnight and positive signals were detected by alkaline phosphatase activity conjugated to anti-digoxigenin antibodies according to the manufacturer's instructions (Boehringer).

Subcloning of the cDNA inserts

Single positive phage plaques were picked and resuspended into 500 μl of λ diluent (10 mM Tris-HCl pH 7.5, 10 mM $MgSO_4$) and 200 μl of chloroform. The inserts of the positive phage were checked by PCR using λgt11 forward and reverse primers (Promega). Recombinant phage DNA were propagated on plates (Sambrook *et al.*, 1989) and the cDNA inserts, obtained from phage DNA by *Eco*RI digestion, were cloned into the pGEM-4Z plasmid vector for sequencing. The vector containing the largest insert is referred to as pλD2.

DNA sequencing

DNA sequencing was performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) according to the manufacturer's instructions except that 55 °C was used for annealing to increase the specificity of the reaction. When needed, oligonucleotides (17–23-mers) were synthesized and used as sequencing primers. Sequencing products were separated on an Applied Biosystems (373) automatic DNA sequencer. Sequence data were analyzed with MacDNASIS (Hitachi) or GeneWorks (IntelliGenetics).

Expression in E. coli

To construct the full-length clone, the 5′ end of the cDNA corresponding to the putative transit peptide was removed from the pλD2 insert by PCR. Primers used for this PCR were PrD12, 5′-GCTGTTCTGAGCGCTTCTGAGGAGAAGCTCGATGC-3′ (207–241), in which a blunt-cut restriction site (*Eco*47III) was created at the target point (underlined); and PrD13, 5′-GAGCAAATTACTGATACCCTCGTCC-3′ (698–722) which corresponds to a region downstream of the *Avr*II and *Sph*I sites of the cDNA. The *Eco*47III/*Sph*I fragment of the PCR product and the *Sph*I/*Bam*HI fragment of the cDNA (pλD2) were then assembled into an *E. coli* expression vector (pProEXHT) at the *Ehe*I/*Bam*HI sites in the multiple cloning site. The resultant plasmid is referred to as pex3-20. The *Pst*I/*Kpn*I fragment of the cDNA (pλD2) was also cloned into this vector at the sites *Pst*I/*Kpn*I resulting in the truncated expression plasmid, pexPst15. *E. coli* DH5α were used as the host cells for all of the expression studies except for the co-expression in which BL21(DE3) were used.

For the co-expression study a new vector with a different (from pProEXHT) origin of replication and selection marker was required. PexMz was constructed by ligating the 1 kb *Bst*EII/*Pst*I fragment of pProEXHT with the 3 kb *Bst*EII/*Pst*I fragment of pACYC177 (American Type Culture Collection). The new vector has an almost identical multiple cloning site, promoter, translation start site and 6× His tag as pProEXHT and confers kanamycin resistance. Thus, the modified full-length expression vector, pexMz3-20, was constructed using the same PCR and restriction fragments of pλD2 and multiple cloning site as for pex3-20 (above).

Cells were cultured to about 0.5 OD_{590} and expression was induced overnight with 0.5 mM IPTG at room temperature for activity studies or for 150 min with 1–2 mM IPTG at 37 °C for SDS-PAGE. For activity measurements, *E. coli* cells were sonicated in lysis buffer (0.3 M glycerol, 100 mM tricine, 25 mM $MgCl_2$, 10 mM DTT, 2 mM ATP pH 7.9) which contained the Complete protease inhibitor cocktail (Boehringer). The lysate was centrifuged at 13 000 × *g* for 20 min at 4 °C and the supernatant saved.

Antigen preparation

Log-phase cells (0.5 OD_{590}) of expression clone pexPst15 (which contains the insert coding for a His-tagged C-terminal fragment of the Mg-chelatase D subunit) was incubated for 2.5 h at 37 °C in the presence of 1 mM IPTG. Cells were harvested and inclusion bodies isolated by lysozyme digestion and Triton X-100 washes. The inclusion bodies were solubilized (6 M urea, 5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9), and purified on a 5 ml Pharmacia Hi-trap metal chelate column preloaded with Ni^{2+} according to the manufacturer's directions. Eluted protein was electrophoresed on a preparative SDS-polyacrylamide gel. The protein band was carefully excised, and the gel slice was used directly as an antigen for polyclonal antibody production in New Zealand white rabbits by Cocalico Biologicals (Reamstown, PA).

SDS-PAGE and western blot analysis

Proteins were electrophoresed on 10% SDS-polyacrylamide gels (Fling and Gregerson, 1986) and transferred onto a polyvinylidene difluoride membrane for protein sequencing (BioRad) using a Mini Genie Electrophoretic blotter (Idea Scientific Company). Incubation with the primary antibody (1:5000 dilution of serum) was overnight at 4 °C. The secondary antibody, affinity-purified peroxidase-conjugated goat anti-rabbit IgG (Cappel, West Chester, PA) was used at a dilution of 1:5000. The Enhanced Chemiluminescence system (Amersham Life Sciences) was used for detection.

Immunoprecipitation

Immunoprecipitation of the chloroplast protein by the anti-D antibody was tested using magnetic Dynabeads M-280 sheep anti-rabbit IgG magnetic beads (Dynal). Duplicate samples of 4 mg of chloroplast soluble proteins (SP; Guo *et al.*, 1998) were pre-cleared with Dynabeads (50 μl) in the presence of Complete protease inhibitor cocktail (Boehringer), and 5% (w/v)

BSA in 1 ml PBS at 4 °C for 2 h. The beads were discarded and either preimmune or immune serum (dilution 1:25) was added to the incubation. After overnight mixing at 4 °C, 50 μl of beads were added for a further 2 h of mixing. Washed beads were immediately boiled in 40 μl SDS-PAGE sample buffer, and the dissociated proteins analyzed by western blotting.

Chloroplast fractionation and Mg-chelatase assays

Pea chloroplast fractions were prepared exactly as described previously (Walker *et al.*, 1992; Guo *et al.*, 1998). Mg-chelatase activity was determined by a stopped or a continuous fluorometric assay (Walker and Weinstein, 1994).

Image analysis

Images were taken by the Scion imaging system, and analyzed on a Macintosh computer using the public domain NIH Image program (fttp://zippy.nimh.nih.gov/pub/nih-image/nih-image_spin-offs/).

Results

Isolation and sequencing of a cDNA coding for the pea Mg-chelatase subunit D

We selected 5 relatively conserved regions among the prokaryotic Mg-chelatase D proteins of *Rhodobacter capsulatus*, *Chlorobium vibrioforme* and *Synechocystis* sp. PCC6803 (protein accession numbers CAA77537, CAB06300 and CAA65418 respectively) and used these to design 6 degenerate primers (see Materials and methods), which could be used to search for the higher-plant D homologue by PCR. Since several of the primers contained very similar sequences at the 3′ ends, we employed a step PCR with annealing temperatures higher in the first few cycles and decreasing gradually (see Materials and methods). This procedure should increase the specificity of the PCR products (Luo *et al.*, 1997). After a second round of PCR with nested primers, using the diluted first PCR products as templates, a DNA fragment of about 300 bp was obtained with the primer combination D2 and D5 (data not shown). Single primers D2 or D5 did not produce this band. Nested PCR with primers D1 and D3, in combination or single primers, also did not produce the expected band (data not shown).

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MGFSLTHTPHTTASPNLQLRFHSLLPPSFTSQPFLSLHSTFPPKRTVPKL   50
RAQSENGAVLQASEEKLDASNYGRQYFPLAAVIGQDAIKTALLLGATDPR  100
IGGIAISGRRGTAKTIMARGMHAILPPIEVVQGSIANADPSCPEEWEDGL  150
YKRVEYDSDGNVKTHIIKSPFVQIPLGVTEDRLIGSVDVEESVKTGTTVF  200
QPGLLAEAHRGVLYVDEINLLDEGISNLLLNVLTEGVNIVEREGISFRHP  250
CRPLLIATYNPDEGSVREHLLDRIAINLSADLPMSFENRVEAVGIATEFQ  300
DNCGQVFKMVDEDTDNAKTQIILAREYLKDVTISKEQLKYLVIEALRGGV  350
QGHRAELYAARVAKCLAALEGREKVYVDDLKKAVELVILPRSIITDTPPE  400
QQNQPPPPPPPPQNQESNEEQNEEEEQEEEEEDDNDEENEQQQDQLPEEF  450
IFDAEGGLVDEKLLFFAQQAQRRRGKAGRAKNVIFSEDRGRYIKPMLPKG  500
PVKRLAVDATLRAAAPYQKLRREKDTENRRKVYVEKTDMRAKRMARKAGA  550
LVIFVVDASGSMALNRMQNAKGAALKLLAESYTSRDQVSIIPFRGDSAEV  600
LLPPSRSIAMARKRLERLPCGGGSPLAHGLTTAVRVGLNAEKSGDVGRIM  650
IVAITDGRANISLKRSNDPEAAAASDAPKPTSQELKDEIIEVAAKIYKTG  700
MSLLVIDTENKFVSTGFAKEIARVAQGKYYYLPNASDAVVSLATREALAA  750
LKSS
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Figure 1. Deduced amino acid sequence of the pea *chlD* cDNA. The filled triangle denotes the position of the putative cleavage site for the chloroplast transit peptide. The boxed areas are nucleotide binding motifs as described by Koonin (1993, 1997). The underlined area is the prominent poly-proline-rich area followed by an acidic region which is conserved in all the known D sequences. A dot underneath indicates that this residue is identical in the sequences from the following organisms: *Rhodobacter capsulatus*, *Synechocystis* sp. PCC6803, *Chlorobium vibrioforme*, *Nicotiana tabacum* and *Pisum sativum* (sequence alignment using Geneworks).

The specific band produced by primers D2 and D5 was the approximate size expected from comparison with the prokaryotic D sequences, and it was subsequently used as a probe to screen a λgt11 pea cDNA library. Six positive phage clones were obtained: three contained inserts of over 2 kb and these inserts were subcloned into plasmids. The largest insert, pλD2, was completely sequenced in both directions. The 2607 bp cDNA sequence (GenBank accession number AF014399) contained an open reading frame (ORF) of 2262 bp starting from nt 36 (ATG) and extending to nt 2300 (TGA), a 5′-untranslated region of 35 bp and a 3′-untranslated region of 307 bp including the poly(A) tail of 11 As. The ORF encoded 754 amino acid residues with a calculated molecular mass of 82 860 Da. Based on a compilation of chloroplast transit peptide cleavage sites and transit peptide domain structure (von Heijne *et al.*, 1989; Gavel and von Heijne, 1990) as well as information on the N-terminal sequences of the prokaryotic Mg-chelatase D homologues (which are transit peptide-free), we predicted that the transit peptide would be cleaved between Gln-61 and Ala-62. The sequence V-L-Q↓A (Figure 1) best fits the loosely conserved consensus motif (V/I)-X-(A/C)↓A (X is any amino acid) of many transit peptide cleavage sites (Gavel and von Heijne, 1990), and this site is located close to the positions corresponding to the first amino acids of the prokaryotic Mg-chelatase D homologues when the deduced amino acid sequences are aligned (3, 14 and 21 amino

acids upstream of the positions corresponding to the first amino acids of *Synechocystis* sp. PCC6803, *C. vibrioforme* and *R. capsulatus* Mg-chelatase D subunit homologues respectively, not shown). The resultant mature protein would contain 693 amino acids with a calculated molecular mass of 76 kDa. The deduced amino acid sequence, excluding the putative transit peptide, showed identities of 58%, 42% and 36% with the Mg-chelatase D sequences of *Synechocystis* sp. PCC6803, *C. vibrioforme* and *R. capsulatus*, respectively. The complete deduced sequence showed an identity of 81% with the Mg-chelatase D sequence of tobacco (Papenbrock *et al.*, 1997) of which the mature protein portions showed an identity of 86% while the transit peptide portions were 41% identical. The deduced amino acid sequence had characteristics similar to other Mg-chelatase D sequences: at the center, a Pro-rich region was surrounded by regions rich in Glu, Asp and their derivatives Gln and Asn, even though more Glu residues were used in plant D sequences (Figure 1). The N-terminal half (excluding the transit peptide) is similar to the Mg-chelatase I subunit (about 40% identity between the N-terminal half of the pea D subunit and the *Arabidopsis thaliana* I subunit, not shown). Mg-chelatase I subunit proteins contain four Mg-ATPase motifs (Caspers *et al.*, 1994; Koonin, 1997). In the deduced pea Mg-chelatase D sequence, all four of these motifs are conserved (Figure 1).

Preliminary sequence data from the other two large insert clones showed that one, pλD10, was 5′-truncated at nt 402 and 3′-truncated at nt 2545, while the other, pλD6, was 5′-truncated at nt 53 and polyadenylated at nt 2511 instead of nt 2596 (not shown).

Heterologous expression of pea Mg-chelatase D in E. coli

The cDNA corresponding to the putative mature protein was constructed into an *E. coli* expression vector, pProEXHT. This vector has a histidine tag fusion at the N-terminal end and expression is IPTG-inducible. To construct the full-length clone, the 5′ end was first modified by PCR to remove the putative transit peptide (see Materials and methods). The resulting construct, pex3-20, encodes a protein which includes the 693 amino acids of the mature protein plus an extra 24 amino acids for the 6× His tag and rTEV protease cleavage site; the calculated molecular mass is 79.2 kDa.

After induction in DH5α cells, no protein with the predicted molecular mass was induced as detected by Coomassie staining of SDS-PAGE; varying culture temperature or induction time did not significantly improve the expression. However, two new proteins were visible after induction, a minor protein at 89 kDa and a major one at 24.5 kDa (not shown). Soluble extracts from these cells could not be substituted for a chloroplast fraction containing the D subunit in a reconstitution of Mg-chelatase activity assay (Guo *et al.*, 1998).

Both of the induced proteins (24.5 and 89 kDa) were confined to inclusion bodies; these were isolated, solubilized in 6 M urea and passed through a Ni-chelate column (data not shown). The smaller protein did not bind to the column, indicating that it did not have the His tag fusion and could not be derived from the extreme N-terminus of the protein. The larger protein did bind to the column, suggesting that it did have the His tag fusion, and might be our target protein despite the anomalous molecular mass.

Overexpression of a truncated D protein or antigen production

Since the N-terminal part of the protein is homologous to the I subunit, we concluded that the C-terminal part of the protein would make a better D-subunit-specific antibody. The cDNA was truncated at the *Pst*I site (amino acid 597) and cloned into the pProEXHT vector. This truncated construct, pexPst15, encodes a protein containing the C-terminal 158 amino acids of the D subunit linked, at the N-terminal end, to a 47 amino acid unit containing the 6× His tag, protease site and a linker region; the expected molecular mass is 22.4 kDa. After induction a protein close to the correct size, 24.6 kDa, was produced at a high level (not shown). The protein resided in the inclusion bodies and was solubilized in buffer containing 6 M urea. The protein was then purified and used as an antigen for the generation of a polyclonal antibody (see Materials and methods).

As expected, the antibody recognized the protein expressed by the pexPst15 vector (Figure 2A, lane c. It also recognized the 24.5 and 89 kDa proteins from the pex3-20 clone (Figure 2A, lane b). Thus, the 24.5 kDa protein from the pex3-20 vector is a truncated form of the Mg-chelatase D subunit derived from the C-terminal portion of the protein, and the 89 kDa protein is most likely a full-length expressed protein

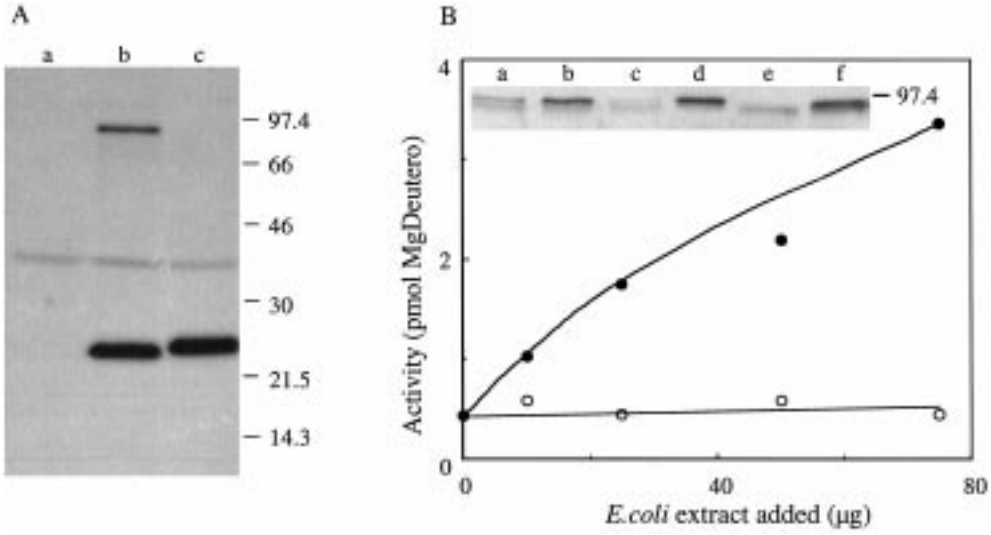

Figure 2. Heterologous expression and co-expression with the chaperonin gene, *dnaK*, of the pea *chlD* cDNA in *E. coli* DH5α. A. Anti-D antibody recognizes heterologously expressed proteins: Lanes: a, vector alone; b, pex3-20; c, pexPst15. In this and all subsequent immunoblots, the membranes were probed with the anti-D antibody, and detection was by means of chemiluminescence from the reaction of peroxidase bound to the secondary antibody. B. Co-expression of the pea *chlD* cDNA with the chaperonin gene, *dnaK*, results in a soluble enzymatically active D subunit. Inset: immunoblot of *E. coli* protein extracts from cells harboring the D expression vector, pexMz3-20 (denoted D), with and without the chaperonin expression vector, pET15b/His-DnaK (denoted K). Lanes: a, D alone not induced; b, D alone induced; c, D plus K not induced; d, D plus K induced; e, soluble extract from induced D alone; f, soluble extract from induced D plus K. Activity graph: Soluble chloroplast extracts were separated into three fractions (Guo *et al.*, 1998). The subunit I- and H-containing fractions were reconstituted with soluble extracts from induced *E. coli* harboring the D expression vector with (●) and without (○) the chaperonin expression vector. The stopped assays were for 2 h and contained 33 μg of the H fraction and 22 μg of the I fraction in 100 μl.

which runs at an anomalously high molecular mass on SDS-PAGE (see Discussion).

Co-expression of the recombinant D subunit with a chaperonin

As reported above, the data from western blotting indicated that the pex3-20 clone was expressing full-length D (Figure 2A, lane b) and our cell fractionation studies suggested that expression was in inclusion bodies (data not shown). Although it is possible with some proteins to recover the protein from the inclusion bodies and refold it *in vitro*, we tested the alternative expression with a chaperonin to facilitate proper folding *in situ* (Caspers *et al.*, 1994). A *dnaK* expression vector (pET15b/His-DnaK), which encodes one of the family of 70 kDa HSP chaperonins (Martin and Hart, 1997) was kindly supplied by Dr Jorge Martin (Brown University, Providence, RI). Since this vector contains the same origin of replication and selection marker as pex3-20, the DNA encoding the full-length recombinant D protein (plus 6× His tag) was moved into a different expression vector (see Materials and methods). Thus, two plasmids, one encoding the chaperonin (pET15b/His-DnaK) and one encoding the recombinant D protein (pexMz3-20), were then used to co-transform *E. coli* BL21(DE3) cells.

A western blot (Figure 2B, inset) shows the effect of the chaperonin on the expression of the recombinant D protein. Co-expression with the chaperonin has a moderate stimulatory effect on the expression level of the D protein when whole-cell extracts from induced cells are compared (lane b versus d). However, when the soluble extracts are compared (lane e versus f), it is apparent that co-expression with the chaperonin results in some of the recombinant D protein remaining soluble.

The soluble proteins from pea chloroplasts have been resolved into three fractions, all of which are required for activity (Guo *et al.*, 1998). These resolved fractions were used to test the recombinant D protein for enzymatic activity in a reconstitution assay (Figure 2B). The soluble extract from cells co-expressing both plasmids was active when combined with chloroplast I and H fractions, whereas the extract from cells expressing only pexMz3-20 was not. For the enzymatically active material, Mg-chelatase activity was proportional to the amount of protein ad-

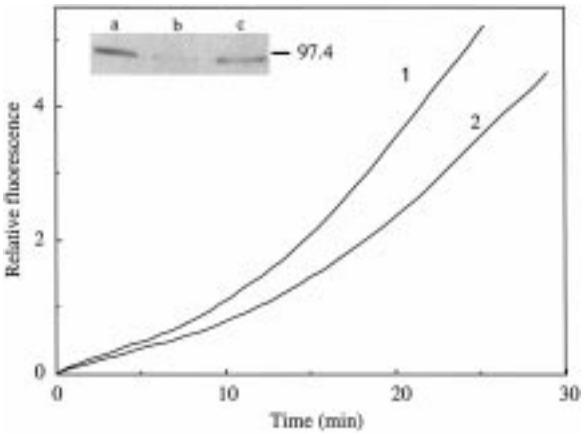

Figure 3. Effect of the anti-D antibody on the native enzyme. Effect on activity: a soluble extract from chloroplasts was preincubated for 1 h at room temperature with preimmune (line 1) or anti-D (line 2) serum at a dilution of 1:12.5. The samples were diluted 6-fold, substrates added, and a continuous assay was run. For line 1 the activity calculated from the linear portion of the curve is 2.31 pmol/min: 600 μg of enzyme was used in a 600 μl assay. Inset: immunoblot of immunoprecipitated proteins. Soluble chloroplast proteins (untreated SP, lane a) were treated with preimmune (lane b) or anti-D (lane c) serum. Antigen-antibody complexes were recovered by precipitation with sheep anti-rabbit IgG coupled to magnetic beads. The recovered D subunit was visualized by western blotting.

ded, and it was dependent on the presence of all the substrates (ATP, Mg^{2+} and deuteroporphyrin).

Immunoprecipitation and inhibition of Mg-chelatase activity with the anti-D antibody

The antibody against the truncated D subunit (referred to as anti-D) was raised against a denatured protein. To test whether the anti-D could recognize the native D subunit, anti-D (or preimmune) serum was incubated in the presence of SP (the soluble protein extract of chloroplasts lysed in a buffer lacking $MgCl_2$; Guo *et al.*, 1998) in a PBS buffer as described in Materials and methods. After overnight incubation, magnetic beads conjugated with sheep anti-rabbit IgG were used to isolate the protein-antibody conjugate; this conjugate was analyzed by western blotting with anti-D (Figure 3, inset). In the sample incubated with anti-D there was a clear immunoreactive band at 89 kDa which was only weakly visible in the sample incubated with preimmune serum indicating that the antibody had recognized the native chloroplast protein.

Immunoprecipitation conditions were used to monitor the effect of the anti-D on Mg-chelatase activity. In a typical continuous fluorometric assay, Mg-chelatase activity has an initial lag time before activity is linear (Walker and Weinstein, 1994). Figure 3 shows the reaction for SP which had been preincubated in PBS in the presence of either preimmune or anti-D serum, prior to dilution with buffer and substrates. In the presence of anti-D (line 2), the reaction lag time was longer and the Mg-chelatase activity measured was lower compared to the sample incubated with preimmune serum (line 1). Typically, the presence of anti-D caused a 24% loss of Mg-chelatase activity when compared with the control of preimmune serum. Similar results of 20–25% inhibition were obtained in the stopped assay when anti-D was compared to preimmune serum (data not shown).

Localization of the D subunit in fractionated chloroplasts

Previously we had published a method for the resolution of SP into three fractions, all of which were required for Mg-chelatase activity (Guo *et al.*, 1998). The presence of the I and H subunits in these fractions were established by western blotting, but since no antibody was available for the D subunit, the presence of this subunit in the fractions had to be inferred. The three fractions were designated: BB (blue-bound protein fraction); FT-lo (low-molecular-mass fraction of the flow-through proteins) and FT-hi (high-molecular-mass fraction of the flow-through proteins). The BB and FT-lo contain H and I subunits respectively (Guo *et al.*, 1998). Western blot analysis with the anti-D antibody showed that, as expected, FT-hi contains the D subunit (Figure 4).

The above fractionation method utilized SP as a starting point; however, we have also described a slightly different fractionation procedure starting from intact Percoll-purified chloroplasts. Figure 4 shows a western blot of fractionated chloroplasts probed with anti-D to determine the behavior of the D subunit under these fractionation conditions. Chloroplasts were lysed and centrifuged to obtain a thylakoid membrane pellet and a supernatant of light membranes and stroma (designated LM/S). As expected from activity profiles, the D subunit was present in the LM/S fraction. LM/S was further fractionated, in the presence of $MgCl_2$, into light membranes (LM) and soluble (S) proteins; the western blot indicated that the D subunit fractionated with the LM. LM were washed with a buffer lacking $MgCl_2$, to yield 'soluble LM' and 'LM-depleted' fractions; immunodetectable protein was observed in both fractions, indicating that

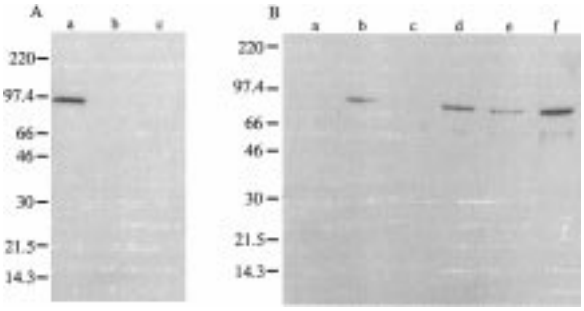

Figure 4. Identification of the D subunit in chloroplast subfractions by western blotting. A. Chloroplast soluble proteins were fractionated into blue-bound and flow-through fractions on a blue-agarose column (Guo *et al.*, 1998). The flow-through was further fractionated by size using centrifugal ultrafiltration units. Lanes: a, high-molecular-mass (>100 kDa) flow-through; b, blue-agarose-bound material; c, low-molecular-mass (<100 kDa) flow-through. B. Intact chloroplasts were fractionated by differential centrifugation in Mg^{2+}-depleted buffer, as described in Results. Lanes: a, thylakoids; b, light membranes and soluble proteins (LM/S); LM/S was made up to 10 mM Mg^{2+} and spun on an ultracentrifuge; c, S supernatant; d, LM pellet. The LM pellet was washed in buffer without Mg^{2+} and spun on an ultracentrifuge. Lane e is the LM wash; lane f is the washed LM pellet. Each lane was loaded with 15 μg of protein.

the washing procedure had dislodged some of the D subunit from the LM.

Discussion

The putative *chlD* cDNA bore all the hallmarks of what is now thought of as a 'typical' D subunit (Walker and Willows, 1997); there were two domains separated by a region with a polyproline stretch and a high abundance of Glu, Asp, Gln and Asn (Figure 1). Overall, the similarity to other D sequences, which was up to 86% with the mature tobacco sequence (Papenbrock *et al.*, 1997) strongly indicated that we had identified the pea *chlD* cDNA. Another feature of a typical D subunit is the clear sequence similarity between the N-terminal domain and the Mg-chelatase I subunit (not shown). While the mechanistic implications of this feature have yet to be determined, it was possible that a polyclonal antibody raised against the full-length D subunit might also recognize the I subunit on a western blot. Subsequent work with an antibody to the soybean I protein (kindly supplied by Dr T. Masuda, Tokyo Institute of Technology, Japan) indicated that this antibody recognized a protein that migrated at the same position on gels as the D protein from chloroplast extracts (C. Walker, unpublished observation). For these reasons the truncated construct (pexPst15) which overexpresses only the C-terminal portion of the protein was used to raise the anti-D antibody. Western blotting of fractionated chloroplasts showed that the anti-D recognized the chloroplast D subunit and did not detect the I subunit (Figure 4). Furthermore, it provided the final piece of evidence that in our previously published fractionation of chloroplasts (Guo *et al.*, 1998), the FT-hi fraction harbored the D subunit as we had predicted. There was no immunodetectable D subunit in either the BB or the FT-lo fractions (H and I respectively) indicating that the separation of the three subunits by our methodology was clean.

The anti-D was also useful in proving that our expressed protein corresponded to the Mg-chelatase D subunit. Although the antibody was raised against a denatured protein, immunoprecipitation studies indicated that it was able to bind to the native protein (Figure 3). Furthermore, the antibody was able to inhibit Mg-chelatase activity (Figure 3). Western blotting confirmed the induction of a higher-molecular-mass protein that corresponded to the full-length protein (Figure 2A). From the derived amino acid sequence of D (Figure 1), the predicted molecular mass of the expressed subunit (including His tag and linker region) is 79.2 kDa, which is significantly smaller than the product observed. The cause for this discrepancy probably lies in the central polyproline and Glu/Gln/Asp/Asn-rich region which has been reported to cause anomalous migration on SDS-PAGE in other proteins (Graceffa *et al.*, 1992; Armstrong and Roman, 1993; John and Keller, 1995). In chloroplast extracts the authentic D subunit also migrates with an anomalously high molecular mass, suggesting that it is unlikely to be an artifact of the clone itself, for example the His tag.

Although the pex3-20 clone expressed full-length protein, this protein was packaged in inclusion bodies. It is thought that proteins form inclusion bodies when they are incorrectly folded after translation (Guise *et al.*, 1996); given that we were attempting the expression of a reasonably large eukaryotic protein in a prokaryotic organism, it was likely that correct folding would be problematic. One way to promote correct folding is co-expression with a chaperonin; in theory, since chaperonins function to maintain and stabilize correctly folded proteins, overexpression of these proteins at the same time as the target protein might aid in forming some correctly folded and active protein. This approach has been successful for several other proteins (Caspers *et al.*, 1994; Martin and Hart, 1997). Our initial success was immediately clear from the

cell lysis and fractionation data which showed that in the presence of the chaperonin we were now observing expression of a soluble D protein (Figure 2B, inset). Final confirmation that the chaperonin had succeeded in promoting correct folding was obtained by reconstituting Mg-chelatase activity when this extract was combined with I- and H-containing fractions from isolated chloroplasts. Thus, we were correct in our putative identification of the *chlD* cDNA, and we also have established a system for the production of soluble, active Mg-chelatase D subunit in an easy manipulatable prokaryotic host.

Our results on the chaperonin requirement for the expression of an active eukaryotic D subunit are not necessarily at odds with those of Papenbrock *et al.* (1997) who demonstrated the activity of the tobacco D subunit in a yeast expression system. In the latter case, expression of activity required the simultaneous co-expression of the I and H subunits in the same host cells. Presumably, co-expression led to subunit-subunit interactions which may have stabilized the newly formed protein. In fact, *in vivo* interactions between the I and D subunits were demonstrated in the same report with the yeast two-hybrid system.

Having an antibody to the D subunit at our disposal, we were also able to address an area which is a long-standing point of confusion about the higher-plant Mg-chelatase, namely its localization (Fuesler *et al.*, 1984; Walker and Weinstein, 1995; Nakayama *et al.*, 1998). There are essentially three fractions to consider in chloroplasts: the stroma (soluble fraction), the thylakoids and the envelopes (membrane fractions). Standard methods for fractionating these components require the use of buffers with low magnesium content (Keegstra and Yousif, 1986; Joyard *et al.*, 1990). Our experiments suggested that the components of Mg-chelatase were soluble under these conditions, and that the presence of magnesium ions caused association of one or more components with the membranes. Recently, it was shown by western blotting that the I subunit is soluble regardless of the $MgCl_2$ concentration in the buffers, whereas H subunit is soluble in the absence of $MgCl_2$ but membrane-associated in its presence (Nakayama *et al.*, 1998). In this paper we have extended these observations to the D subunit. When chloroplasts were lysed in the presence of $MgCl_2$, the D subunit associated with the membranes, but could be partially 'washed off' by a buffer lacking $MgCl_2$. This implied that its behavior is much like the H subunit (Nakayama *et al.*, 1998). In terms of indicating whether the enzyme is associated with thylakoids or envelopes *in vivo*, it seems that both the D and H subunits are too loosely membrane-associated to pin this down by standard fractionation techniques and that the answer to the localization question will require the application of some *in situ* labelling methodology.

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