REVIEW ARTICLE

Mechanism and regulation of Mg-chelatase

Caroline J. WALKER*[1] and Robert D. WILLOWS†

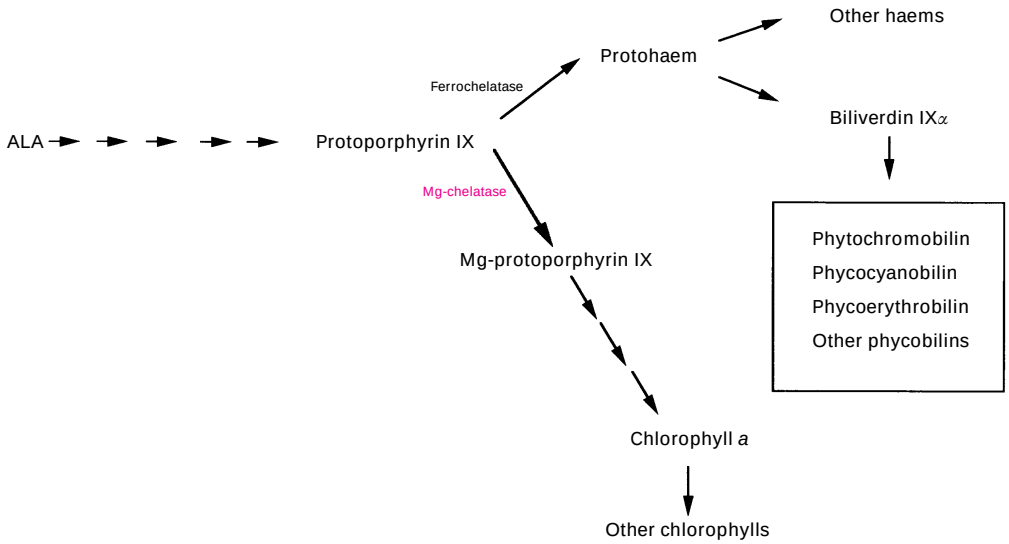
*Department of Biological Sciences, Clemson University, Clemson, SC 29634-1903, U.S.A., and †Division of Biology and Medicine, Brown University, Providence, RI 02912, U.S.A.

Mg-chelatase catalyses the insertion of Mg into protoporphyrin IX (Proto). This seemingly simple reaction also is potentially one of the most interesting and crucial steps in the (bacterio)-chlorophyll (Bchl/Chl)-synthesis pathway, owing to its position at the branch-point between haem and Bchl/Chl synthesis. Up until the level of Proto, haem and Bchl/Chl synthesis share a common pathway. However, at the point of metal-ion insertion there are two choices: Mg^{2+} insertion to make Bchl/Chl (catalysed by Mg-chelatase) or Fe^{2+} insertion to make haem (catalysed by ferrochelatase). Thus the relative activities of Mg-chelatase and ferrochelatase must be regulated with respect to the organism's requirements for these end products . How is this regulation achieved? For Mg-chelatase, the recent design of an *in vitro* assay combined with the identification of Bchl-biosynthetic enzyme genes has now made it possible to address this question. In all photosynthetic organisms studied to date, Mg-chelatase is a three-component enzyme, and in several species these proteins have been cloned and expressed in an active form. The reaction takes place in two steps, with an ATP-dependent activation followed by an ATP-dependent chelation step. The activation step may be the key to regulation, although variations in subunit levels during diurnal growth may also play a role in determining the flux through the Bchl/Chl and haem branches of the pathway.

Mg-CHELATASE: A BRANCH-POINT ENZYME

Enzymes at the branch-point of biosynthetic pathways have always been of interest to biochemists because, as a general rule, they are tightly regulated according to the prevailing metabolic conditions. In photosynthetic organisms, a large part of their biosynthetic effort is put into the synthesis of chlorophyll (Chl) or bacteriochlorophyll (Bchl). However, Bchl/Chl synthesis shares a common biosynthetic pathway with a second end product, haem (Scheme 1). Since haem and Bchl/Chl are each needed in various amounts according to developmental stage and/or light conditions, the co-ordination of haem and Bchl/Chl synthesis has been an area of special interest.

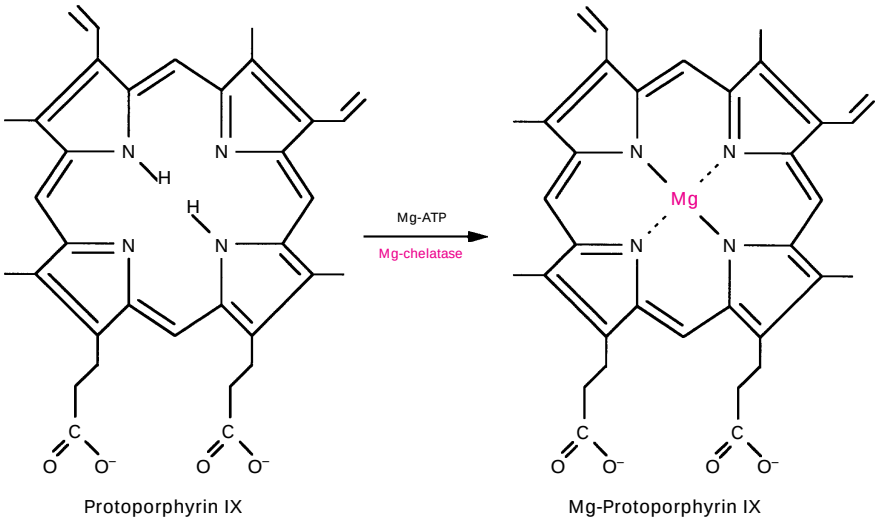
The branch-point of haem and Bchl/Chl synthesis is at the level of protoporphyrin IX (Proto) utilization for metal-ion chelation. In haem synthesis, a ferrous ion is inserted into the tetrapyrrole ring by the enzyme ferrochelatase. In Bchl/Chl synthesis, an Mg ion is inserted into Proto by Mg-chelatase



Scheme 1 Position of Mg-chelatase at the branch-point of haem and Bchl/Chl biosynthesis

Abbreviations used: Chl, chlorophyll; Bchl, bacteriochlorophyll; Proto, protoporphyrin IX; MgProto, Mg-protoporphyrin; ATP[S], adenosine 5′-[γ-thio]triphosphate; PCMB, *p*-chloromercuribenzoate; PCMBS, *p*-chloromercuribenzenesulphonate; ALA, 5-aminolaevulinic acid; Pchlide, protochlorophyllide; Chlide, chlorophyllide.

[1] To whom correspondence should be addressed.



Scheme 2 Reaction catalysed by Mg-chelatase

(Scheme 2). Characterization of the properties of the two branch-point chelatases should throw light on how the levels of haem and Bchl/Chl are co-ordinated in photosynthetic cells.

Ferrochelatase has been characterized in both photosynthetic and non-photosynthetic organisms: it is a membrane-associated protein of about 50 kDa. Catalysis by this enzyme appears to be straightforward, requiring only Proto and ferrous iron as substrates [1]. In contrast, Mg-chelatase has resisted attempts at characterization for many years and has proved to be a much more difficult enzyme to analyse. The major impediment was the lack of an *in vitro* assay, which was only achieved 6 years ago [2]. Since that time our knowledge of Mg-chelatase has expanded dramatically to the extent that the enzyme has been cloned and sequenced from several photosynthetic species [3–10].

The purpose of this Review is to bring together the information on Mg-chelatase that has been published since the review by Beale and Weinstein in 1990 [11], in which the knowledge of Mg-chelatase at that time was described in great detail. With apologies and acknowledgments to those authors who did the pioneering studies on Mg-chelatase, this review will only touch briefly upon their early work. The first half of this review will concentrate on the enzymology, structure and mechanism of Mg-chelatase; the second half will discuss physiological aspects such as localization of the enzyme and the regulation of the branch-point with respect to both enzyme and gene regulation.

DEMONSTRATION OF Mg-CHELATASE ACTIVITY *IN VITRO*

In the 1970s and early 1980s, attempts were being made to assay Mg-chelatase activity in both photosynthetic bacteria and higher plants. In both systems, researchers found two features in common. First, activity could be measured in chloroplasts (higher plants) or whole cells (bacteria), but breakage of the chloroplast or cell invariably resulted in either a complete loss or approx. 98% loss of activity [12–17]. Secondly, in these systems, Mg-chelation was only observed in the presence of ATP [13,15].

The reason for the loss of activity on breakage became apparent when high *in vitro* levels of Mg-chelatase activity were reported for lysed pea (*Pisum sativum*) chloroplasts [2]. The key to preserving activity was to lyse chloroplasts at a high protein concentration so as not to dilute the chloroplast contents. When this lysed preparation was separated into a stromal and a membrane fraction, the activity was lost. However, recombination of these two fractions restored activity. The authors concluded that Mg-chelatase must be a multicomponent enzyme, requiring at least two proteins, one membrane-associated and one soluble. Interestingly, the activity was still absolutely dependent on ATP, eliminating any possibility that the ATP requirement was an artifact of assaying the enzyme in whole chloroplasts or sphaeroplasts.

It is worth noting that, initially, these results were considered surprising. Since ferrochelatase and Mg-chelatase carry out virtually identical reactions, there was the assumption that these enzymes would be similar (i.e. a single membrane-bound protein). Shortly after the report of *in vitro* activity from peas was published, the measurement of high *in vitro* Mg-chelatase activity from lysed cucumber (*Cucumis sativus*) etioplasts was described in which activity was confined to the membrane fraction [18]. In that study the authors gently lysed the etioplasts in a fortified medium containing high concentrations of $MgCl_2$, ATP and Proto. Interestingly, the addition of soluble proteins back to the membranes had a slight inhibitory effect on Mg-chelatase activity, reinforcing the authors' conclusion that Mg-chelatase was a membrane-bound enzyme.

Mg-chelatase has now been confirmed to be a multicomponent enzyme in several organisms by both biochemical assay and molecular genetics [4,6,19]. While the conclusions of Lee et al. [18] may initially seem invalid, more recent data has suggested that Mg-chelatase functions as a multicomponent complex [20,21]. If this proves to be the case, Lee et al.'s data can be reinterpreted to suggest that the presence of high concentrations of Proto, ATP and Mg^{2+} during gentle lysis may stabilise the entire Mg-chelatase complex.

Mg-CHELATASE HAS THREE COMPONENTS

The *in vitro* assay of Walker and Weinstein [2] established that a minimum of two proteins were required for Mg-chelatase activity. Since either the membrane or the soluble fraction could contain multiple Mg-chelatase subunits, the exact number of individual proteins or subunits involved in the reaction could not be established.

Progress on identifying the subunit structure of Mg-chelatase came from work on the photosynthetic bacteria *Rhodobacter capsulatus* and *R. sphaeroides*. In these organisms, the genes for Bchl synthesis are present in a single 45 kb region of the chromosome, termed 'the photosynthesis gene cluster', which has now been completely sequenced (Reviewed in [22]). Assigning functions to the genes in the cluster has been achieved in many cases by insertional mutagenesis studies in which an open reading frame is disrupted and the biosynthetic intermediate is identified [23]. In theory, the enzyme which utilizes this intermediate as a substrate can be assigned to the mutated gene.

The three genes, *bchI*, *bchD* and *bchH*, were expected to code for Mg-chelatase subunits, as the Mg-chelatase precursor, Proto, accumulated when they were each disrupted [24]. Gibson et al. confirmed this assignment biochemically, by cloning these genes into expression vectors [4]. The expressed proteins were all soluble, and when combined had Mg-chelatase activity. The authors found that they only observed Mg-chelatase activity when all three proteins were present. The levels of activity were high (2 nmol/h per mg of protein), and again, the activity required ATP. The *bchD* gene is found immediately after the *bchI* gene as part of the same operon in the photosynthesis gene cluster, and the authors found that they were only able to get expression from *bchD* if it was cloned into an expression vector with *bchI* as an *ID* construct. Even in these clones, expression from *bchD* was not visible on Coomassie Blue-stained protein gels. The lack of activity with the combined extracts from *Escherichia coli* cells that were expressing BchI and BchH was a persuasive argument that BchD was a required subunit.

The best evidence for a three-component Mg-chelatase enzyme was obtained when expressed proteins were purified [21]. In crude extracts from clones containing the *bchID* construct, an initial gel-filtration step separated the BchI and BchD subunits. The recombinant BchI and BchH were subsequently purified to homogeneity, and the BchD was highly purified, but could not be separated from contaminating *E. coli* GroEL. The net result of this work was to obtain three highly purified Mg-chelatase subunit fractions that were all required for Mg-chelatase activity, providing convincing evidence that the enzyme has three subunits.

A similar result has been obtained from the cyanobacterium *Synechocystis* [6] where, on the basis of sequence similarities to the *Rhodobacter* Mg-chelatase genes, three putative Mg-chelatase genes were isolated and cloned. Since *Synechocystis* is a Chl- rather than a Bchl-containing organism, these three genes were denoted *chlI*, *chlH* and *chlD* [6,7]. Expression clones were constructed for all three subunits, with the I and D subunits in separate vectors. All three expressed proteins were soluble and all were necessary for Mg-chelatase activity.

FRACTIONATION OF Mg-CHELATASE IN HIGHER-PLANT EXTRACTS

Are there also three proteins for Mg-chelatase in higher plants? Although there has been less success with isolating Mg-chelatase genes from higher plants (potential Mg-chelatase genes that have been isolated so far will be discussed in detail below), there is evidence that Mg-chelatase also has at least three subunits in these organisms. This evidence comes from mutant studies and protein fractionation work.

In barley (*Hordeum vulgare*), there is a series of Chl-synthesis mutants which have been identified on the basis of the accumulation of intermediates when fed the Chl precursor 5-aminolaevulinic acid (ALA).

Three non-allelic mutants, *Xantha-f*, *Xantha-g* and *Xantha-h*, accumulate Proto under these conditions [25,26]. In developing chloroplasts isolated from these mutants, no Mg-chelatase activity was detectable [7]. However, the pairwise combination of lysed chloroplast extracts from these mutants restored activity [19]. The deduction from these assays is that each mutant has a lesion in a protein component of Mg-chelatase and, by implication, Mg-chelatase has at least three components.

Protein-fractionation work has also suggested that there are three components. Mg-chelatase is readily solubilized from membranes in the absence of Mg [5,19,27]. The solubilized proteins have been separated into three fractions by pseudo-affinity chromatography and ultrafiltration [28], and all three fractions are necessary for Mg-chelatase activity.

Until three proteins are either purified to homogeneity or cloned and expressed from higher plants, there will always be the possibility that more than three proteins are involved in Mg-chelatase. To date, the sequence information we have on Mg-chelatase genes indicates that the enzyme is conserved in all photosynthetic species and it is most probable that higher plants will also have a three-component enzyme.

Mg-CHELATASE MUTANTS

As discussed above, Bchl/Chl-deficient mutants of photosynthetic organisms have been invaluable in the identification of the structural genes responsible for Mg-chelatase. These Mg-chelatase mutants have generally been identified by their inability to make Bchl/Chl and by their accumulation of the enzyme's substrate, Proto. Several examples of Mg-chelatase mutants are described in this Review, and for these mutants it has been possible to identify which of the three subunits is mutated. However, there are also several other mutants which satisfy these criteria and have been suggested in the literature to be Mg-chelatase mutants. It should be noted that these putative Mg-chelatase mutants have not been confirmed either by biochemical assay or by the sequences of the mutated genes, and the importance of such additional identification cannot be overstated. For example, mutations in protoporphyrinogen oxidase also display the phenotype of accumulating Proto, due to the oxidation of the accumulated protoporphyrinogen [29]. Additionally, it has been noted that the *brs-2* mutant of *Chlamydomonas* is defective in chloroplast-membrane development and has a pleiotropic effect that causes the accumulation of Proto [30]; these authors also noted that some carotenoid synthesis mutants also accumulate some Proto.

For the sake of completeness, and as a general reference, Table 1 lists all of the Mg-chelatase mutants, both putative and confirmed. In each case the reason for classification is given and where possible, the mutant subunit is identified. With the current availability of probes and antibodies it is possible that some of these putative mutants will be identified in the near future.

MECHANISM OF Mg-CHELATASE

The first significant progress towards understanding the mechanism of Mg-chelatase came from the development of a continuous fluorimetric assay in which chloroplast fractions with a low Chl content were used [31]. With the improved time resolution of this assay, it became apparent that there was a lag of several minutes before Mg-chelatase activity was observed after the addition of all the substrates. This lag in activity could be eliminated if the enzyme was preincubated with ATP, which suggested that there is a two-stage process: an ATP-dependent activation followed by Mg-chelation. Therefore, the further discussion of the mechanism of Mg-chelatase will be described in terms of the 'activation' and 'chelation' steps.

Table 1 Bchl/Chl-deficient mutants of photosynthetic bacteria, algae and plants with the demonstrated or probable defect at the Mg-chelatase step

These mutants accumulate Proto either under normal growth conditions or when fed ALA.

Species	Mutant	Subunit effected	Comments	References
R. capsulatus	ZY6	H	Interposon mutant of *bchH*	[73]
	DB561	D	Interposon mutant of *bchD*	[23]
	DB350	I	Interposon mutant of *bchI*	[23]
R. sphaeroides	D61	D	Total of nine transposon mutants of *bchD* some of which may be in *bchI*	[65,74]
	I55	I	Transposon mutant of *bchI*	[74]
	H340, H335, H241	H	Transposon mutants of *bchH*; total of 20 H mutants	[65,74]
H. vulgare (barley)	*xantha-f*	H	*xantha-f26* is leaky; other alleles are completely blocked	[7,19,26,75]
	xantha-g	D	*xantha-g45* is leaky; other alleles are completely blocked	[7,19,26,75]
	xantha-h	I	All mutant alleles are completely blocked	[7,19,26,75]
	chlorina-104	Unknown	May be regulatory mutant	[77]
Zea mays (maize)	*oil yellow 1*	Unknown	*oy-1040* completely blocked; *oy-1039* leaky; non allellic to *I13* or *I*Blandy4*	[78]
	I-Blandy4*	Unknown	Completely blocked; non allellic with *oy1* or *I13*	[78]
	I13	Unknown	Leaky mutant; non-allellic with *oy1* or *I*Blandy4*	[78]
Triticum sp.(wheat)	chlorina-214, CD3, chlorina-1	Unknown	All mutants leaky; reduced amounts of chlorophyll *b*	[77]
Antirrhinum majus	*olive*	H	Transposon-tagged *chlH*	[8]
A. thaliana	*chlorata-42*	I	T-DNA-tagged *chlI*	[9]
	xantha-2	Unknown	Non-allelic to *xantha-3*	[79]
	xantha-3	Unknown	Non-allelic to *xantha-2*	[79]
Chlorella vulgaris	W5B	Unknown		[80]
Chlamydomonas reinhardtii	*brs-2*	Unknown	Lethal when grown in light; probably defective in plastid membrane development	[30,81]
	brs-1	Unknown	Lethal when grown in light; trace of Chl in the dark; non-allelic to *brs-2* or *brc-1*	[30,81]
	brc-1	Unknown	Normal phenotype in light; trace of Chl in dark and Proto accumulates; non allelic to *brs-2* or *brs-1*	[30,81]
	y-y	Unknown	May be pleiotrophic mutant analogous to *brs-2*	[82]

In pea chloroplast extracts, either adenosine 5′-[γ-thio]- PN4 triphosphate (ATP[S]) or ATP could overcome the lag period, implying that either substrate could be utilized for activation [20]. ATP[S] is typically used as a protein kinase substrate, raising the possibility that protein phosphorylation may play a part in the activation process. However, when ATP[S] was used for activation, the subsequent addition of ATP was required for Mg^{2+} chelation to take place. This implied that ATP was also required for the Mg-chelation step and that the role of ATP may be different in each of these two steps. The optimal ATP concentration for both steps was determined and found to be higher for activation than for Mg-chelation. The activation step was also characterized as requiring high protein concentrations, and after activation the sample could be diluted without loss of activity. Put together, these observations suggested that activation required a concentration-dependent protein–protein interaction and either ATP or ATP[S], and that Mg-chelation had an absolute requirement for ATP and was not sensitive to protein concentration.

With the cloning and expression of the *Rhodobacter* Mg-chelatase, a more detailed analysis of the mechanism could be made with purified protein fractions [21]. In a continuous assay, the *Rhodobacter* Mg-chelatase kinetics resembled the higher-plant-enzyme kinetics in that there was an activation step and Mg-chelation step. However, the activation could be achieved by incubating only the I and D subunits with ATP, while H was only required for the chelation step. This result has recently been confirmed in fractionated pea chloroplasts, where the fractions of the higher-plant I and D counterparts are required for activation [28].

The roles of the I, D and H proteins can only be guessed at. When the H protein is expressed in host cells, it has Proto non-covalently bound [4,21]. In the purified protein, the Proto/H subunit ratio is 1:1 [21]. Dynamic-light-scattering studies have shown that purified H is present as a monomer, whereas I is present as a dimer. For optimal activity a ratio of one I dimer to one H monomer is required [21]. The lack of pure D protein did not enable this study to be extended to all subunits, although it would seem that D is only needed in very small amounts [6,21].

CHEMICAL CONSIDERATIONS FOR Mg^{2+} INSERTION

It is possible to gain an insight into the mechanism of an enzyme by examining how the reaction takes place in the absence of biological catalysts. The chemistry of the metal-ion insertion into porphyrins, termed ‘metalation’, in aqueous and non-aqueous systems has been extensively reviewed [32,33], so the discussion will be limited to aspects which bear directly on Mg-chelatase.

The mechanism of metalation of porphyrins with bivalent metal ions requires at least four steps which may occur sequentially or in concert: (I) deprotonation of the pyrrole nitrogen atoms; (II) removal of ligands that are co-ordinated to the metal ion or metal-ion carrier, for example, water molecules; (III) co-ordination of the metal ion with the four pyrrole nitrogen atoms; (IV) completion of the co-ordination sphere, if required, with an axial ligand [32]. The ease with which a metal is inserted into a porphyrin will depend in part on all of these parameters. For example, Cu^{2+} is inserted very easily, and the deprotonation of the pyrrole nitrogens occurs in concert with metal-ion insertion [33].

For Mg^{2+} chelation, the initial step is most likely to involve a rate-dependent dissociation of a hydrogen ion to yield a porphyrin anion, followed by rapid reaction with the Mg^{2+} cation [34]. Pyridine and other nitrogenous bases with pK_a values in the range 4–7 act as catalysts, probably by forming a pyridine–Mg^{2+}-porphyrin complex in which the co-ordinated Mg^{2+} aids in the dissociation of the hydrogen ions [34]. The activation energy is reduced from 152.7 kJ (36.5 kcal)/mol for the uncatalysed reaction to 66.1 kJ (15.8 kcal)/mol) for the catalysed reaction. Given the energetic advantages of using the catalysed reaction, it

Figure 1 Consensus sequence derived from the deduced amino acid sequences of the H-subunit genes

The consensus was created from an alignment of the H subunits from *Arabidopsis thaliana*, *Hordeum vulgare*, *Antirrhinum majus*, *Synechocystis* P.C.C. 6803 and *Rhodobacter capsulatus* with GenBank accession numbers Z68495, U26916, X73144, U29131 and M74001 respectively. '*' Indicates completely conserved residues and '.' indicates conservative substitutions. Lower-case 'x' indicate amino acids that are not conserved. The numbering is for the *Arabidopsis sequence* and starts from position 51, which is after the chloroplast transit sequence. Gaps created by pile-up are indicated by '—'. Residues that are underlined are conserved in the other H-subunit counterparts; the Co-chelatase CobN from *Pseudomonas denitrificans*, accession number M62866; the putative cobaltochelatases and nickle chelatase homologues from *Methanococcus jannaschii*, accession numbers U67585 and U67534, and *Synechocystis* PCC 6803, accession number D90905. The three conserved histidine residues are coloured red. The programs in the GCG program package (GCG Inc., Madison, WI, U.S.A.) were used to help create this diagram.

is probable that the biological insertion of Mg^{2+} into Proto may also require the formation of a nitrogenous base–Mg^{2+}-porphyrin complex. The most likely candidate for the nitrogenous base is a histidine residue, and in the H subunit (which binds Proto) there are three histidine residues which are conserved in all of the subunit counterparts (H^{664}, H^{668} and H^{813}; Figure 1).

As Mg-porphyrins are invariably five-co-ordinate [33], the final stage in Mg^{2+} insertion is likely to be the addition of an axial ligand. If a nitrogenous base is involved in the insertion of Mg^{2+}, this base probably also acts as the axial ligand. Again, on the Mg-chelatase this base would also probably be a histidine residue and when the enzyme releases the Mg-protoporphyrin (MgProto), the fifth ligand on the protein may be replaced by a water molecule.

So what is the function of ATP in the Mg-chelatase reaction? A clue to the role of ATP may be found in the relative rates of bivalent-metal-ion insertion into porphyrins, which follow the order [33,35]:

$$Cu > Zn > Mn, Co, Fe > Ni > Cd \gg Mg$$

The above rates correlate with the ability of these metal ions to render labile a co-ordinated water molecule, and the position of Mg^{2+} in this rate order suggests that one of the functions of the Mg-chelatase may be the active removal of one or more co-ordinated water molecules; this could be achieved by the use of this water for the hydrolysis of ATP.

On the basis of the work described above we can speculate that the Mg^{2+} is probably bound to the H subunit via a histidine residue to form a histidine–Mg^{2+}-Proto complex. In order for the Mg^{2+} to bind, the enzyme must first remove one or more co-ordinated water molecules from the Mg^{2+} ion's co-ordination sphere, and this may be achieved by utilizing the water in the hydrolysis of ATP. As the histidine–Mg^{2+}-Proto complex is formed, the dissociation of hydrogen ions from the pyrrole nitrogens is promoted and the Mg^{2+} is sequentially co-ordinated to the pyrrole nitrogen atoms and moves it into the plane of the ring [33]. The Mg^{2+} remains co-ordinated by the pyrrole nitrogen atoms and the histidine residue until the MgProto is released from the active site, at which point a water molecule will replace the histidine residue in the co-ordination sphere.

It should be emphasized that there is no direct experimental evidence that the Mg^{2+} which is inserted into Proto is bound to the H subunit. The mechanism above is based on comparisons with Co-chelatase (see below) and the observation that Mg^{2+} is required in excess of the ATP concentration; the mechanism is also consistent with the chemical properties of the system. However, a second possibility is that ATP-bound Mg^{2+} is inserted into Proto. Establishing the function of the conserved histidine

residues on the H subunit may well help to distinguish between these two possiblities

GENE SEQUENCES FOR D, I AND H SUBUNITS

The I, D and H subunits of Mg-chelatase have been cloned, sequenced and assayed for activity in several organisms. By searching the database for similar sequences it is possible to find many more I-, D- and H-like sequences and, on the bais of conserved motifs, speculate as to the function of the subunits.

There are currently 13 counterparts of the I subunit of Mg-chelatase in the GenBank Nucleotide Sequence Database that have at least 49 %, and up to 86 %, identity with each other at the amino acid level. These proteins range in size from 37 kDa for the chloroplast-encoded subunit I of *Olisthodiscus luteus* (a unicellular alga) to 46 kDa for the nuclear-encoded *Arabidopsis thaliana* (thale cress) subunit I counterpart which includes a N-terminal chloroplast transit peptide [5]. It is interesting that only in the cases of the non-chlorophyte algae are the subunit I sequences encoded by the chloroplast genome. An alignment of these sequences was used to create the consensus sequence shown in Figure 2. It is noteworthy that the similarity dendogram that was generated by the Genetics Computer Group (GCG) program 'pileup' when these sequences were aligned was similar to the phylogenetic relationship of these organisms deduced by a comparison of *tufA* genes [36].

All of the subunit I sequences have the three Mg-ATPase motifs (Figure 2) identified by Koonin, and by this grouping the I subunit sequences are in a superfamily of putative DNA-dependent ATPases [37,38]. The I subunit sequences can be further grouped to a subfamily whose members are involved in the initiation of DNA replication or in transcription regulation. However, since there is no experimental evidence to indicate that Mg-chelatase requires DNA for activity, it is most probable that the sequence similarity is due solely to structural and functional conservation of the ATPase domain in this subunit. It is expected that one of the Mg-chelatases will have an ATPase activity, since ATP hydrolysis is required for activity. It therefore seems reasonable to predict that the I subunit binds to, and hydrolyses, ATP during the reaction.

D subunit sequences are less abundant in the GenBank Database, with only four counterparts of this subunit. Only three of these sequences encode for Mg-chelatase subunits, with the fourth being a putative Ni-chelatase subunit. The D subunits have calculated molecular masses of 87, 73 and 60 kDa for the pea, *Synechocystis* and *Rhodobacter* sequences respectively [4,6,39]. One interesting feature of all the D subunits is the proline-rich region at the centre, followed by a highly charged stretch of amino acids. Proline residues are thought to be important in protein–protein interactions [40] and this region may be important in interacting with the H or I subunits. Figure 3 shows a Dot-plot comparison [41] of the subunit I consensus sequence shown in Figure 1 with a subunit D consensus generated from the three D subunit sequences. Diagonal lines on this Figure show regions of similarity between the two compared sequences, and it is obvious that the N-terminal of the D subunits show similarity to the I subunit. The D subunits from pea and *Synechocystis* (i.e. organisms that make Chl) have retained all three Mg-ATPase motifs (see Figure 2), while the *Rhodobacter* D subunit appears to have lost all except the second motif. The MgATPase motifs on the D subunits may be very significant in that they give the first indication that enzymes from Bchl- and Chl-forming organisms might have substantial differences. In fact, as discussed previously, higher plants have a requirement for ATP in both the activation and chelation steps and, since only the activation step may utilize ATP[S] instead of ATP, the data suggest that these ATP requirements are separate. Possibly the D-bound ATP is used in one step and I-bound ATP for the other. Why does the bacterial enzyme not have the second ATP-binding site? If the D subunit participates in regulation, there is likely to be a difference in its properties between *Rhodobacter* and Chl-producing species which face substantial differences in controlling haem and Bchl/Chl synthesis; possibly the variation in D sequence reflects this.

There are nine counterparts of the H subunit in GenBank that range in size from 123 to 153 kDa. Only five of these are Mg-chelatase subunits, with the remainder encoding Co-chelatase subunits or putative Ni-chelatase subunits (see below). The sequence identities of the Mg-chelatase H subunits range from between 38 %, comparing *Rhodobacter* with barley, and up to 86 %, comparing *Arabidopsis* with *Antirrhinum* (snapdragon). Figure 1 shows the consensus sequence for the Mg-chelatase H subunit and the residues that are 100 % conserved in the sequence of all nine counterparts of the H subunit. A function for these proteins cannot be surmised from a comparison with other sequences of known function. However, the function of H is thought to involve both the binding of Proto, based on biochemical observations [4,21] and the binding of the Mg^{2+} used for chelation, based on evolutionary parsimony with the Co-chelatase H counterpart which binds both the tetrapyrrole and

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                                          ____A____
86    PVFPFTAIVG QDEMKLxLLL NVIDPKIGGV MIMGDRGTGK STTIRALxDL LPEIxVVxxD PFNSxPxDxE MMxEEVRxxx xxGExLxxxx xKVxMVDLPL
      .......... ...... ... ....*.**** ...*..**.* **..*.. .. **.* ..  . .... . . . .. ....      .. .      .. ...**.
                                     ____B____                                                 ____C____
186   GATEDRVCGT IDIEKALSEG VKAFEPGLLA KANRGILYVD EVNLLDDHLV DVLLDSAASG WNTVEREGIS IRHPARFVLV GSGNPEEGEL RPQLLDRFGM
      *..****.*. .**..*...* .*..***.** .***  **.* *.***.**.. *.***.... .*..**.*.. ..*..**.*. *..*****.* ***.*****.

286   HAxIxTVRDx xLRVxIVExR xxFDxxPxxF xDxYxxxQxx LxxxIVxAxx xLxxVxIxxD LRVKISxVCx ELxVDGLRGD IVTNRAxAKA xAAxxGRxxV
      .. . ....   .** ... .   .. *  .  . .   .   .  *. *    .  . . .  ..... .*  .* ....*.. ....*. *..  ..  ..  *

386   TxDDIxxVIx LCLRHRLRKD PLESIDxSGx KVxxxFxxVF
      . ...  ..  ..*.**.*.. *..... ..  ..   .  ..
```

Figure 2 Consensus of I subunits showing the three conserved ATPase-binding motifs [37]

The sequence numbering is for the *Arabidopsis thaliana* sequence and starts from position 86, which is after the chloroplast transit sequence. The consensus is from an alignment of the I subunits from *Cryptomonas phi, Methanococcus jannaschii, Arabidopsis thaliana, Euglena gracilis, Cyanophora paradoxa, Olisthodiscus luteus, Hordeum vulgare, Rhodobacter capsulatus, Synechocystis* sp. P.C.C. 6803, *Anabaena variabilis, Odontella sinensis, Porphyra purpurea* and *Glycine max* with GenBank accession numbers Z21976, U67534, X51799, X65484, U30821, S32166, U26545, Z11165, U35144, D49426, Z67753, U38804, D45857 respectively. The three MgATPase motifs are indicated (A, B and C) and the most important residues are shown in red. '*' Indicates completely conserved residues and '.' indicates conservative substitutions. Lower-case 'x' indicates amino acids that are not conserved. This alignment was created using programs in the GCG program package.

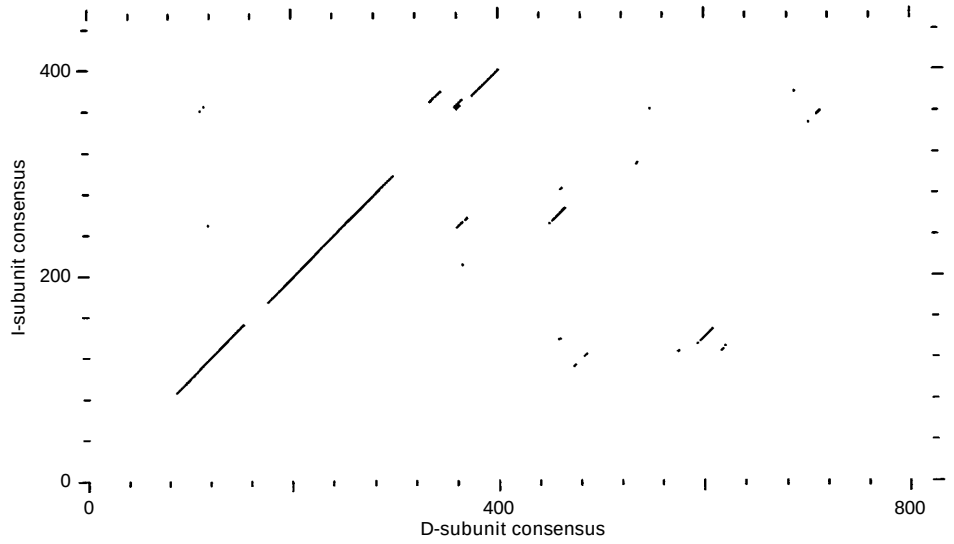


Figure 3 Dot-plot of the D-subunit consensus with the I-subunit consensus

The axis numbers are the amino acid residues starting from the N-terminus. The programs in the GCG program package were used to help create this diagram.

Co^{2+} substrates (see the discussion below) [42]. Therefore, some of the conserved sequences must be involved with porphyrin and Mg^{2+} binding, and possibly also catalysis, if it takes place on this subunit. On the basis of chemical studies (see above) we have suggested that three conserved histidine residues might participate in the reaction.

While functions to proteins based on sequence similarities must be assigned with caution, it seems likely that the I subunit binds MgATP. In the Chl-containing organisms studied so far, the D subunit may also bind MgATP. Given that the biochemical data suggest that, mechanistically, the Mg-chelatases of *Rhodobacter* and pea are the same, the sequence differences in the D subunit may be related to regulation rather than mechanism. This information can be incorporated into models of how Mg-chelatase is operating, and has been used below in the discussion of the mechanism.

A MODEL FOR Mg-CHELATASE ACTIVITY?

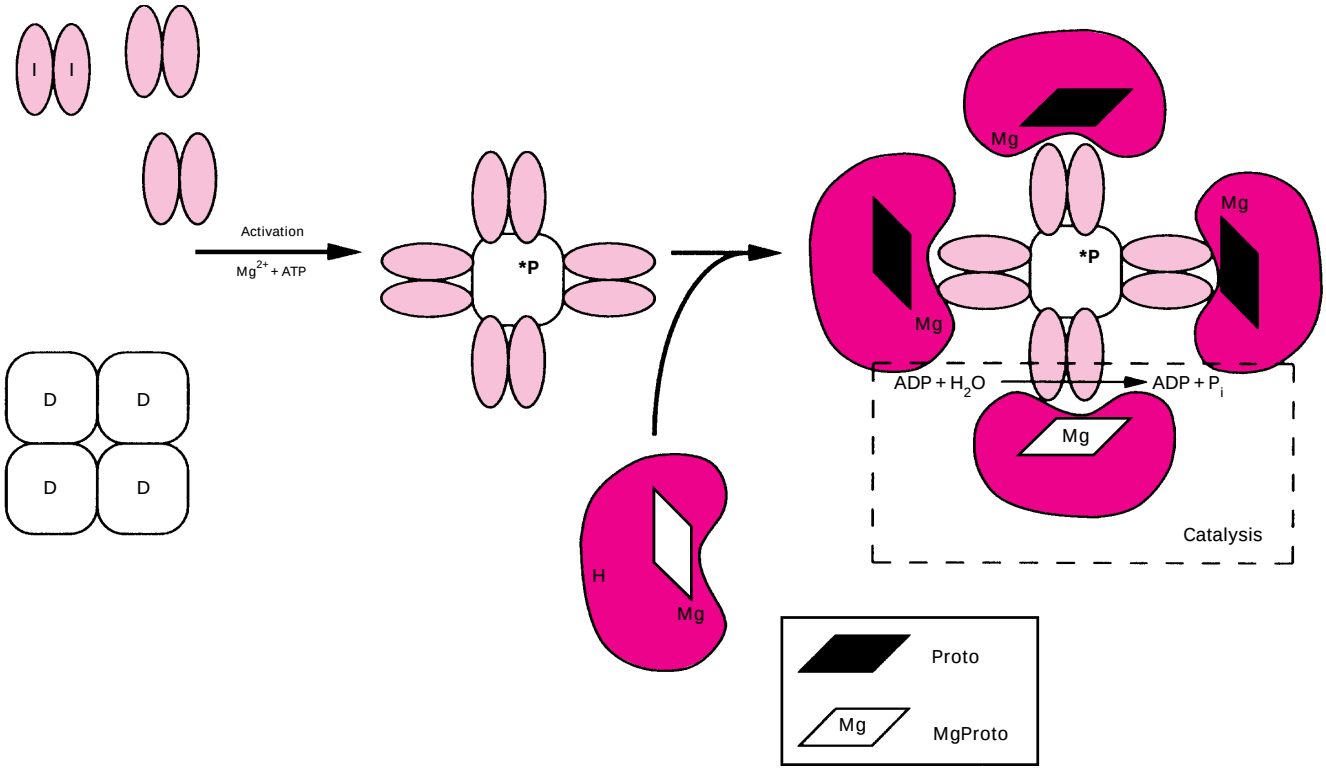
Combining information from biochemical assays, chemical models and gene sequence comparisons of an enzyme can give some insights into how that enzyme is working. While this is true for Mg-chelatase, research on this enzyme is at an early stage and the gaps in our knowledge of this enzyme are considerable; any model proposed under these circumstances should therefore be considered as highly speculative. Therefore, the model that we present in Scheme 3 is provisional, but is consistent with the data that are available in the current literature.

The first step in the reaction is activation. In this step, we suggest that I is a dimer which interacts with a D subunit. Since D appears to behave as an aggregate [19,28], this step is shown as involving the dissociation of a single D subunit from the aggregate: a process which may be precipitated by the presence of I dimers and ATP. This step requires ATP or ATP[S] and could involve protein phosphorylation. However, it should be noted that there is no direct evidence for protein phosphorylation in the activation step at this time; the participation of a protein kinase has been included solely in an attempt to incorporate the ATP[S] data into the model. Since both D and I have ATP-binding sites (at least in Chl-containing organisms), either subunit could be acting as a protein kinase; in the Scheme, ***P** denotes a phosphorylated $I \cdot D$ complex and is not intended to suggest which of the two subunits is phosphorylated. The product of the first step is an activated $I_2 \cdot D$ complex, which is shown with a low ratio of D to I to accommodate experimental observations [6,21]. However, the number of I_2 dimers which bind to D (shown as four in the Scheme) is not yet known. The second step of catalysis is Mg^{2+} insertion, and involves the H subunit (with its bound Proto and Mg^{2+}) coming together with the $I_2 \cdot D$ complex. The I subunit of the $I_2 \cdot D$ complex then drives the release of a water molecule from the Mg^{2+} ion's co-ordination sphere, by using the water as a substrate for the hydrolysis of ATP. The released Mg^{2+}, co-ordinated to a histidine residue on the H subunit, promotes the dissociation of hydrogen ions from the pyrrole nitrogen atoms and is then itself co-ordinated into the porphyrin macrocycle.

This model suggests that the I and H subunits participate in catalysis, and the D subunit only in activation; this is based solely on the stiochiometry of the subunits [6,21] rather than properties of the subunits. If this model is correct, one would expect a large complex to form in the presence of Proto, ATP and Mg^{2+}. Possibly this has been observed experimentally by Lee et al., who isolated their etioplast membranes under these conditions and reported Mg-chelatase to be a single membrane-bound protein [18].

COMPARISON OF OTHER METAL-ION CHELATASES WITH Mg-CHELATASE

Comparing the insertion of divalent-metal ions into tetrapyrroles is also interesting from the standpoint of the enzyme-catalysed reactions. In living organisms there are six other metal ions, namely Fe(II), Co(II), Ni(II), Zn(II), Mn(II) and Cu(II) that are found in tetrapyrroles. There is nothing presently known about the enzymic formation of the Cu(II) tetrapyrroles found in the wing feathers of *Taracus indicus* (violet turaco), the Zn-protoporphyrin found in yeast mutants or the Mn-porphyrins found in blood, and it may be that these compounds are formed non-



Scheme 3 Possible model for the mechanism of Mg-chelatase

During activation, a D subunit dissociates from a D aggregate and associates with I dimers to form an $I_2 \cdot D$ complex. ATP is involved in the formation of the $I_2 \cdot D$ complex and the model shows phosphorylation by a protein kinase in this step; the involvement of a kinase activity is speculative, and in the model ***P** denotes a phosphorylated $I_2 \cdot D$ complex where the position of the phosphorylated residue with regard to either subunit is unknown. The activation step does not require the presence of the H subunit or Proto and, for the sake of clarity, these are not shown in this part of the model; however, during activation under physiological conditions, H and Proto would be present. In the second step of the reaction, the $I_2 \cdot D$ complex associates with H subunits and the $H \cdot I_2 \cdot D$ complex catalyses the insertion of Mg^{2+} into Proto. Insertion of Mg^{2+} is accompanied by ATP hydrolysis (shown for one of the H subunits in the box surrounded by the broken line); the water molecule for hydrolysis is proposed to come from the co-ordination sphere of the Mg^{2+} ion. The stoichiometry of $H:I_2 \cdot D$ complex is not known, but has been shown as 4:1 to maintain the reported ratio of $1H:1I_2$ [21].

enzymically. However, the two classes of Fe(II)-containing tetrapyrroles, which are the haems and sirohaem, are formed enzymically with separate ferrochelatases catalysing the insertion of the Fe^{2+}. These ferrochelatases bear no resemblance to Mg-chelatase at either the enzymic or DNA-sequence levels and do not require ATP for activity [1]. The lack of an energetic requirement for Fe^{2+} insertion may reflect the relative ease of Fe^{2+} insertion compared with Mg^{2+} insertion for the chemical reaction as discussed above.

The Co(II)-containing tetrapyrroles are intermediates in the biosynthesis of cobalamins, such as vitamin B-12, and there are at least two separate biosynthetic pathways for the synthesis of these tetrapyrroles. The essential difference between the two pathways is the stage at which Co^{2+} is inserted with respect to ring contraction [43]. In *Propionbacterium shermanii*, a Co-chelatase acts before the contracted corrin ring is formed, and the enzyme appears to be a single polypeptide that does not require ATP [44]. In contrast, in *Pseudomonas denitrificans* the metal ion is inserted after the contraction of the corrin ring, which, due to the smaller size of the ring, is a less favourable reaction in energetic terms; this Co-chelatase is ATP-requiring and consists of two separate proteins, CobN and CobST, made up from three different subunits [42]. The CobN of the cobalto-chelatase, which is a counterpart of BchH [6,8], binds both the tetrapyrrole and the Co^{2+} substrates for the reaction, forming a ternary enzyme$\cdot Co^{2+} \cdot$corrinoid complex [42]. Like the I subunit of Mg-chelatase, the N-terminal of the CobS subunit also has an ATP-binding consensus and Mg^{2+}-binding consensus [37,38], but the remainder of the protein shows no further sequence similarity to I. The third CobT subunit has no sequence similarity to the D subunit of Mg-chelatase. At the enzyme level, the K_m values for the Co-chelatase were 85 nM for hydrogenobyrinic acid *a*,*c* diamide, 220 μM for ATP [42] which are comparable with K_m values of Mg-chelatase of 360 nM or 13 nM for Proto, and 166 μM or 350 μM for ATP (bacterial and plant respectively) [21,28]. The K_m value for the Mg^{2+} of the Mg-chelatase is not directly comparable with the K_m value for Co^{2+}, since both enzymes utilize ATP as the Mg$\cdot$ATP complex.

The F430 cofactor is an Ni(II) tetrapyrrole that is required for the metabolism of methane in methanogenic bacteria [45]. These bacteria also contain cobalt-containing corrinoids [46]. Nothing is known about the enzyme that inserts Ni^{2+} to make the F430 cofactor. However, based on the tetrapyrroles excreted by mutants, it is surmised that Ni^{2+} is inserted into a dihydrosiro-hydrochlorin precursor which has three hydrogen atoms at its centre and is thus more sterically hindered than the comparable porphyrin macrocycle, which has two hydrogen atoms [45]. This may necessitate an energy-requiring system similar to the Mg-chelatase reaction for the insertion of Ni^{2+}. The recent sequencing of the genome of the archaeal bacterium *Methanococcus jannaschii* revealed four open reading frames, namely MJ0908, MJ1441, MJ0911 and MJ0910, that show sequence similarity to

CobN, BchH, BchI and BchD respectively [40]. However, there were no sequences that show similarity to the Co-chelatase subunits CobS or CobT. Since there have been no Mg-containing tetrapyrroles reported in methanogenic bacteria, it is reasonable to assume that the counterparts of Mg-chelatase are in fact subunits of the Ni-chelatase involved in the biosynthesis of the F430 cofactor. Assuming evolutionary parsimony, the BchI and BchD counterparts may also be involved in the insertion of Co^{2+} with the CobN homologue. It remains to be seen if this is in fact the case.

To summarize, there appear to be two classes of metal-ion chelatases in Nature. Members of the first class do not require ATP and consist of a single protein. The enzymes of the second class catalyse reactions that are less favorable energetically, require ATP and have multiple (three) subunits. The Ni-chelatase, Mg-chelatase and the Co-chelatase of *P. denitrificans* all fall into the second class. The similarities among these ATP-requiring chelatases can also be seen at the level of DNA sequence similarities, indicating that they share a common ancestry.

LOCALIZATION OF Mg-CHELATASE WITHIN THE CHLOROPLAST

There is a debate as to whether the enzymes for Chl and haem synthesis are spatially separated from each other within the chloroplast, by compartmentation of one part of the Chl pathway to the thylakoids and another part to the envelope membranes [47–51]. Such a division could potentially be useful in regulating the relative amounts of haem and Chl synthesized in the chloroplast. This point was initially raised by Fuesler et al., who compared the inhibition patterns of Mg-chelatase and the Mg-Proto monomethyl ester cyclase (cyclase) in intact chloroplasts [47]. This work showed that cyclase was more protected from the non-permeant thiol-group inhibitor *p*-chloromercuribenzene-sulphonate (PCMBS) than Mg-chelatase. One possible interpretation of these data was that Mg-chelatase is more accessible to the outside of the chloroplast than the cyclase, which could be consistent with Mg-chelatase being localized to the envelope and the cyclase to the thylakoids. However, more recent work has shown that when the inhibition of Mg-chelatase by PCMBS and *p*-chloromercuribenzoate (PCMB) was directly compared in intact chloroplasts, PCMB was a more effective inhibitor [27]. Since PCMBS is less permeant than PCMB, the data indicated that the Mg-chelatase must lie within a permeability barrier, presumably the chloroplast inner envelope. Furthermore, Mg-chelatase activity was supported by internally generated ATP in the presence of an external ATP trap, again pointing to a location within the inner envelope. The two reports can be rationalized by concluding that both Mg-chelatase and the cyclase lie within the chloroplast inner envelope, but that the cyclase is in a more hydrophobic environment and is therefore less accessible to PCMBS.

It has been confusing that the *in vitro* pea chloroplast Mg-chelatase assay was refined to include a crude separation of thylakoids from envelopes; the chloroplasts were lysed in buffer without Mg^{2+} and the thylakoids removed by a low-speed spin [31]. The remaining crude envelope and stromal proteins were highly active, indicating that Mg-chelatase was associated with the envelope membranes. Now, with the information that Mg-chelatase is a soluble enzyme in the absence of Mg [5,19,27], these data can be reinterpreted: during lysis and removal of the thylakoids, Mg-chelatase would have been soluble: on the subsequent addition of Mg^{2+} the enzyme would have been driven on to the envelope membranes.

In vivo, there is sufficient Mg^{2+} in the chloroplasts to cause Mg-chelatase to be associated with a membrane. However, since envelope and thylakoid separation techniques require low Mg concentrations to obtain membranes with little cross-contamination [52,53], it is not possible to localize Mg-chelatase within the chloroplast by these methods. This question may be settled by *in vitro* import studies and immunogold labelling. Already the import of the H and I subunit into the stroma of *Arabidopsis* chloroplasts has been reported, although their definitive association with either the envelopes and thylakoids has not yet been shown [5].

In summary, while there is no evidence to show that Mg-chelatase associates with either the thylakoids or the envelopes, the enzyme is certainly transported through the envelopes and into the stroma. The likelihood that its final destination is the thylakoids is increased by the recent reassignment of another Chl-biosynthetic enzyme, Pchlide reductase, to the thylakoids [54,55]. Interestingly, Pchlide reductase had previously been reported to be on the chloroplast envelopes [48], but this has now been explained as an import intermediate [56], and the final destination of this enzyme is thought to be the thylakoids [54,55]. Only two other porphyrin-biosynthetic enzymes have been reported to be on the envelope: protoporphyrinogen oxidase (which functions in both haem and Chl synthesis) [49] and ferrochelatase [57]. Since these enzymes are also found in large amounts on the thylakoids [49,50], it begs the question of whether their presence on the envelope might also be explained as import intermediates. Thus any separate porphyrin-biosynthetic capacity of the envelopes is not certain at this time.

REGULATION OF Mg-CHELATASE AND FERROCHELATASE AT THE BRANCH-POINT

The position of Mg-chelatase at the branch-point of haem and Chl synthesis, as well as its status as the enzyme for the first committed step of Chl synthesis, makes Mg-chelatase a prime candidate for tight regulation. Initial ideas of how Mg-chelatase and the Chl branch were regulated centred on Mg-chelatase being regulated by feedback inhibition by one of the intermediates on the Mg branch of the pathway [11]. This is supported by the recent observation that the artificial *in vivo* elevation of Mg-Proto and Mg-Proto monomethyl ester by feeding ALA to pea seedlings caused a 95% decrease in the *in-vitro*-measurable Mg-chelatase activity in isolated pea chloroplasts [58]. However, when these intermediates were directly tested in chloroplasts, and more recently in a solubilized Mg-chelatase preparation, no evidence for feedback inhibition by Mg-Proto, Mg-Proto monomethyl ester or Pchlide could be found [16,28]. Since these intermediates do not directly inhibit Mg-chelatase, the only explanation for their apparent inhibition in the ALA-feeding experiments is by reducing the amount of one or more of the Mg-chelatase subunits present. This could be accomplished by either reducing the transcript level of the Mg-chelatase genes or adversely affecting the import of the subunits into the chloroplast and/or increasing the turnover of one or more of the Mg-chelatase subunits.

The advent of the *in vitro* assay and determination of the two-step nature of the reaction presents another option for the control of the Mg-chelatase. Could the initial activation, which can be catalysed by ATP[S], involve a protein kinase [20]? With pure, expressed recombinant proteins this idea is now readily testable by the incubation of I and D subunits with radiolabelled ATP[S].

Although no mechanism for the direct regulation of Mg-chelatase has been reported, the overall regulation of Mg-chelatase and ferrochelatase has been addressed. In theory, if ferrochelatase and Mg-chelatase compete for the same pool of

substrates, their relative activities should be co-ordinated to favour one or the other pathway according to the prevailing demands of the chloroplast. There has been only one recent study in which the relative activities of these two enzymes were simultaneously estimated in intact chloroplasts, allowing this aspect of regulation to be examined [59]. When haem and MgProto synthesis were compared in pea chloroplasts, it was clear that in the presence of ATP there was a drastic reduction in haem synthesis and MgProto synthesis was favoured over haem synthesis by a factor of about 5. In addition, besides allowing Mg^{2+} chelation to occur, ATP caused an increase in the level of haem + Proto + MgProto. The authors suggested that ATP may be the switch between Chl and haem synthesis. A possible mechanism for this switch would be for ATP to have a direct, negative effect on ferrochelatase activity.

There was an initial report of ATP-inhibiting ferrochelatase at millimolar concentrations (3 mM ATP inhibiting by around 67 %), in which ferrochelatase was also reported to be a soluble enzyme of 55–65 kDa (based on gel filtration) [60]. This work has now been followed up with studies on recombinant ferrochelatase cloned from cucumber (T. Masuda, personal communication). Again, this enzyme was directly inhibited by ATP, with 1 mM ATP inhibiting by 40 %. The expressed fusion protein was soluble, and immunolocalization studies on cucumber and spinach (*Spinacia oleracea*) chloroplasts suggested that it was localized to the chloroplast stroma *in vivo*. ATP inhibition of ferrochelatase was competitive with respect to both porphyrin and Fe^{2+} substrates, and the authors proposed that ATP was directly binding the Fe^{2+} and it was the Fe·ATP complex which was blocking the substrate-binding sites.

The purpose of the present Review is not to cover ferrochelatase in detail, but it should be noted that this enzyme has also been localized to both the chloroplast thylakoids [50] and the envelopes [57]. Is there more than one form of ferrochelatase in higher plants? There is a good physiological argument that this may be the case: mitochondria cannot synthesize haem, although they do contain ferrochelatase, and therefore chloroplasts must supply either haem or haem precursors to mitochondria and the rest of the cell. The efflux of haem and other porphyrins from isolated chloroplasts has been observed [61–63] . Possibly there are two haem-synthesis pathways in the chloroplast: one for photosynthetic uses and the other for non-photosynthetic and/or extrachloroplastidic haem. Perhaps the more soluble form of the enzyme is associated with the photosynthetic pathway. While highly speculative, it begs the question of whether the membrane-bound ferrochelatase is also inhibited by ATP.

If ferrochelatase and Mg-chelatase compete for the same pool of Proto, another factor which must be taken into account is their relative kinetic constants. Ferrochelatase in pea has a K_m for Proto of 2.4 μM and a V_{max} of about 5.2 nmol·min^{-1}·mg of Chl^{-1} [50]. Mg-chelatase in pea has about the same V_{max} as ferrochelatase [59], and initially the K_m for Proto was thought to be about the same as that for ferrochelatase [59], but more recently assay conditions were further optimized with solubilized preparations to obtain a better estimate of the kinetic constants and the K_m for Proto was re-estimated as about 13 nM [28].

To bring these data together, it seems that we can be confident of two mechanisms which control the flux of intermediates through the branch-point. First, the presence of ATP, which is absolutely required for Mg-chelatase activity, also inhibits ferrochelatase. Secondly, the K_m of Mg-chelatase for Proto is low enough that it should out-compete ferrochelatase for their common substrate. This picture of the regulation of the branch-point is far from complete, since the possibility that Mg-chelatase is regulated by protein phosphorylation may yet have to be taken into account. In addition, there is evidence to suggest that the D subunit of the enzyme associates with ribosomes in both higher plants and *Rhodobacter* [19]. This result was not confirmed by Gorchein, who reported that lysed *Rhodobacter* cells had activity in the high-speed supernatant [64]; however, the lysis buffer included ATP, which could potentially affect the fractionation of the D component. While the association of D with the ribosomes could be fortuitous, the enzyme was also inhibited by chloramphenicol, raising the interesting possibility that there may be some connection between the regulation of protein synthesis and of Mg-chelatase. Another level of control may be gene transcription, and before an overall model for regulation is discussed, current work on the regulation of transcription will be discussed.

REGULATION OF BACTERIAL GENE TRANSCRIPTION

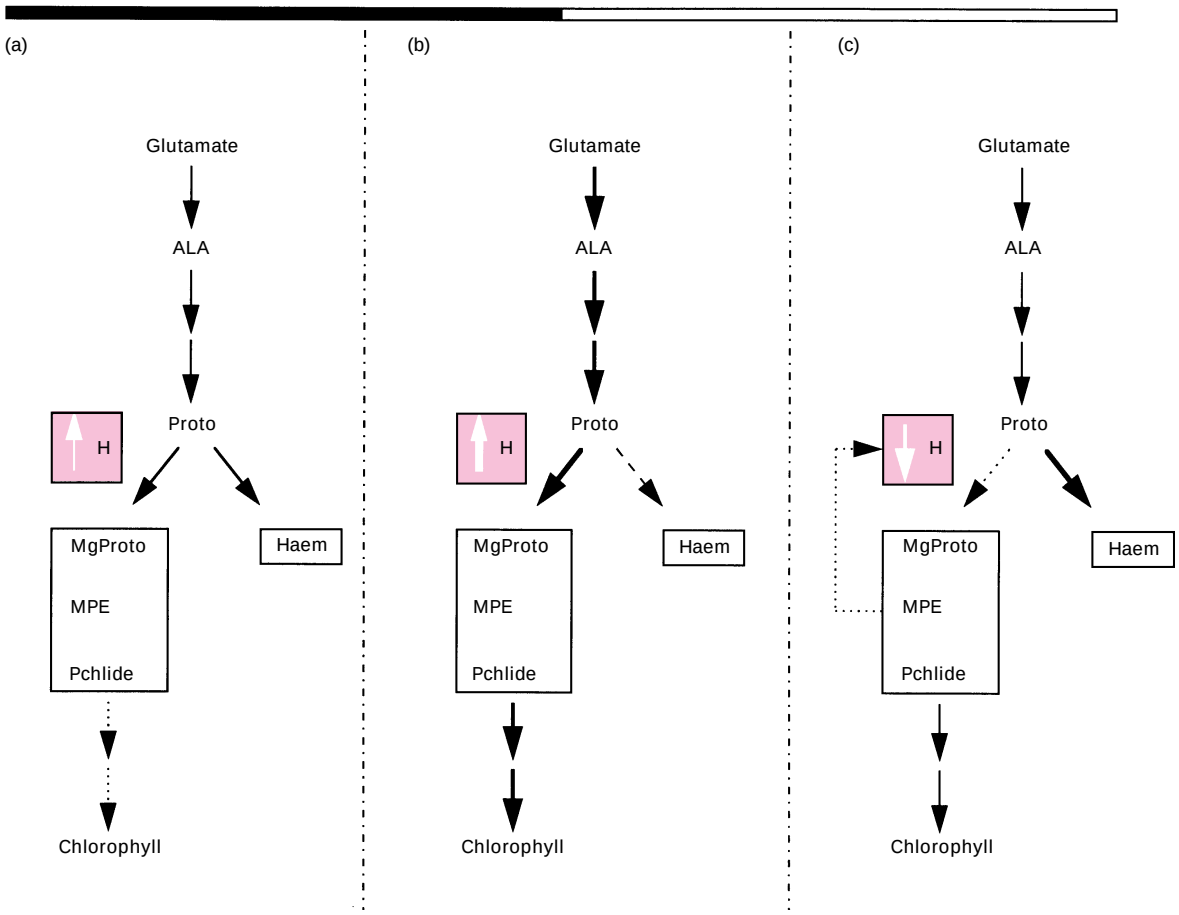
The genes for Mg-chelatase in *R. capsulatus*, *R. sphaeroides* and *Chlorobium vibrioforme* are clustered with other Bchl-biosynthetic genes and, for *Rhodobacter* at least, with the photosystem biosynthetic genes [3,65,66]. In *Rhodobacter* , the *bchI* and *bchD* genes are part of the same operon, whereas the *bchH* is transcribed separately. In *Chlorobium vibrioforme* the *bchH*, *bchI* and *bchD* are on the same operon [3]. *Rhodobacter* can grow either photosynthetically or aerobically using a fermentable carbon source, in which case the photosystems and pigments are not synthesized. The regulation of the genes in the *Rhodobacter* photosynthetic gene cluster has been reviewed recently [67,68] and will only be discussed briefly. Under anaerobic conditions, expression of these pigment-biosynthetic genes is coupled with other genes involved in photosynthesis as part of overlapping transcriptional units termed superoperons. The regulation of the photosystem genes is by an anaerobic induction circuit, whereas the regulation of the pigment-biosynthetic genes is by an aerobic repression circuit involving a DNA-binding redox-sensing protein named CrtJ.

At the protein level not much is known about the regulation of Mg-chelatase in photosynthetic bacteria. One possible control mechanism has been suggested by the finding that, when the H subunit from *Rhodobacter capsulatus* is exposed to light and oxygen, the bound Proto is converted into photoprotoporphyrin. This porphyrin forms a very tight (possibly covalent) attachment to the H subunit and renders it inactive; this may be an additional mechanism for the inhibition of Bchl biosynthesis by high oxygen tension and light [69]. Additionally, it has been observed that, *in vitro*, the presence of the H subunit increases the activity of the next enzyme in the pathway, namely the methyltransferase [70]. Interestingly, in whole *Rhodobacter* cells, it was found that Mg-chelatase activity was only measurable if *S*-adenosylmethionine was included to allow the methyltransferase to operate [12]. These data suggest that there may be a close linkage between these enzymes *in vivo* and raises the possibly that the H subunit is involved in regulating another enzyme in the pathway.

HIGHER-PLANT GENE REGULATION

Only the *chlH* and *I* genes have been available for transcriptional studies to date, and so information on the regulation of the subunits at this level is not complete. However, transcriptional regulation of the I and H subunits has been looked at in both greening tissue and as a function of diurnal variation.

The expression of the *chlH* and *chlI* genes in developing plants is induced by light. When dark-adapted *Arabidopsis* and soybean (*Glycine max*) were transferred to light for 24 h, the *chlI* transcript was induced up to 64-fold compared with the light-adapted plants [9,10]. When etiolated barley seedlings were transferred to light, the *chlH* transcript increased 25-fold after 4 h and remained



Scheme 4 Model for the regulation of Chl and haem biosynthesis in the chloroplast

The bar at the top of the Scheme represents the time of day ranging from dark to light conditions. The widths of the arrows going down represent the relative flux of intermediates, while the red arrows pointing to a position in the pathway show the level of repression at that point in the pathway. The red arrow in the pink box containing H indicates the amount of functional H subunit entering the chloroplast. Dotted lines indicate a negligible flux or effect. Abbreviation: MPE, Mg-protoporphyrin monomethyl ester. (**a**) Shows the situation just after the start of the dark period. (**b**) shows the situation just after the start of the light period, and (**c**) shows the situation at the end of the light period, as described in the text.

at this level; in this system, the induction of the transcription of I was not so pronounced, with only a 2-fold increase after 8 h and a return to the uninduced level after 24 h [7]. This correlated with a 4-fold increase in measurable Mg-chelatase activity after 6 h in the light. To date, the one exception to this general pattern of light induction is the expression of *chlI* gene of *Euglena gracilis*, which was reported to be constitutive; however, this exception may be due to the *chlI* gene being chloroplast-encoded in this organism [71].

The expression of these genes in plants grown in a light/dark cycle contrasts with the light-induced expression in etiolated tissue. In both barley and *Arabidopsis*, the *chlH* transcript levels had about a 40-fold diurnal oscillation with the transcript level beginning to rise approx. 4 h before dawn, reaching a maximum 1–3 h into the light phase, then falling again [5,7]. In barley, a diurnal fluctuation in Mg-chelatase activity was measured with a tripling of activity from pre-dawn to maximal activity 1–3 h post-dawn [7]; no activity measurements were made in the *Arabidopsis* study [5]. In *Antirrhinum majus* a similar situation was observed, except that transcript levels decreased to almost zero by the end of the light period; however, as *in Arabidopsis* and barley, transcript levels came up during the dark phase [8]. In barley, this work was extended, and continued fluctuations in transcript levels were observed if the plants were transferred to continuous light, indicating that the transcription was under the control of a circadian clock. The behaviour of the I transcript under diurnal conditions was quite different from that of the H transcript: in both barley and *Arabidopsis* there was little or no oscillation [5,7].

With the assumption that the higher plants described above are regulating Mg-chelatase in similar ways, two themes emerge from this work. Firstly, the H subunit seems to fluctuate more than the I subunit under greening conditions or during diurnal growth. Secondly, the I subunit is induced by light if the tissue is etiolated, but once the chloroplasts have developed, there is very little effect of light on this subunit's transcription. While it is not possible to fit D into this pattern as yet, it is reasonable to assume that the regulation of this enzyme is in part achieved by regulation of the amounts of the H subunit in the chloroplast.

MODEL FOR THE REGULATION OF Mg-CHELATASE IN HIGHER PLANTS

The data above can be used to suggest a model for the overall regulation of the Mg-chelatase pathway in higher plants grown in light–dark cycles. Scheme 4 shows the situation at the end of

the light phase, where the *chlH* transcript level is low and begins to rise at the start of the dark phase. This results in slightly higher Mg-chelatase activity and a slight shift in the flux of the intermediates from the haem branch to the Mg branch. However, Mg containing intermediates begin to accumulate because the conversion of Pchlide to Chlide requires light [55]. As Mg porphyrins accumulate they decrease the amount of the H subunit that can enter the chloroplast, preventing their own synthesis. In Scheme 4(b), at the start of the light phase, these remaining Mg intermediates are utilized to make Chl now that the conversion of Pchlide into Chlide can take place. This releases the block on H subunit accumulation. The *chlH* transcript levels and levels of H subunit reach a maximum early in this phase. As H subunits accumulate in the chloroplast, they cause the diversion of nearly all the Proto into the Mg branch; and, since haem turns over in the chloroplast, this will also cause a decrease in free haem levels. In the presence of haem, ALA synthesis is inhibited [72] and so as the haem levels fall in this phase, so will the inhibition of ALA synthesis be removed, resulting in a net increase in the flux of intermediates into the tetrapyrrole pathway. In Scheme 4(c), towards the end of the light phase, the *chlH* transcript falls rapidly to almost zero with the resultant decrease in H subunit levels and a block in the flow of intermediates into the Mg branch of the pathway. This allows Proto to be diverted back to haem synthesis again, and the increased levels of free haem inhibit ALA synthesis and shut down the tetrapyrrole pathway.

The regulation described above is a dynamic process and Schemes 4(a)–4(c) only represent specific stages through the day. The model is also incomplete and will probably need to be extended to incorporate other factors such as the possibility of the phosphorylation of Mg-chelatase subunits and the ATP/ADP ratio of the chloroplast.

CONCLUSIONS AND PERSPECTIVES

In the 1990s, work on Mg-chelatase has made a huge leap from the first *in vitro* assay to cloning and expression of the genes involved. The current status of our knowledge can be summarized by concluding that there are three proteins involved in Mg-chelatase and that the reaction itself takes place in two steps. With the recent availability of purified recombinant proteins, we anticipate that establishing the mechanism of Mg-chelatase will happen very soon. It will be interesting to solve finally the puzzle of why ATP is needed in the reaction, which may be solved by *in vitro* labelling studies of the subunits. Also, it should be possible to determine the role of each subunit in the catalytic mechanism. This work will have repercussions for those researchers looking into both Co-chelatase and Ni-chelatase, which are expected to have similar mechanisms.

We stated at the outset that the major interest in Mg-chelatase centres on regulation of the branch-point between haem and Chl biosynthesis: the challenge now is for researchers to come up with a model of how this is achieved. We expect that regulation is achieved at many levels. There is some preliminary evidence that a protein kinase might be involved in the reaction; the regulation of the gene transcription; and possibly control of the translation and import of the subunits. With the current availability of probes for gene-expression analysis (for both Mg-chelatase and ferrochelatase) and with assays and antibodies available for protein analysis, we anticipate that this challenge will be met in the near future.

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REFERENCES

1 Dailey, H. A. (1990) in Conversion of Coproporphyrinogen to Protohaem in Higher Eukaryotes and Bacteria: Terminal Three Enzymes (Dailey, H. A., ed.), pp. 123–162, McGraw-Hill, New York
2 Walker, C. J. and Weinstein, J. D. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 5789–5793
3 Petersen, B., Møller, M., Stummann, B. and Henningsen, K. (1996) Hereditas **125**, 93–96
4 Gibson, L. C. D., Willows, R. D., Kannangara, C. G., von Wettstein, D. and Hunter, C. N. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 1941–1944
5 Gibson, L. C. D., Marrison, J. L., Leech, R. M., Jensen, P. E., Bassham, D. C., Gibson, M. and Hunter, C. N. (1996) Plant Physiol. **111**, 61–71
6 Jensen, P. E., Gibson, L. E., Henningsen, K. W. and Hunter, C. N. (1996) J. Biol. Chem. **271**, 16662–16667
7 Jensen, P. E., Willows, R. D., Petersen, B. L., Vothknecht, U. C., Stummann, B. M., Kannangara, C. G., von Wettstein, D. and Henningsen, K. W. (1996) Mol. Gen. Genet. **250**, 383–394
8 Hudson, A., Carpenter, R., Doyle, S. and Coen, E. S. (1993) EMBO J. **12**, 3711–3719
9 Koncz, C., Mayerhofer, R., Koncz-Kalman, Z., Nawrath, C., Reiss, B., Redei, G. P. and Schell, J. (1990) EMBO J. **9**, 1337–1346
10 Nakayama, M., Masuda, T., Sato, N., Yamagata, H., Bowler, C., Ohta, H., Shioi, Y. and Takamiya, K. (1995) Biochem. Biophys. Res. Commun. **215**, 422–428
11 Beale, S. I. and Weinstein, J. D. (1990) in Tetrapyrrole Metabolism in Photosynthetic Organisms (Dailey, H. A., ed.), pp. 287–391, McGraw-Hill, New York
12 Gorchein, A. (1972) Biochem. J. **127**, 97–106
13 Gorchein, A. (1973) Biochem J. **134**, 833–845
14 Castelfranco, P. A., Weinstein, J. D., Schwarcz, S., Pardo, A. D. and Wezelman, B. E. (1979) Arch. Biochem. Biophys. **192**, 592–598
15 Pardo, A. D., Chereskin, B. M., Castelfranco, P. A., Franceschi, V. R. and Wezelman, B. E. (1980) Plant Physiol. **65**, 956–960
16 Walker, C. J. and Weinstein, J. D. (1991) Plant Physiol. **95**, 1189–1196
17 Richter, M. L. and Rienits, K. G. (1982) Biochim. Biophys. Acta **717**, 255–264
18 Lee, H. J., Ball, M. D., Parham, R. and Rebeiz, C. A. (1992) Plant Physiol. **99**, 1134–1140
19 Kannangara, C., Vothknecht, U., Hansson, M. and von Wettstein, D. (1997) Mol. Gen. Genet. **254**, 85–92
20 Walker, C. J. and Weinstein, J. D. (1994) Biochem. J. **299**, 277–284
21 Willows, R. D., Gibson, L. C. D., Kanangara, G. C., Hunter, C. N. and von Wettstein, D. (1996) Eur. J. Biochem. **235**, 438–443
22 Alberti, M., Burke, D. H. and Hearst, J. E. (1995) in Anoxygenic photosynthetic bacteria, (Blankenship, R. E., Madigan, M. T. and Bauer, C. E., eds.), pp. 1083–1106, Kluwer Academic Publishers, Dordrecht
23 Bollivar, D. W., Suzuki, J. Y., Beatty, J. T., Dobrowolski, J. M. and Bauer, C. E. (1994) J. Mol. Biol. **237**, 622–640
24 Bollivar, D., Jiang, Z., Bauer, C. and Beale, S. (1994) J. Bacteriol. **176**, 5290–5296
25 Von Wettstein, D., Henningsen, K., Boynton, J., Kannangara, C. and Nielsen, O. (1971) in The Genetic Control of Chloroplast Development in Barley (Boardmann, N., Linnana, A. and Smillie, R., eds.), pp. 205–223, North-Holland, Amsterdam
26 Henningsen, K. W., Boynton, J. E. and von Wettstein, D. (1993) Biol. Skr. K. Dan. Vidensk. Selsk. **42**, 1–349
27 Walker, C. J. and Weinstein, J. D. (1995) Physiol. Plant. **94**, 419–424
28 Guo, R., Luo, M. and Weinstein, J. D. (1997) Plant Phys. Electron. Abstr. 743
29 Jacobs, J. M., Jacobs, J. J., Borotz, S. E. and Guerinot, M. L. (1990) Arch. Biochem. Biophys. **280**, 1–7
30 Crawford, M., Wang, W.-Y. and Jensen, K. (1982) Mol. Gen. Genet. **188**, 1–6
31 Walker, C. J., Hupp, L. R. and Weinstein, J. D. (1992) Plant Physiol. Biochem. **30**, 263–269
32 Buchler (1975) in Porphyrins and Metalloporphyrins, (Smith, K., ed.), pp. 157–232, Elsevier Scientific Publishers, Amsterdam
33 Hambright, P. (1975) in Dynamic Co-ordination Chemistry of Metalloporphyrins, (Smith, K., ed.), pp. 233–278, Elsevier/North Holland, Amsterdam
34 Baum, S. and Plane, R. (1966) J. Am. Chem. Soc. **88**, 910–913
35 Fleischer, E., Choi, E., Hambright, P. and Stone, A. (1964) Inorg. Chem. **3**, 1284–1287
36 Köhler, S., Delwiche, C., Denny, P., Tilney, L., Webster, P., Wilson, R., Palmer, J. and Roos, D. (1997) Science **275**, 1485–1489
37 Koonin, E. V. (1993) Nucleic Acids Res. **21**, 2541–2547
38 Koonin, E. V. (1997) Science **275**, 1489–1490

39 Luo, M., Walker, C. J. and Weinstein, J. D. (1997) Plant Physiol. Electron. Abstr. 737
40 Bult, C., White, O., Olsen, G., Zhou, L., Fleischmann, R., Sutton, G., Blake, J., Fitzgerald, L., Clayton, R., Gocayne, J. et al. (1996) Science **273**, 1058–1073
41 Maizel, J. and Lenk, R. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 7665–7669
42 Debussche, L., Couder, M., Thibaut, D., Cameron, B., Crouzet, J. and Blanche, F. (1992) J. Bacteriol. **174**, 7445–7451
43 Blanche, F., Thibaut, D., Debussche, L., Hertle, R., Zipfel, F. and Müller, G. (1993) Angew. Chem. Int. Ed. Engl. **32**, 1651–1653
44 Müller, G., Zipfel, F., Hlineny, K., Savvidis, E., Hertle, R., Traub-Eberhard, U., Scott, A., Williams, H., Stolowich, N., Santander, P. et al. (1991) J. Am. Chem. Soc. **113**, 9893–9895
45 Thauer, R. and Bonacker, L. (1995) in The Biosynthesis of the Tetrapyrrole Pigments (Chadwick, D. and Ackrill, K., eds.), pp. 210–227, John Wiley, Chichester
46 Stupperich, F., Eisinger, H. and Schurr, S. (1990) FEMS Microbiol. Rev. **87**, 355–360
47 Fuesler, T. P., Wong, Y.-S. and Castelfranco, P. A. (1984) Plant Physiol. **75**, 662–664
48 Joyard, J., Block, M., Pineau, B., Albrieux, C. and Douce, R. (1990) J. Biol. Chem. **265**, 21820–21827
49 Matringe, M., Camadro, J.-M., Block, M. A., Scalla, R., Labbe, P. and Douce, R. (1992) J. Biol. Chem. **267**, 4646–4651
50 Matringe, M., Camadro, J.-M., Joyard, J. and Douce, R. (1994) J. Biol. Chem. **269**, 15010–15015
51 Pineau, B., Gérard-Hirne, C., Douce, R. and Joyard, J. (1993) Plant Physiol. **102**, 821–828
52 Keegstra, K. and Yousif, A. E. (1986) Methods Enzymol. **118**, 316–325
53 Douce, R., Holtz, R. B. and Benson, A. A. (1973) J. Biol. Chem. **248**, 7215–7222
54 Reinbothe, S. and Reinbothe, C. (1996) Eur. J. Biochem. **237**, 323–343
55 Reinbothe, S. and Reinbothe, C. (1996) Plant Physiol. **111**, 1–7
56 Reinbothe, S., Runge, S., Reinbothe, C., van Cleve, B. and Apel, K. (1995) Plant Cell **7**, 161–172
57 Roper, J. M. and Smith, A. G. (1997) Eur. J. Biochem. **246**, 32–37
58 Averina, N., Yaranskaya, E., Rassadina, V. and Walter, G. (1996) J. Photochem. Photobiol. **36**, 17–22
59 Walker, C. J., Yu, G. and Weinstein, J. D. (1997) Plant Physiol. Biochem. **35**, 213–221
60 Goldin, B. R. and Little, H. N. (1969) Biochim. Biophys. Acta **171**, 321–332
61 Thomas, J. and Weinstein, J. D. (1990) Plant Physiol. **94**, 1414–1423
62 Jacobs, J. M. and Jacobs, N. J. (1993) Plant Physiol. **101**, 1181–1187
63 Jacobs, J. and Jacobs, N. (1995) Arch. Biochem. Biophys. **323**, 274–278
64 Gorchein, A. (1997) Biochem. Soc. Trans. **82S**, 25
65 Coomber, S., Chaundri, M., Connor, A., Britton, G. and Hunter, C. (1990) Mol. Microbiol. **4**, 977–989
66 Marrs, B. (1981) J. Bacteriol. **146**, 1003–1012
67 Bauer, C. E. (1995) in Regulation of Photosynthesis Gene Expression (Blankenship, R. E., Madigan, M. T. and Bauer, C. E., eds.), pp. 1221–1234, Kluwer Academic Publishers, Dordrecht and Boston
68 Bauer, C. E. and Bird, T. H. (1996) Cell **85**, 5–8
69 Willows, R. D. and Beale, S. I. (1997) Plant Physiol. Electron. Abstr. 742
70 Hinchigeri, S. B., Hundle, B. and Richards, W. R. (1997) FEBS Lett. **407**, 337–342
71 Orsat, B., Montfort, A., Chatellard, P. and Stutz, E. (1992) FEBS Lett. **303**, 181–184
72 Pontoppidan, B. and Kannangara, C. G. (1994) Eur. J. Biochem. **225**, 529–537
73 Yang, Z. and Bauer, C. E. (1990) J. Bacteriol. **172**, 5001–5010
74 Gorchein, A., Gibson, L. C. D. and Hunter, C. N. (1993) Biochem. Soc. Trans. **21**, 201S
75 Gough, S. (1972) Biochim. Biophys. Acta **286**, 36–54
76 Reference deleted
77 Falbel, T. G. and Staehelin, L. A. (1994) Plant Physiol. **104**, 639–648
78 Mascia, P. (1978) Mol. Gen. Genet. **161**, 237–244
79 Runge, S., van Cleve, B., Lebedev, N., Armstrong, G. and Apel, K. (1995) Plants **197**, 490–500
80 Granick, S. (1948) J. Biol. Chem. **175**, 333
81 Wang, W., Wang, W. L., Boynton, J. E. and Gillham, N. W. (1974) J. Cell. Biol. **63**, 806–823
82 Nicholson-Guthrie, C. S. and Guthrie, G. D. (1987) Arch. Biochem. Biophys. **252**, 570–573