Stabilization of chlorophyll $\alpha$-binding apoproteins P700, CP47, CP43, D2, and D1 by Chlorophyll $\alpha$ or Zn-pheophytin $\alpha$*

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The biogenesis of higher plant photosystems I and II requires assembly of nuclear- and plastid-encoded apoproteins with cofactors (e.g. chlorophyll, carotenoid, heme, quinone, iron, and manganese) within the inner plastid membrane system. Chlorophyll $\alpha$ (Chl)$\alpha$ is the key chromophore for higher plants to carry out the photosynthetic light reactions and is known to regulate the accumulation of the nuclear- and plastid-encoded apoproteins of the photosystems (1–4). Etioplasts isolated from 4-day-old, dark-grown barley are ideal to study the Chl-dependent accumulation of plastid-encoded photosystem proteins. Etioplasts in barley are formed from proplastids, during early primary leaf and plastid development, which proceeds uninhibited in the absence of light (5, 6).

In the dark, etioplasts do not synthesize Chl and neither accumulate plastid-encoded Chl $\alpha$-binding proteins (Chl $\alpha$P) (7, 8) nor nuclear-encoded Chl $\alpha/b$-binding apoproteins (1, 2), although they accumulate protochlorophyllide (Pchlide), a Chl precursor. When plants are illuminated, Pchlide is reduced to chlorophyllide (Chlide) in the plastid by protochlorophyllide oxidoreductase (9) in a light- and NADPH-dependent reaction. Illumination leads to disintegration of the prolamellar body and its dispersal into the primary lamellar layers of the thylakoid membrane (10). Chlide is esterified with geranylgeranylpyrophosphate (GGPP) to yield Chl$\gamma$(11) in a light-independent enzymatic step catalyzed by chlorophyll synthase (11–13). In addition to the prenylation of the natural substrates Chlide $a$ and $b$, chlorophyll synthase prenylates modified tetrapyrrole derivatives (14). Pentacoordinate metals (e.g. magnesium or zinc) are accepted as central atoms of the tetrapyrrole substrate, whereas metal-free phosphorhoebides or typical tetra-coordinate central atoms (e.g. copper, nickel) do not act as a substrate for the enzyme (15). In isolated plastids Chl formation is accompanied by the accumulation of the plastid-encoded Chl-binding apoproteins (8) and assembly of the photosynthetic apparatus (16). Plastid DNA encodes at least six Chl $\alpha$P, including the proteins encoded by psaA (P700-appoprotein), psaB (P700- appoprotein), psbA (D1), psbB (CP47), psbC (CP43), and psbD (D2) (17). Transcripts for these Chl $\alpha$P are present in etioplasts from dark grown seedlings (7, 8, 18); however on the protein level, only D2 accumulates (19). Upon illumination of etiolated seedlings, no change in the amount of the mRNA for the D1 protein or in the distribution of its mRNA in polysomes could be measured (20). Furthermore, while Chl synthesis in the dark did not affect initiation or elongation of translation, it did trigger posttranslational accumulation of chloroplast encoded Chl $\alpha$P by enhancement of Chl $\alpha$P stability (21). In the absence of Chl synthesis, the D1, CP47, and CP43 polypeptides bound to polysomes were stable; upon release to the membrane phase, they were rapidly degraded unless Chl was synthesized (21).

Although an indirect effect of Chl synthesis on stabilization of the Chl $\alpha$P against proteolytic digestion has never been ruled out, most authors assume a direct interaction of Chl with the apoproteins, leading to folding and stable integration of the apoproteins into the thylakoid membrane (22). Chl-binding reaction center and core proteins were predicted to each contain either nine (P700 A and B), six (CP47 and CP43), or five (D2 and D1) $\alpha$-helices and to bind 45 (P700 A and B), at least 10 (CP47 or CP43), or between 4 and 6 (D2 and D1) Chl molecules (23–26). With respect to folding of the chloroplast-encoded apoproteins, the hydropathy, the distribution of charged amino acids (Asp, Arg), and the distribution of the Chl ligands (e.g. His, Gln, Asn) have been used as major determinants to predict the orientation of the folded apoproteins according to the "positive-inside-rule" (27). Binding of Chl $a$ and $b$ and a mixture of xanthophylls to light-harvesting chlorophyll-binding protein have been shown in in vitro reconstitution studies to lead to a rearrangement of the N-terminal portion of the apoprotein that rendered the Chl $a/b$ protein protease inaccessible (28). In
order to render the Chl aP protease inaccessible, the plastid-encoded nascent apoprotein chains could scavenge newly synthesized Chl late in translation elongation or after release of apoproteins from the ribosome, and the Chl may facilitate the folding of the apoprotein within the membrane phase (21).

Here, we used Zn-pheophytin a (Zn-phe) as a tool to investigate the stabilization of the Chl aP. We show that the central atom of the Chl has a strong influence on the stabilization of higher plant Chl aP against proteolytic degradation. The use of Zn-phe may help us understand the structural interactions between Chl and Chl aP that lead to the stabilization of the Chl-binding proteins.

MATERIALS AND METHODS

Plant Growth and Plastid Isolation—Barley (Hordeum vulgare, L. var. Steffi) seeds were planted in moist vermiculite and grown for 4.25 days in a light-tight growth chamber at 25°C. At this stage of development, seedlings were about 95% of the maximum height achieved. The etioplasts plus other cellular material pelleted by a brief centrifugation (4097 g, 3 min). The pellet was resuspended in 5 ml of buffer B (0.4 M sorbitol, 2 mM EDTA, 50 mM HEPES, pH 8.0) and the etioplasts plus other cellular material pelleted by a brief centrifugation (4097 g × 3 min). The pellet was resuspended in 5 ml of buffer B and loaded onto a Percoll™ step gradient (35%/65% Percoll, 0.4 M sorbitol, 2 mM EDTA, 50 mM HEPES, pH 8.0). Intact etioplasts were centrifuged at 10,000 × g for 30 min. Intact etioplasts were then fractionated into a membrane and a soluble fraction, as described (30).

RESULTS

Synthesis of Chl and Chl aP—Chl and Chl aP synthesis in etioplasts (1.4 × 106 plastids assay) was performed at 25°C in 50 mM HEPES/KOH, pH 8.0, 0.2 mM ATP, 0.2 mM GTP, 7 mM magnesium acetate, boric acid, pH 7.0, 115 mM potassium acetate, pH 7.0, 10 mM dithiothreitol, 100 μM of each amino acid (minus methionine) and 2.96 × 106 Bq of [35S]methionine. The radioactivity was measured using a liquid scintillation counter.

The pigment was isolated and purified as described (30). The pigments were then etherified as described (30)

Incubation of Zn-Bpheide and GGPP to the intact etioplasts, Chl or Zn-phe is performed to test the ability of Zn-phe to scavenge newly synthesized Chl. The incubation was performed at 20°C in 50 mM HEPES/KOH, pH 8.0, with or without Zn-Bpheide and GGPP. The incubation was performed in the presence of [14C]Zn-Bpheide and GGPP. The radioactivity was measured using a liquid scintillation counter.

For addition to plastid-based esterification and translation assays, the pigment was dissolved in peroxide-free ether to a concentration of 1 μM and stored under argon at −20°C. For esterification of Chlide or Zn-pheide to Chl or Zn-phe, 10–15 nmol of the M-phenide in diethyl ether were mixed with 50 mM HEPES/KOH, pH 8.0, and the diethyl ether was evaporated under an argon stream (final concentration: 0.1 mM of HEPES solution). The M-phenide/HEPES solution was sonified for 2 min. Undissolved pigment aggregates were pelleted by microcentrifugation (15 s, room temperature) and discarded. The pigment concentration of the clear supernatant was determined by UV/VIS spectroscopy in 80% acetone using the molar extinction coefficients of 76.8 × 10^3 (B, graph 2) and 77.3 × 10^3 (B, graph 2) mol−1 cm−1 at 659 nm for Zn-phe a (32). Chl or Zn-pheide was synthesized during the in vitro reactions was determined by extraction of the pigments into 75% acetone. The separation of esterified and nonesterified pigment and UV/VIS determinations of pigment concentrations were as described (30).

 Determination of the Coordination State of Bacteriochlorophyll a and Zn-bacterial phorphophytin a—Transmetallated bacteriochlorophylls were kindly prepared by I. Katheder in the laboratory of H. Scheer, Munich, Germany after isolation of bacteriochlorophyll a (BChl) from Rhodobacter sphaeroides. Determination of the pigment coordination was done according to Hartwich (33). UV/VIS spectra were recorded on a lambda 2 spectrophotometer and data analyzed with LabCalc (version 2.23, 1991, Galactic Industries Corp.). An equal concentration of BChl or Zn-bacterial phorphophytin a (Zn-Bphe) was dissolved in dichloromethane (CH2Cl2) and titrated with either a 12.4 mM solution of pyridine (BChl) or a 12.4 mM solution of pyridine in CH2Cl2 (Zn-Bphe). Extinction coefficients ε and extinction coefficients e (liter−1 mol−1 cm−1) for the Qa band of BChl and Zn-Bphe were measured in 10 mM and 10 mM (18, 19, 16.5) were taken from Hartwich to calculate the coordination state and the equilibrium constants for the transformation of the monopyridinate into the dipyridinate form of BChl or Zn-Bphe. The coordination state C of the pigment was calculated according to the equation (2), for the extinction measured at 473 or 433 for the fifth ligand and for the fourth ligand (Fig. 1, A–C).

RESULTS

Synthesis of Chl or Zn-phe in Vitro—Etioplasts from 4-day-old dark-grown etiolated barley were used to examine the effect of the de novo synthesized Chl or Zn-phe on the accumulation of plastid-encoded proteins. Upon addition of Chl or Zn-phe and GGPP to the intact etioplasts, Chl or Zn-phe was formed through esterification of the substrates (Fig. 1, A–C). Hardly any difference could be detected between the accumulation of Chl and Zn-phe after a reaction time of 80 min (Fig. 1A).

We investigated the esterification over a substrate concentration range of 50 to 4000 pmol/4.2 × 106 etioplasts (pt*2) (where pt* = 4.2 × 106 etioplasts). About 70% of the M-phenide was converted to M-phe at a concentration of 75 and 150 pmol. At concentrations of M-phenide of 150–4000 pmol, the percentage of esterification declined continuously to a yield of 25% at the highest concentration (Fig. 1A). The decrease in the total yield of esterified substrate could indicate that Chl synthase in the intact etioplasts was already saturated at concentrations of substrate greater than 150 pmol.

To obtain additional information about the esterification of Chl-synthase for Chl or Zn-phe, we measured the kinetic of Chl (Fig. 1B) or Zn-phe (Fig. 1C) accumulation at low (350 or 450 pmol; Fig. 1, B or C, graph 1) and high (3700 or 2500 pmol; Fig. 1, B or C, graph 2) substrate concentrations. At low concentrations, Chl or Zn-pheide was esterified at an initial rate of about 20 pmol/pt*min and 100% of 350 pmol of Chl or Zn-pheide was converted to Chl or Zn-phe after 40 min (Fig. 1B, graph 1). At these higher substrate concentrations, up to 80% of 1000 pmol of Chl or Zn-pheide was converted to Chl, although the initial rate of esterification of Chl was unchanged (20 pmol/pt*min) (Fig. 1B, graph 2). These data indicated that the maximum rate of esterification
was achieved at the low substrate concentration (350 pmol), but the rate of esterification declined after a reaction time of 10 min, when about 60% of the substrate was esterified. At the high Chl concentration (3700 pmol) the esterification rate was unchanged within the reaction time of 40 min (Fig. 1B, graphs 1 and 2).

For 450 pmol of Zn-pheide, the initial rate of esterification was about 10 pmol/pt*/min during the first 10 min; after 40 min about 80% was esterified (Fig. 1C, graph 1). At 2500 pmol the initial esterification rate was about 40 pmol/pt*/min (Fig. 1C, graph 2). After 10 min the esterification rate declined and upon completion of the 40-min incubation only 25% of the Zn-pheide was converted to Zn-phe (Fig. 1C, graph 2). Based on the different kinetic of Chl and Zn-phe formation, Chl-synthase has a different specificity for the esterification of the Zn(II)- and Mg(II)-containing substrates.

Effect of Chl and Zn-phe on Accumulation of Chl αP—We investigated the effect of Chl or Zn-phe levels on the accumulation of Chl αP (P700, CP47, CP43, D2, pD1, and D1) within the membranes of intact etioplasts during pulse-chase radiolabeling. The intactness of etioplasts was evaluated from measurements of the amount of leakage of radiolabeled large subunit of ribulose-1,5-bisphosphate carboxylase (LSU) from the plastids. Essentially, no release of LSU could be measured during incubation of etioplasts in the esterification/translation assays (data not shown). Upon exposure of etioplasts to a buffer of low osmolarity, most of the plastids were lysed and the LSU released (data not shown). Hence, the etioplasts remained largely intact during esterification of the tetrapyrrols and in organello translation.

The accumulation of radiolabeled LSU- and α- and β-subunits of CP43 in the stromal or membrane phase of the plastid was not dependent on the concentration of substrates used for Chl synthesis (Fig. 2A). The contamination of the membrane fraction with LSU was coupled to the washing procedure (see "Materials and Methods," data not shown). With a standardized washing procedure, contamination with LSU was the same at all concentrations of Zn-phe synthesized except perhaps for the highest value (Fig. 2A, lanes 9, LSU).

Chl or Zn-phe altered the accumulation of the Chl αP in the membrane fraction in a concentration-dependent manner (Fig. 2A, Chl or Zn-phe: lanes 2–9). At concentrations of 13–75 pmol/pt* of the M-phe, only synthesis of Zn-phe appeared to stabilize the Chl αP (Fig. 2A, Chl, Zn-phe: lanes 2–5). A high yield of CP47, D2, and pD1 accumulation was already observed at the lowest Zn-phe concentration of 13 pmol/pt*; at this Zn-phe concentration the yield of stabilized CP43 corresponded to the yield of CP43 stabilized during synthesis of 300–500 pmol/pt* Chl (Fig. 2A, Zn-phe: lane 2; Chl: lanes 7 and 8 and Fig. 2B).

P700 exhibited a very sharp response to the accumulation of Zn-phe and Chl (Fig. 2A, Zn-phe: lanes 2–5; Chl: lanes 6–9). Quantitative determination of P700 accumulation showed that a 6-fold increase in the Zn-phe yield (15–90 pmol) paralleled a 15-fold increase in the yield of radiolabeled P700 (arbitrary units of radioactivity of 1–15) (Fig. 2B, P700). Synthesis of Chl stabilized P700 at a Chl concentration of about 200 pmol/pt*; and stabilization was saturated at about 500 pmol Chl/pt* (Fig. 2B). About 10-fold less Zn-phe was required to obtain similar levels of accumulation of P700 (Fig. 2B, P700). Generally, considerably less Zn-phe than Chl was required to stabilize a similar amount of de novo accumulated P700.

Similar patterns of apoprotein stabilization were observed for CP47, CP43, and D1; a stepwise increase in the Chl concentration revealed apoprotein specific concentrations for both the initiation and saturation of apoprotein stabilization (Fig. 2, A and B). The saturation level for CP47 and D1 was the same as that for P700 (500 pmol Chl/pt*), while higher Chl concentrations were required to stabilize CP43 (saturation was not achieved even at 900 pmol Chl/pt*) (Fig. 2A, Chl: lanes 7–9, and 2B, CP43). CP47 appeared to require the least amount of Chl or Zn-phe for the initiation of concentration-dependent stabilization, followed by CP43 and P700, indicating that CP47 was stabilized by Chl and also by Zn-phe with the highest efficiency of all Chl αP investigated.

Interestingly, both pD1 and D2 were present in etioplasts prior to treatments. In the presence of 13 pmol/pt* Zn-phe, stabilization of pD1 and D2 was increased; however, further increases in the Zn-phe concentration had little effect on the
the accumulation of Chl concentration of Zn-phe was increased above the point of optimum stabilization were obtained (Fig. 2A, Chl a, Zn-phe: lanes 1–9). After the labeling period, translation was arrested using chloramphenicol (100 μg/ml) and plastids incubated for an additional 40 min. Accumulation of Chl and radiolabeled polypeptides were analyzed as described under “Materials and Methods.” Equal levels of plastid membranes were loaded onto the SDS-polyacrylamide gels. Lanes 2 through 9 show proteins radiolabeled in the presence of increasing amounts of either Chl a or Zn-phe in pmol: Chl a, 12, 20, 50, 70, 230, 320, 520, 900 or Zn-phe a, 15, 30, 60, 90, 230, 300, 600, 1050. Fluorographs were exposed to x-ray film for 24 h. The autoradiograms of A were scanned with a laser densitometer as described under “Materials and Methods.” The arbitrary units of radiolabel incorporation into the Chl aP P700, CP47, CP43, and D1 were plotted versus the yield of Chl a or Zn-phe a, determined following a total pulse/chase incubation of 90 min.

pD1 and D2 levels (Fig. 2C, Zn-phe: lanes 1–5). In contrast, the stepwise increase in the Chl or Zn-phe levels resulted in increased stabilization of D1 (Fig. 2A, Chl a, Zn-phe: lanes 1 and 2–7). This suggests that in the absence of Chl synthesis pD1 and D2 are more stable to proteolytic digestion than D1 or the other Chl aP (Fig. 2A, Chl a or Zn-phe: lane 1 versus 2–9). The protection of D2 and pD1 to proteolysis was lost at the highest concentration of Zn-phe tested, while some protection of D1 remained (Fig. 2A, Zn-phe: lane 9).

Decreased Chl aP Accumulation at Increased Zn-phe Concentrations—In the case of Zn-phe, different optima for apoprotein stabilization were obtained (Fig. 2A, Chl a and Zn-phe: lanes 2–9, and 2B, P700, CP47, CP43, D1). Optimal stabilization of P700 was at about 90 pmol/ppt, while CP47, CP43, and D1 required about 200 and 300 pmol/ppt, respectively (Fig. 2B). As the concentration of Zn-phe was increased above the point of optimum stabilization, the accumulation of Chl aP decreased (Fig. 2B). This decrease was observed over a wide concentration range (90–300 pmol/ppt for P700, 230-1050 pmol/ppt for CP47 and CP43, and 300-1050 pmol/ppt for D1; Fig. 2B). At a Zn-phe concentration that no longer supported P700 stabilization and that started to destabilize CP47, CP43, D2, and D1, the accumulation of LSU and the α- and β-subunits of CF1 were constant (Fig. 2A, Zn-phe: lanes 6–9). These data demonstrate that synthesis of Chl or Zn-phe did not affect translation in general, but selectively affected the accumulation of the Chl aP.

Chl Counteracts Destabilization of Chl aP by Zn-phe—The higher affinity of Zn-phe than Chl for the Chl aP, suggested by the data presented in Fig. 2, was tested in a competition study (Fig. 3A). Etioplasts (400 pmol of PChlide) were phototransformed in vitro to yield 350 pmol of Chl for stabilization of the Chl aP P700, CP47, CP43, D2, and D1. By exogenous addition of different amounts of Zn-phe, the stabilization of Chl aP was increased to a maximum level (Fig. 3A, lanes 3–6). Further increases in Zn-phe resulted in decreased accumulation of the
Chl a P CP47, CP43, D2, and D1; at 600 pmol Zn-phe no accumulation of P700 was detected (Fig. 3, A, lanes 7 and 8, and B). As seen in Fig. 3B, more than 300 pmol of Zn-phe was required to inhibit stabilization of P700 in the presence of 350 pmol of Chl (Fig. 3B, graph 2). In contrast, optimum P700 stabilization was at 90 pmol if Zn-phe were the only source of a M-phe (Fig. 2, A and B, graph 1). The higher amount of Zn-phe required to inhibit stabilization of P700 in the presence of Chl suggests that low concentrations of Zn-phe were not sufficient to displace Chl from binding sites on the Chl a P. At higher Zn-phe concentrations, the Chl bound to P700 may have been displaced (Fig. 3, A, lanes 7 and 8, and B, graphs 1 and 2).

The Coordination State of Zn(II) or Mg(II) Is Different in Bacteriochlorophyll—For stabilization of the Chl a P, the coordination state of the central metal ion of Chl with the Chl a P may be critical. We used a chemical approach to demonstrate differences between the central Zn(II) versus Mg(II) atom of the tetrapyrrolyl, e.g. for the binding affinity to a ligand (Fig. 4). Central atom BCHl derivatives were chosen as a model for analysis of the ligation characteristic of Chl or Zn-phe, because a change in the coordination state of the BCHl could be measured by UV/VIS Spectroscopy via the Q_a band. Upon titration of the Zn-Bphe or BCHl dissolved in dichloromethane with pyridine the Q_a absorption of the metallobacteriopheides was shifted from λ_{555}, or λ_{581.6} or λ_{576} or λ_{510.2}, respectively (data not shown). This shift indicated that the coordination number of Zn-Bphe was changed from 4 to 5 via pyridine binding at the 5th ligand position. In BCHl the transformation of the Q_a band indicated that the coordination number shifted from 5 to 6. The percentage of the specific extinction at the 5th or the 6th coordination state of Zn-Bphe or BCHl was plotted versus the concentration of pyridine required to transform the spectra of the pigment, respectively. The data indicates that a 1.5 × 10^4-fold higher concentration of pyridine is required to transform BCHl from the 5th to the 6th coordination state than for transformation of Zn-Bphe from the 4th to the 5th coordination state. In Zn-Bphe a ligation of pyridine to the 6th ligand position could not be measured. Hence, Zn-Bphe and BCHl differed substantial in their capacity to ligate an electron donor to the 5th or the 6th ligand positions. We therefore conclude that the coordination state of the central metal ion of Chl may be critical for stabilization of the Chl a P.

**DISCUSSION**

We had shown previously in lysed etioplasts that the accumulation of Chl a P is induced via de novo synthesis of Chl (4). There was no effect of Chl synthesis on the regulation of initiation or elongation of translation (21). Hence, it was concluded that the Chl-dependent accumulation of Chl a P was a consequence of stabilization of Chl a P (21). However, it remained an open question whether stabilization was achieved by direct interaction of newly synthesized Chl with Chl a P or by a Chl a P specific protease that acts as sensor for newly synthesized Chl. In the experimental system described here, the M-phe-dependent esterification and the M-phe-dependent stabilization of Chl a P were investigated in intact etioplasts. Chlide and Zn-pheide were shown to differ with respect to concentration-dependent esterification by Chl-synthase. Furthermore, the products Chl and Zn-pheide differed with respect to the concentrations at which they stabilized the Chl a P. In vivo, no Zn-pheide has been detected in higher plant photosystems. During Chl biogenesis incorporation of Zn(II) into the protoporphyrin-9-structure can occur in vitro in a nonenzymatic manner. The enzyme Mg-chelatase has been reported to strongly select Mg(II) over Zn(II) (34). The chemical synthesis of Zn-pheide allowed us to circumvent this enzymatic control and to study the influence of the central metal ion on the accumulation of the Chl a P. Zn-pheide was clearly able to stabilize of the Chl a P against proteolytic degradation.

Stabilization of Chl a P Could Be Regulated Directly through Binding of Chl or Zn-phe—In vitro, reconstitution studies demonstrate that protection of light-harvesting chlorophyll-binding protein against proteolysis depends on the binding of pigments (28). Protection of the Chl a P by Chl or Zn-phe may be a direct consequence of binding between the metal ion of the tetrapyrrolyl and an electron donor from an amino acid group on the apoprotein. A nitrogen-containing amino acid side chain in His, Asn, or Gln may act as the 5th ligand during the binding of Chl or Zn-phe to the Chl a P (35). If each of the Chl a P had differential binding constants for Chl or Zn-phe, the apoprotein-specific stabilization of the Chl a P by Chl or Zn-phe could be explained. CP47 would have the highest affinity for Chl or Zn-phe, followed by P700 and CP43; D1 would have the lowest affinity for Chl or Zn-phe.

The selective decrease of apoprotein stabilization at high Zn-phe concentrations may reflect a concentration-dependent coordination of ligand positions on the Chl a P. At low concentrations, Zn-phe could stabilize Chl a P by binding to specific binding sites on the proteins. At higher concentrations, additional sites could bind Zn-phe leading to destabilization of Chl a P, Zn-phe-dependent stabilization of the Chl a P could be explained. CP47 would have the highest affinity for Chl or Zn-phe, followed by P700 and CP43; D1 would have the lowest affinity for Chl or Zn-phe.

Zn-phe could lead to the inactivation of a protease degrading the Chl a P-specific protease.
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Chl αP. The observed apoprotein-specific threshold values for the Chl αP accumulation argue against a M-phe-dependent inactivation of a single protease that degrades all of the Chl αP. Furthermore, the apoprotein-specific decrease in Chl αP stability at high Zn-phe (Fig. 2, A and B) would require protease reactivation, which is unlikely. Hence, indirect control of Chl αP accumulation seems most unlikely and we favor the hypothesis that the Chl αP are stabilized by direct interaction with Chl (and Zn-phe).

Intact Etioplasts Take Up Exogenously Added Substrates for Chl Synthesis—The esterification of M-pheides by Chl-synthesizing enzyme (Cis) occurred at high Zn-phe (Fig. 2, A and B) would require protease reactivation, which is unlikely. Hence, indirect control of Chl αP accumulation seems most unlikely and we favor the hypothesis that the Chl αP are stabilized by direct interaction with Chl (and Zn-phe).

Esterification or Stabilization Is Altered in the Presence of Zn-phe—The electronegativity of Zn-phe is increased by 0.4 unit relative to Chlide, while the electron density of the chlorin-Zn-phe system and the redox potential of Zn-phe is decreased (38). Hydrogen bonding and van der Waals contacts, which were described to be important for a correct positioning of BCChl within the bacterial reaction center proteins, may be affected (39).

The coordination behavior of the central metal for binding of a 5th ligand is affected (Fig. 4). Usually Mg(II) and Zn(II) ions are coordinated between the four nitrogen atoms in the chlorin or bacteriochlorin structure (40, 41). In bacterial reaction centers BCChl was found in a 5-fold coordination state with the imidazol group from His as electron donor, and also Zn-BPhe has been shown in bacterial reaction centers to coordinate a 5th ligand (42). The differences determined between BCChl and Zn-BPhe to coordinate a 5th ligand could result from an increased selectivity of the central Mg(II) atom to ligate oxygen, e.g. from water (40, 43).

For the esterification reaction and the stabilization of the Chl αP, this could indicate that water, bound to the 5th ligand position in Chl or Chl, would have to be displaced by an electron donor from the enzyme or the protein to allow binding of Chl or Chl, whereas the Zn-containing analog could be bound directly. Hence, binding of Zn-phe or Chl to the esterifying enzyme or to the Chl αP may be affected by the different ligation characteristics of the central Zn(II) or Mg(II) ion.

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