Enzymes of the Last Steps of Chlorophyll Biosynthesis: Modification of the Substrate Structure Helps To Understand the Topology of the Active Centers†

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Received March 4, 2005; Revised Manuscript Received April 25, 2005

ABSTRACT: Enzymes catalyzing two of the late steps of chlorophyll biosynthesis are NADPH: protochlorophyllide oxidoreductase (POR), responsible for the light-dependent reduction of protochlorophyllide to chlorophyllide, and chlorophyll synthase that catalyses the esterification of chlorophyllide to chlorophyll. Inhibitors of these enzymes are of interest as potential herbicides. Both enzymes presumably form a complex, and the question arose whether chlorophyll synthase can react with chlorophyllide while it is still bound to POR. Here, we describe the chemical modification of protochlorophyllides and chlorophyllides with space-filling substituents at rings A, B, and E of the tetrapyrrole macrocycle and the reactivity of the modified substrates. Both enzymes tolerate the large and flexible phenylamino substituent at ring B, indicating that ring B points toward the enzyme surface while the substrate is bound. On the basis of the standard compound zinc protopheophorbide *a* (100% activity), the 7^1-phenylamino derivative shows a comparable activity (83%) with POR that is higher than that of the parent formyl derivative zinc protopheophorbide *b* (58% activity). In contrast, the 3^1-phenylamino derivative is less active (12%) than the parent formyl compound zinc protopheophorbide *d* (49% activity), indicating that the binding pocket leaves less space around ring A than around ring B. Almost no space must be left around ring E because substitution of the 13^2-carboxymethyl ester (100% activity) by the 13^2-carboxyethyl ester reduces the activity to 0.2%. Chlorophyll synthase leaves somewhat more space around ring E on the A side of the tetrapyrrole in the binding pocket; substitution of the 13^2-proton (100% activity) by a methoxy group (53% activity) and an ethoxy group (11% activity) is tolerated to a certain extent, while the carbomethoxy group in this position is not accepted. Opening of ring E to a chlorin e6 dimethylester is tolerated (39% activity), while the large benzylamide residue at this site leads to the loss of activity. We conclude that the tetrapyrroles bind to both enzymes in the same direction: rings C, D, and E are oriented to the interior of the binding cleft, and rings A and B are oriented to the surface of the enzyme; this excludes simultaneous binding to both enzymes.

In angiosperms, chlorophyll (Chl)[1] synthesis requires light and is arrested in darkness after formation of protochlorophyllide *a* (Pchlide *a*, **1**). The reactions that take place after the transfer of dark-grown angiosperms to light have been named the last steps of Chl synthesis (*1*). The crucial light-dependent step is the hydrogenation of **1** to Chlide *a* (**2**, see Scheme 1), catalyzed by NADPH:Pchlide oxidoreductase (POR, *1*−*5*). POR is a member of the short chain dehydrogenase/reductase family characterized by a $\beta-\alpha-\beta$ fold involved in cofactor binding at the bottom of the substrate-binding pocket and a YxxxK sequence as part of the active center (*5*−*7*). POR occurs in all organisms that carry out oxygenic photosynthesis (*8*); it has been postulated that the photosynthetic eukaryotes obtained their POR homologues from endosymbiotic gene transfer (*9*). Several isoforms of POR were found in barley (*10*) and *Arabidopsis thaliana* (*11*−*13*), while only one POR gene and protein was detectable in other angiosperms such as cucumber (*14*) and pea (*15*, *16*). Seedlings lacking POR undergo photooxidative damage on transfer from darkness to light, mediated by free tetrapyrroles and singlet oxygen that switches on a genetic lethality program (*17*−*19*). There is interest in inhibitors of POR aimed at finding new herbicides (*20*). The finding that Chl c_1, which is structurally identical with Pchlide *a* except for an acrylate side chain instead of a propionate side chain at C-17, is a competitive inhibitor of POR (*21*) encouraged us to synthesize several Pchlide analogues with modified side chains; their synthesis and substrate activity is described in this paper.

Helpful for inhibitor design of an enzyme is the knowledge of its three-dimensional structure. Because an X-ray structure of POR was not available, Townley et al. (*5*) modeled the structure of POR as a homologue to the known structure of 7α-hydroxysteroid dehydrogenase and confirmed the cofactor-binding site by site-directed mutagenesis. Further, the authors tried to locate the substrate in the binding cleft using published data on the activity of substrate analogues; however, some of their conclusions must be reconsidered.

† This work was supported by the Deutsche Forschungsgemeinschaft (SFB 533) and the Fonds der Chemischen Industrie.

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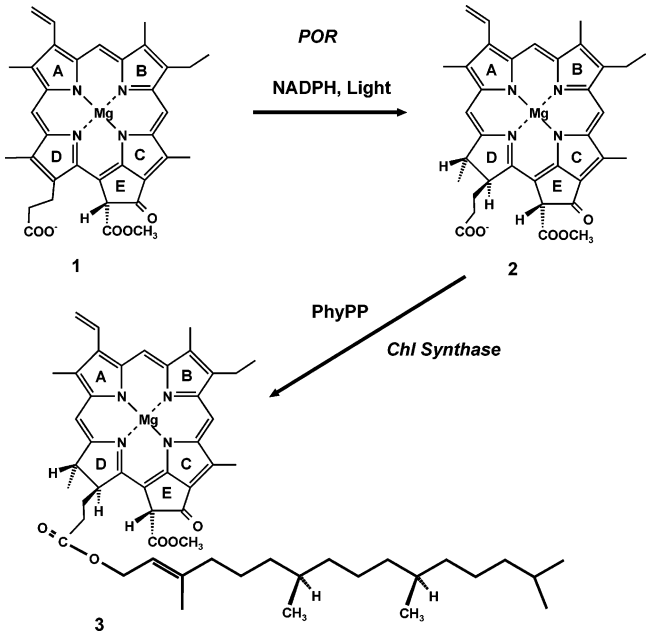
[1] Abbreviations: Chl, chlorophyll; Chlide, chlorophyllide; DDQ, 2,3-dichloro-5,6-dicyanobenzoquinone; PhyPP, phytyl diphosphate; Pchlide, protochlorophyllide; POR, NADPH:protochlorophyllide oxidoreductase; TFA, trifluoroacetic acid.

10.1021/bi0504198 CCC: $30.25
Published on Web 07/21/2005

Scheme 1



Their conclusion that "rings C, D, and E of the pigment must be embedded in and in intimate specific contact with protein" was based on the observation that "any modification to this part of the molecule leads to loss of activity as substrate". Apparently, the authors assumed a high-steric specificity and mentioned as an example "that even the minimal alteration of the C_{17} propionate group to an acrylate chain, as present in chlorophyll c_1, renders it inactive as a substrate for POR" (*5*). In the meantime, it is known that Chl c_1 binds to the active center of POR and is a competitive inhibitor of Pchlide photoreduction; it was hypothesized that the additional double bond alters the photoreactivity resulting in lack of activity despite the correct binding to the enzyme (*21*). Likewise, the modifications at ring E, leading to inactive substrate analogues cited by Townley et al. (*5*), throughout included a change in the chemical reactivity such that enol formation at ring E was abolished. Therefore, it was desirable to change the size of substituents at ring E without a change in the reactivity. Such a modification is described in this paper.

In dark-grown angiosperm seedlings, a ternary complex consisting of the enzyme protein, the cosubstrate NADPH, and the substrate Pchlide *a* accumulates in the inner membranes of etioplasts, and the conversion to Chlide *a* requires one photon per molecule enzyme-bound Pchlide *a* (*22*). Photoactivation probably involves formation of a Pchlide radical (*7*, *23*, *24*) followed by hydride transfer from the *pro-S* face of NADPH (*25*) and proton transfer from a conserved Tyr residue (*6*). The POR−substrate complex can be considered as a photoreceptor for chloroplast development because the photoconversion is the precondition for the subsequent esterification of Chlide *a* at the side chain of ring D, and the esterified Chl *a* (**3**, see Scheme 1) in turn is required for accumulation of functional pigment−protein complexes of the thylakoid membrane (*26*). The esterification step is the prenylation of Chlide *a* with geranylgeranyl diphosphate or phytyl diphosphate, catalyzed by Chl synthase (Scheme 1). Recently, a rapid phase of Chlide prenylation (*27*, *28*) was explained by the preloading of Chl synthase with geranylgeranyl diphosphate or phytyl diphosphate, and it was hypothesized that POR and Chl synthase form a complex (*29*). In this connection, the question arose whether the prenylation can occur while Chlide is still bound to POR; the side chain at ring D must in this case protrude from the binding pocket. This prompted us to probe the topology of substrate binding in the active center not only of POR but also of Chl synthase with modified substrates. Several modified substrates have already been used to test the substrate specificity of Chl synthase (*30*−*32*) and POR (*21*, *33*−*37*); however, the data were not sufficient to answer the question which part of the tetrapyrrole macrocycle is buried in the binding pockets of POR and Chl synthase, respectively, and whether some parts protrude from the enzyme surface during catalysis. Here, we concentrated on artificial substrates with different space-filling substituents at rings A, B, and E of the tetrapyrrole macrocycle and tested the activity as substrates to gain information on the topology of substrate binding to POR and Chl synthase, respectively, and on the flexibility of the active site.

MATERIALS AND METHODS

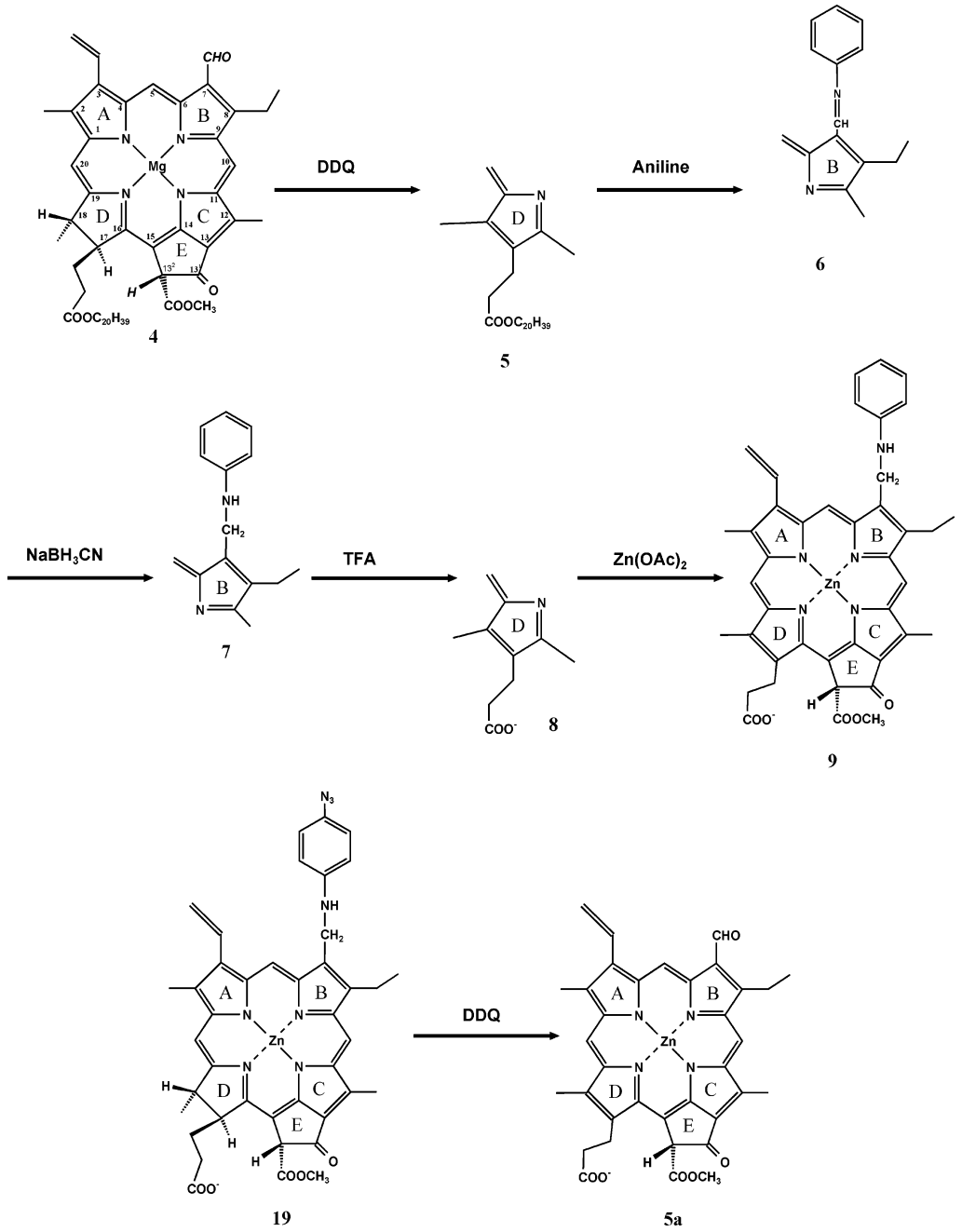
UV/vis absorption spectra were recorded at room temperature in acetone with a diode array spectrophotometer (HP 8451 A, Hewlett−Packard, Palo Alto, CA). ^{1}H NMR spectra were measured on a 360 MHz instrument (AM-360, Bruker, Karlsruhe, Germany) in pyridine-d_5 at room temperature. Mass spectra were recorded by electrospray ionization with a system consisting of a HPLC unit (HP 1100, Hewlett−Packard) and a mass spectrometer unit (LCQ, Finnigan, Bremen, Germany). The HPLC system used for analytical separations consisted of a Waters 600E pump and controller, a diode array detector (Tidas, J&M, Aalen, Germany) and a fluorescence detector (RF-551, Shimadzu, Duisburg, Germany). The solvents used were A, 25 mM ammonium acetate; B, acetone; and C, methanol. The columns were filled with C_{18} reverse-phase Grom Sil 120 ODS-4HE (250 × 4 mm). The analyses were run with flow rates of 1−1.5 mL/min and the following linear gradient: time (min) (vol % A/B/C): 0 (34:15:51), 20 (16:60:24), and 34 (0:100:0).

If not stated otherwise, the reactions were carried out at room temperature under dim green light.

Enzyme Preparation. As a source of POR, we used its fusion protein with the maltose-binding protein (MBP). The plasmid pMAL-POR, containing the POR sequence of pea (*Pisum sativum* L.) was transformed into *Escherichia coli* ER2508, and the MBP−POR fusion protein was expressed and purified by affinity chromatography as described before (*21*, *38*). Pilot experiments, performed with POR after cleavage of the MBP according to ref *7*, showed the same activity as with MBP−POR.

Enzyme Assay. To 50 μg of MBP−POR in 1 mL buffer (50 mM Tris/HCl at pH 7.5, 1 mM EDTA, 1 mM DDT, 0.1% Triton X-100, 0.3 mM OG, and 1 mM NADPH) 2 μL of a 1 mM stock solution of pigment in DMSO was added, and the mixture was incubated under gentle shaking for 15−20 min in the dark at room temperature. The absorption spectra were monitored in intervals after irradiating the samples with white light (100 μmol s^{-1} m^{-2}, cold light lamp KL 2500, Schott, Mainz, Germany). Samples to be analyzed by HPLC were extracted into ethyl acetate, and the organic

Scheme 2



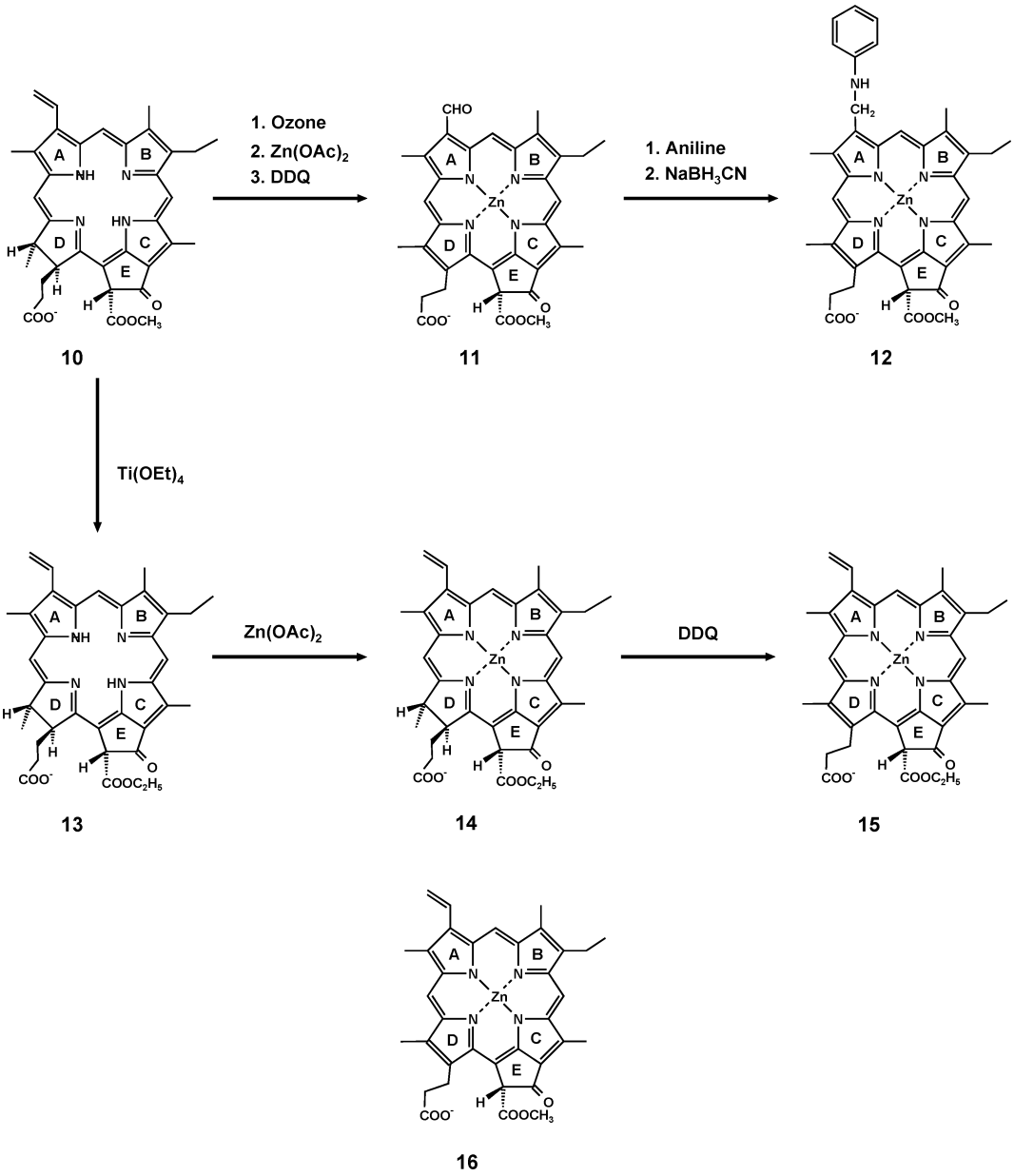
phase was washed with water and evaporated to dryness. The residue was dissolved in 20 μL of acetone for HPLC analysis.

Chl Synthase Activity. Chl synthase activity was determined with the inner-membrane fraction of oat etioplasts (*39*). For the standard assay, 10 μL aliquots of this fraction containing 30 μg of protein were diluted with 210 μL of reaction buffer (40 nM tricine, 10% glycerol, 4 mM KH_2PO_4, 10 mM $MgCl_2$, 10 mM NaF, 2 mM ascorbate, and 0.1% β-mercaptoethanol), 10 μL of 130 mM ATP, and 10 μL of 4 mM phytyl diphosphate. The reaction was started by addition of 2−3 nmol of the zinc−pheophorbide, dissolved in 10 μL of acetone; this amount of pigment had been shown to saturate the enzyme reaction. The mixture was incubated for 45 min at 26 °C, and the reaction was stopped by addition of 750 μL of acetone, 50 mg of diethylamino-ethyl-cellulose (DE 52, Whatman), and 500 μL of *n*-hexane. The mixture was given an intense shake, and the phases were then separated by centrifugation. The amount of esterified pigment was determined using the absorption spectrum of the upper phase.

RESULTS AND DISCUSSION

Substrates for POR. Modified substrates for POR are not commercially available, and only a limited selection of chemical reactions can be applied to keep the core macrocycle and sensitive side chains intact. The following schemes 2−4 describe the successful chemical reactions; detailed information and characterization of the products by NMR and mass spectra are described in the Supporting Information. Starting point for the variation of the substrate structure was the observation that the 3-formyl derivative (Zn−protopheophorbide *d*) and the 7-formyl derivative (Pchlide *b*) *a*

Scheme 3

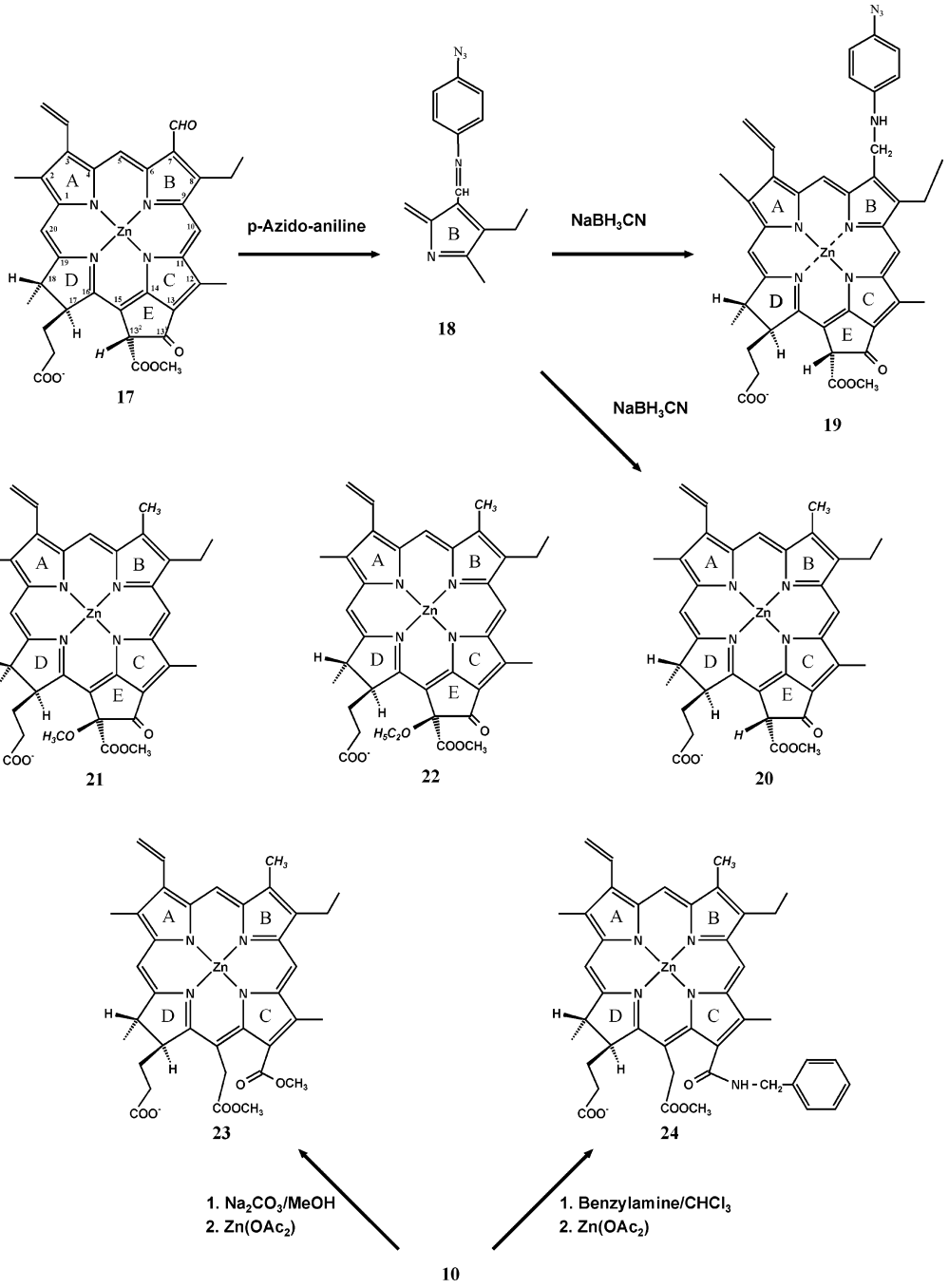


are suitable substrates for POR (*35*). The V_{max} value of Pchlide *b* was in the same order of magnitude as that of Pchlide *a*, and the V_{max} value of Zn−protopheophorbide *d* was about half of the V_{max} value of Zn−protopheophorbide *a*. For introduction of space-filling substituents, we applied reductive amination of the formyl groups with aniline. This weak base was chosen to avoid opening of the isocyclic ring E, the preferred reaction with strong bases (see structure **24**, Scheme 4). We used the Zn complexes throughout, which are more stable than the Mg complexes, and insertion of Zn occurs under milder conditions than insertion of Mg. An important aspect if sensitive side chains are present.

Chl *b* (**4**) was the starting compound for preparation of the presumptive substrate Zn−7^1-phenylamino-protopheophorbide *a* (**9**) with a bulky side chain at ring B (Scheme 2). As the first step, **4** was dehydrogenated to protochlorophyll *b* (**5**). The subsequent 4 steps include formation of the Schiff base **6** with aniline, reduction with $NaBH_3CN$ to the amino derivative **7**, removal of the phytol chain (and Mg) with trifluoroacetic acid, forming the protopheophorbide **8**, and insertion of Zn to the desired product **9**. The reaction with aniline was controlled by HPLC. The subsequent reduction was best monitored by the shift of the Soret band from 450 to 436 nm, while the Qy band shows only a blue shift of 3 nm. Product **9** was purified and characterized by its UV−vis, mass, and ^{1}H NMR spectra. The reaction with DDQ must occur before modification of the side chain; when we treated Zn−7^1-[4-azido-phenyl]amino-pheophorbide *a* (**19**, see Scheme 4) with DDQ, we obtained only Zn−protopheophorbide *b* (**5a**) (see Scheme 2). The amino compound was presumably dehydrogenated to the Schiff base, which underwent hydrolysis under the reaction conditions.

The second putative substrate Zn−3^1-phenylamino-protopheophorbide *a* (**12**) with a bulky side chain at ring A was then synthesized in 5 steps from pheophorbide *a* (**10**, Scheme 3). The progress of ozonolysis of **10** (λ_{max}, 668 nm) to pheophorbide *d* (λ_{max}, 693 nm) was monitored by a change in the absorption spectra. Zn had to be inserted for dehydrogenation to the porphyrin **11**. The hydrogenation of the Schiff base (λ_{max}, 636 nm) to the amino derivative **12** (λ_{max},

Scheme 4



618 nm) was also monitored by the absorption spectra. Finally, **12** was characterized by its UV−vis, mass, and ^{1}H NMR spectra.

Finally, transesterification of **10** with ethanol yielded the 13^3-ethyl derivative **13**, which carries a substituent at the isocyclic ring E that is only slightly larger than the natural substituent (Scheme 3). The chemical reactivity of the ethyl ester should be about the same as that of the methyl ester. The reaction mixture contained, besides the educt **10** and the desired product **13**, the 17^3-ethyl ester of educt and product and the diastereomeric 13^2-(*S*) compounds (Figure 1). After purification, the identity of **13** was shown by the ^{1}H NMR spectrum (see the Supporting Information); decisive are the signals for the 13^3-OC_2H_5 group at 4.41 ppm (q) and 1.21 ppm (t), instead of the signals for the 13^3-OCH_3 group of **10** at 3.88 (s). Zn insertion resulted in **14**, and dehydrogenation yielded the proto derivative **15** (Scheme 3), which was tested as a possible substrate for POR.

Substrates for Chl Synthase. While previous investigations concentrated on variation of the central metal and substituents at ring E (*30*, *31*), we prepared here chlorin derivatives with space-filling substituents at rings B and C (Scheme 4). Starting material for the modification at ring B was Zn−pheophorbide *b* (**17**), which had already been shown to be a suitable substrate for Chl synthase (*32*). Formation of the Schiff base **18** with an excess of *p*-azido-aniline required the presence of small amounts of the hydrochloride; the reaction was then almost quantitative. Reduction with $NaBH_3CN$ yielded the expected amino derivative **19** as the main product and Zn−pheophorbide *a* (**20**) as a byproduct. With the strong base benzylamine, opening of ring E was the preferred reaction. To avoid complications by competing

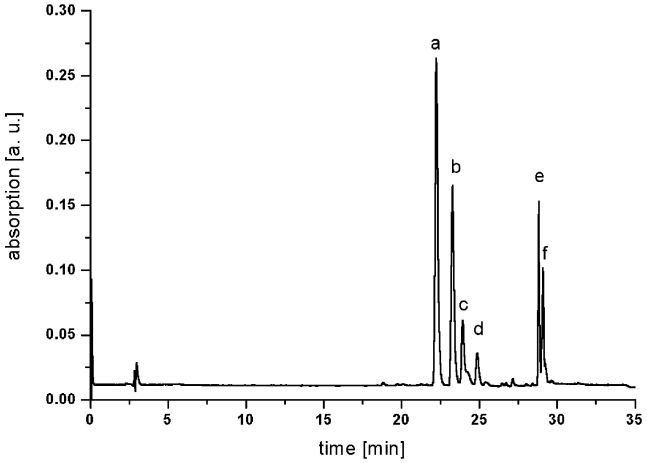


FIGURE 1: HPLC analysis of the products of trans-esterification of pheophorbide *a* (**10**) with ethanol. The identity of the reaction products was confirmed by comparison of retention time and absorption spectrum with those of authentic compounds or, if not available, by mass and NMR spectroscopy (see the Supporting Information) as follows: (a), pheophorbide *a* (**10**); (b), [13^2-ethoxycarbonyl]pheophorbide *a* (**13**); (c), pheophorbide *a*′; (d), [13^2-ethoxycarbonyl]pheophorbide *a*′; (e), [17^2-ethoxycarbonyl]pheophorbide *a*; and (f), [13^2,17^2-diethoxycarbonyl]pheophorbide *a*.

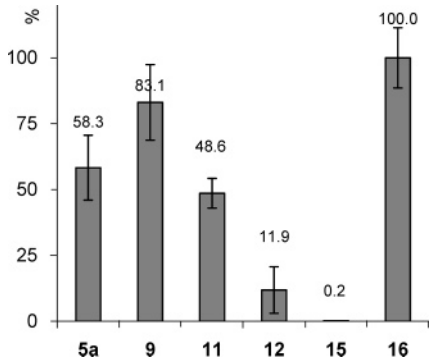


FIGURE 2: Relative reaction rate of different modified substrates with MBP−POR, compared with Zn protopheophorbide *a* (**16**, 100%). For structures of **5a**, **9**, **11**, **12**, and **15**, see Schemes 2 and 3.

reactions at side chains, we used pheophorbide *a* (**10**) as starting material; the product **24**, obtained after insertion of Zn, carries a bulky side chain at ring C of about the same size as the side chain at ring B in **19**. For comparison, we prepared compound **23** (Scheme 4), which lacks the bulky side chain but has ring E opened like **24**.

Enzyme Reaction of POR. As a source of the enzyme, POR from pea was used as a fusion protein with the MBP (*38*). The enzyme reaction was monitored by changes in the absorption spectra (*35*). Because the reaction is light-dependent, we mixed the enzyme, substrate, and cosubstrate in the dark and started the reaction by saturating irradiation with white light. Under the conditions applied here, the turnover number for the standard model substrate Zn−protopheophorbide *a* (**16**) is 0.053 mol of product (mol of enzyme)$^{-1}$ sec^{-1}. We confirmed that the compounds with formyl group, **5a** and **11**, are good substrates; under the present conditions, they exhibit about 58 and 49%, respectively, of the standard activity (Figure 2). As previously outlined (*35*), this reduction in activity is probably due to the electronic rather than to the steric influence of the formyl groups in positions 3 and 7. In accordance with this

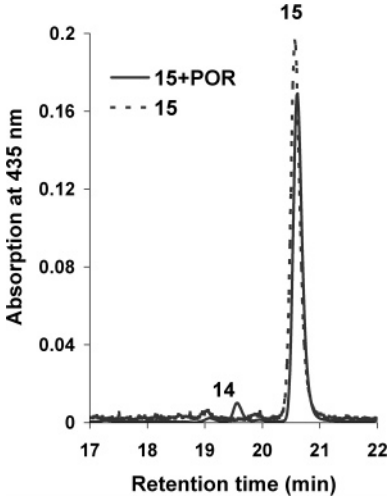


FIGURE 3: HPLC analysis of the photoconversion of Zn 13^3[ethyl]-protopheophorbide *a* (**15**, retention time of 20.6 min) to that of Zn 13^3[ethyl]pheophorbide *a* (**14**). The educt (- - -) does not contain any traces of Zn protopheophorbide *a*, (**16**; R_t, 19.7 min). After photoconversion (—), a new peak is detected (**14**; R_t, 19.5 min) with a typical chlorin absorption spectrum. The retention time differs from that of Zn pheophorbide *a* (**20**, 18.5 min).

assumption, the 7^1-phenylamino derivative **9**, the electronic spectrum of which resembles the *a* and not the *b* compound, is an even better substrate (83% of the standard) than Zn−protopheophorbide *b* (**5a**). The 3^1-phenylamino derivative **12** is reasonably accepted as a substrate (12% of the standard) while the 13^3-ethyl derivative **15** is a very poor substrate (0.2% of the standard).

To show that such a small activity of **15**, monitored by the increase in absorption at 655 nm, was not due to traces of **16**, which is a much better substrate, the reaction was also monitored by HPLC (Figure 3). No trace of **16** was detectable in the solution of the applied substrate **15**, and while the absorption spectrum of the product **14** (maxima at 423 and 654 nm) was identical with that of Zn−pheophorbide *a* (**20**), its retention time (19.56 min) differed from that of **20** (18.60 min), the expected product of the enzymatic reduction of **16**.

Enzyme Reaction of Chl Synthase. The source of the enzyme was a preparation of inner membranes from wheat etioplasts (*39*). The products of the enzymatic reaction were extracted in *n*-hexane and quantified by spectrometry. The best of the tested substrates was Zn−pheophorbide *b* (**17**), which yielded about 0.6 nmol of product at substrate saturation within 45 min under standard conditions; this value was set at 100% (Figure 4). Zn−pheophorbide *a* (**20**) showed about 90%, and the phenylamino derivative **19** showed almost 60% of this activity. Interestingly, the 7^1-phenylimino derivative **18** shows a significant decrease in activity to 27%, although its substituent at ring B requires about the same space as that of **19**; however, it is more rigid because of the C═N double bond. There must be some limited space left around ring E because substitution of the 13^2 proton on the A side of the macrocycle by a methoxy group (compound **21**) still leaves 53% activity; this is reduced to 11% for the 13^2-ethoxy compound **22** (Figure 4) and reduced to complete inactivity when the 13^2-carbomethoxy group is on the A side as in Chlide *a*′ (*31*).

Surprising is the significant activity (39%) of compound **23** that originated from the opening of ring E; the side chain

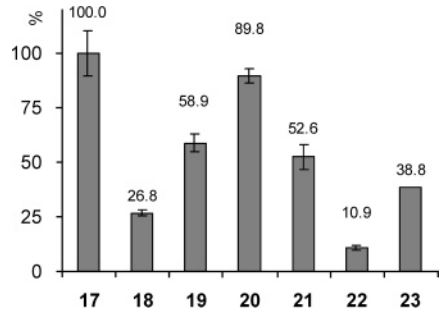


FIGURE 4: Esterification rates of different modified substrates with Chl synthase, compared with Zn−pheophorbide *b* (**17**, 100%). For structures **18**−**23**, see Scheme 4. Compound **24** does not react with Chl synthase (not shown).

carrying the 15^1-carbomethoxy group (corresponding to the 13^2-carbomethoxy group of **20**) is apparently flexible enough to accommodate the requirements of the binding cleft. Further, the acceptance of **23** as a substrate means that a closed ring E and its keto group are not essential for binding. In contrast, compound **24** is not accepted as a substrate; the bulky substituent at ring C probably prevents **24** from binding to the active site.

Topology of the Active Centers. The data of this paper extend previous studies on the substrate specificity of POR and Chl synthase (*30*−*37*). When the data are taken together, two features for the substrate specificity common to both enzymes become apparent. (1) Both enzymes are able to accommodate the bulky phenylamino substituent at C-7^1, indicating that there is some free space around ring B, while the substrate is enzyme-bound. We propose that this substituent remains at the surface of the enzyme protein or protrudes out of the binding pocket into the medium. (2) On the other hand, both enzymes do not tolerate much steric variation around ring E, indicating that this part of the substrate is tightly surrounded by the peptide in the binding pocket.

Despite this common feature, there are differences between POR and Chl synthase. In the case of POR, the 7^1-phenylamino compound **9** shows a higher activity than Zn−protopheophorbide *b* (see Figure 2), while the 7^1-phenylamino compound **19**, the corresponding substrate of Chl synthase, shows a lower activity than Zn−pheophorbide *b* (see Figure 4). This could indicate some steric restriction specific for Chl synthase. Indirect indication for such restriction is the low activity of the phenylimino compound **18** with the rigid C═N bond at C-7^1 (27% activity) compared to the phenylamino compound **19** (59% activity) and the lack of activity of bacteriochlorophyllide with its "out-of-plane" substituents at ring B (*40*).

It had been demonstrated that the 13^2 enantiomers, Chlide *a*′ and Pchlide *a*′, are not accepted by Chl synthase and POR, respectively (*31*, *34*). For Chl synthase, this has been explained as a steric constraint: the A side of Chlides that naturally carries a proton at C-13^2 can be substituted by small residues such as a hydroxy or methoxy group without the loss of activity but not by residues as large as the carbomethoxy group (*31*). The lower activity (11%) of the ethoxy compound **22** compared to the methoxy compound **21** (53% activity, see Figure 4) fits into this picture. The chemical nature of the substituent at C-13^2 seems to play only a marginal role; Zn−pyropheophorbide *a* that carries two protons at C-13^2 shows about the same activity as the methoxy compound **21** (*31*).

This feature is different for POR: the carbomethoxy group on the B side seems to be essential for substrate binding because Zn−pyroprotopheophorbide *a* that lacks the carbomethoxy group and carries protons on the A and the B side at C-13^2 is neither accepted as a substrate nor does it inhibit binding and activity of Zn−protopheophorbide *a* (*34*). Likewise, substitution of the carbomethoxy group on the B side by the smaller methoxy group results in the lack of acceptance by POR (*34*). Thus, the lack of activity as a substrate cannot be taken as evidence for steric constraint. It seems reasonable to assume that the chemical or photochemical reactivity of the carbomethoxy group is required for being a substrate of POR. It is remarkable that all modifications at ring E that resulted in the loss of activity also resulted in the loss of ability for enol formation at ring E. Here, we prepared the carboethoxy compound **15** (see Scheme 3) that is only one CH_2 residue larger than the "natural" carbomethoxy substrate **16** without significant change of the electrostatic properties. Surprisingly, this minor change resulted in almost complete loss of activity in the reaction with POR (see Figure 2). We conclude that there is scarcely any free space around the 13^2-carbomethoxy group of the substrate in the binding pocket of POR.

When our results with artificial substrates are taken together with data from the literature, they suggest a likely position of the substrate within the active center of POR where the "southern part" of the tetrapyrrole (rings C, D, and E) is deeply buried in the binding pocket, while the "northern part" (rings A and B) points to the surface or even protrudes from the enzyme surface. While rings A and B and their bulky substituents of the artificial substrates are possibly exposed to the medium in the recombinant enzyme, this must not necessarily be the case for the native enzyme in the plastids: at least in etioplasts, POR is highly aggregated, which may not leave any free space at the enzyme surface. We propose, nevertheless, the same basic position of the substrate within the binding pocket in the recombinant and native enzyme. In particular, it is highly unlikely that ring D with its propionic acid side chain extrudes from the binding pocket into the surrounding medium or to Chl synthase for esterification while still bound to POR. In accordance with this view, POR does not accept the propionic acid methyl ester as a substrate (*35*−*37*); this means either that there is only limited space around the propionic acid group in the binding pocket or that the negative charge of the propionic acid anion is involved in binding. The latter possibility is supported by the finding that Chl c_1, the compound with an acrylic side chain at ring D, binds to POR and is a competitive inhibitor for Pchlide photoreduction (*21*). Ring D is anyhow most likely located close to the dihydropyridine ring of NADPH, which is probably located at the bottom of the binding cleft of POR like in other members of the tyrosine-dependent oxidoreductase family (*5*), such that hydride transfer can take place at C-17 of Pchlide. Because the phototransformation to Chlide takes place in the submicrosecond time scale (*41*, *42*), it can be hypothesized that the location of the propionic acid side chain is still the same after phototransformation, and this view implies that the release of Chlide from POR before esterification can occur at the propionic side chain.

ACKNOWLEDGMENT

We thank Dr. Margareta Ryberg for a preparation of wheat etioplasts, Dr. Hugo Scheer for a gift of 13^2-(*S*)-ethoxyethylpheophorbide, and Dr. Edmund Cmiel for the NMR spectra.

SUPPORTING INFORMATION AVAILABLE

Experimental details of the synthesis of modified substrates and their characterization by UV/vis, NMR, and mass spectrometry. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI0504198