Absolute Configuration of Protochlorophyllide *a* and Substrate Specificity of NADPH−Protochlorophyllide Oxidoreductase[||]

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Abstract: Protochlorophyllide *a* was isolated from dark-grown barley (*Hordeum vulgare* L.) seedlings. The circular dichroism spectrum was identical with that of protochlorophyll *a* prepared from chlorophyll *a* by dehydrogenation. The mirror image was obtained for the circular dichroism spectrum of protochlorophyllide *a*′ that had been prepared from chlorophyllide *a*′. These results prove the $13^2(R)$ configuration of natural protochlorophyllide *a*. Incubation of the seedlings with 5-aminolevulinate resulted in accumulation of protochlorophyllide *a*. This proved to be partially racemized already within the plants. Circular dichroism spectra were also obtained for $13^2(R)$-methoxy and $13^2(S)$-methoxy derivatives of zinc protopheophorbide *a* that cannot racemize. Using the increment of the methoxy group by comparison with the respective methoxypyro compound, the circular dichroism spectrum of zinc protopheophorbide *a* was calculated. Kinetics of racemization indicated a faster reaction for zinc than for magnesium complexes. The enzyme NADPH−protochlorophyllide oxidoreductase solubilized from isolated prolamellar bodies of dark-grown wheat (*Triticum aestivum* L.) does not accept protochlorophyllide *a*′ or any compound with substituents at C-13^2 different from protochlorophyllide *a*. The substrate specificity is compared with that of chlorophyll synthase.

Introduction

Chlorophyll (Chl) biosynthesis is light-dependent in angiosperms due to the light requirement of NADPH−protochlorophyllide oxidoreductase (POR) for its enzymatic activity. This key enzyme in the greening process exhibits some interesting properties.[1] It forms a ternary complex with both substrates, protochlorophyllide *a* (Pchlide *a*) and NADPH.[2] The complex is accumulated in form of large aggregates mainly in prolamellar bodies (PLBs), highly regular three-dimensional membrane networks characteristic of etioplasts.[3,4] The enzymatic reduction of Pchlide *a* to chlorophyllide *a* (Chlide *a*) does not occur in the dark but requires at least one photon per Chlide formed. Thus the assumption seems justified that not Pchlide in its S_o state, but a photoproduct derived from the S_1 state, is the proper substrate. The presumed photoproduct is possibly identical with the intermediate that can be trapped at liquid nitrogen temperature.[5,6] More recently, it was shown that at least two intermediates are present after irradiation at low temperature.[7] In intact plastids, the POR protein is degraded after the enzymatic reaction. It is however relatively stable in isolated PLBs after irradiation.[8] This experimental system has been used to reload the apoenzyme with chemically modified substrates and test the enzymatic reaction, e.g. with zinc protopheophorbide (Ppheide) *b*.[9] It turned out later, that zinc Ppheide *b* used in these experiments was racemic, i.e. the $13^2(R,S)$ pigment. It was not clear whether both enantiomers were accepted as substrate. We had already shown that chlorophyll synthase, the enzyme that catalyzes the subsequent step of Chl biosynthesis, only accepts $13^2(R)$-Chlide *a* and not the epimeric $13^2(S)$-Chlide *a* as substrate.[10]

Two questions arise from these observations: (1) Is Pchlide *a*, the precursor molecule of chlorophyll *a* as accumulated in dark-grown plants, racemic or optically active, and (2) does POR have the same steric requirements at C-13^2 of its substrate as chlorophyll synthase or does it use the racemate as well and what is the configuration of its product?

To our knowledge, the configuration at C-13^2 of natural Pchlide has not yet been elucidated. Houssier and Sauer[11] found optical activity of protochlorophyll *a* (Pchl *a*) isolated from pumpkin seed coats. The authors assumed without any proof that this Pchl *a* should have the same configuration at C-13^2 as Chl *a*. This Pchl is, however, not metabolized to Chl and hence could have the $13^2(S)$ configuration or the $13^2(R)$ configuration. We describe here the determination of the configuration of natural Pchlide *a* at C-13^2 and the steric requirements of POR.

Experimental Section

All reactions were carried out at room temperature under dim green light. Electronic absorption spectra were recorded in diethyl ether at

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[||] List of abbreviations: Chl, chlorophyll; Chlide, chlorophyllide; DDQ, 2,3-dichloro-5,6-dicyanobenzoquinone; Pchl, protochlorophyll; Pchlide, protochlorophyllide; Phe, pheophytin; Pheide, pheophorbide; PLB, prolamellar body; POR, NADPH:protochlorophyllide oxidoreductase; Pphe, protopheophytin; Ppheide, protopheophorbide.
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room temperature with a Lambda 2 UV/vis spectrophotometer (Perkin Elmer). ^{1}H-NMR spectra were measured in pyridine-d_5 with a Bruker model AM 360-MHz instrument. Liquid secondary-ion mass spectra were recorded in a *m*-nitrobenzyl alcohol matrix with a Finnigan model MAT900 and a cesium gun (20 kV, 4 μA emission). After detection of a survey spectrum going up to 1200 Da, the molecular ion region was scanned 15−25-fold and then averaged. Since porphyrins tend to form varying amounts of $[M + H]^+$ and $[M - H]^+$ ions beside the $M^{\bullet+}$ ion, the measured molecular ion distribution is not identical with the intensity distribution based on the natural abundance. We therefore state both values together with the calculated percentage of $[M + H]^+$ and $[M - H]^+$ ions. CD spectra were measured with a Dichrograph CD6 (Jobin Yvon) at 277 K in diethyl ether if not otherwise stated. All spectra were normalized to $A_{soret} = 1$. HPLC analysis was performed using a 480 model (Gynkothek) fitted with a UV/vis detector (SP-6V, bandwidth 8 nm, Gynkothek). The pigments were separated on a Rosil C_{18} reversed phase column (5 μm, 250 × 4.6 mm) at a flow rate of 1.5 mL min^{-1} with 66% acetone/water (pH 3.5, HOAc) for 12 min, followed by a linear gradient to 100% acetone (5 min). Pigments were purified by isocratic chromatography on a preparative column (reversed phase material, C_{18}, 55−105 μm, Waters) with 60% acetone/40% buffer (50 mM Hepes/KOH, pH 7.85, containing 1 mM $Na_2S_2O_4$) for Ppheides or 85% acetone/15% buffer (10 mM Hepes/KOH, pH 7.85, 1 mM $Na_2S_2O_4$) for protopheophytins (Pphes). The collected fractions containing the desired pigment were extracted into diethyl ether if racemization had to be avoided. Otherwise ethyl acetate, which is a better solvent for zinc Pheides, was used instead. The organic phase was washed with water, dried over Na_2SO_4, and evaporated to dryness. Some of the dry zinc Ppheides could not be redissolved in diethyl ether. For spectral measurements, these pigments were dissolved in small amounts of pyridine and then diluted with diethyl ether to a final concentration of ≥99% diethyl ether.

Enzyme Assay. Prolamellar bodies were isolated from dark-grown wheat seedlings (*Triticum aestivum*) as previously described by Ryberg and Sundqvist.[12] The enzyme assay was performed as described by Schoch et al.[9] Briefly, after phototransformation of endogenous Pchlide in the presence of NADPH, Triton X-100 and exogenous substrate were added. The mixture was irradiated, and the pigments were extracted and analyzed by UV/vis spectra and HPLC.

Infiltration of Leaves. Six-day-old dark-grown barley leaves were excised from the seedlings and placed in small tubes in either 0.035 M phosphate buffer (pH 6.5) or 5 mM 5-aminolevulinic acid in 0.035 M phosphate buffer (pH 6.5) for 22 h at 298 K in the dark according to Sundqvist.[13]

Preparation of Pigments. 13^2(*R*)- and 13^2(*S*)-Chlorophyllide *a* (Chlide *a* and *a*′) were prepared as described by Helfrich et al.[10]

13^2(*R*)-Protochlorophyllide *a* (Pchlide *a*) (1a). Partial purification: 20 g of 7-day-old dark-grown barley leaves was frozen in liquid N_2, and the pigment was extracted with 80 mL of acetone. After filtration, 40 mL of petroleum ether (30−50 °C) was added. The upper layer, containing extracted carotenoids, was discarded. The Pchlide *a* was extracted into diethyl ether. The organic layer was washed several times with water. This solution (partially purified Pchlide *a*) was used without further purification for CD measurements. Pure Pchlide *a* was prepared according to Schoch et al.[9] This procedure caused more racemization than the partial purification described above.

13^2(*S*)-Protochlorophyllide *a* (Pchlide *a*′) (1b) was prepared from 13^2(*S*)-chlorophyllide *a* by dehydrogenation with DDQ (see below). The UV/vis spectrum was identical to that of **1a**.

13^2(*R*)-Protochlorophyll *a* (Pchl *a*) (2a) was prepared from Chl *a* as described by Schoch et al.[9]

Dehydrogenation of Metallopheophytins (Metallo-Phes) and Metallopheophorbides (Metallo-Pheides) to the Corresponding Protopigments. This reaction, which required a central metal in the tetrapyrrole, was achieved in a rapid reaction with a large excess (about 10 000-fold) of 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ). Preparation of the educts used in this work are described by Helfrich et al.;[10] preparation of Zn 13^2(*R*,*S*)-hydroxy-Pheide *a* is described by Helfrich.[14] The educt (0.5−2 μmol) was dissolved in 5 mL of acetone. DDQ (0.1 M in acetone) was added in portions of 10−100 μL. The progress of the reaction was checked after each addition by determining the disappearance of the chlorin band (at 652−660 nm) and appearance of the porphyrin band (at 612−620 nm). When the reaction was nearly completed, further oxidation was stopped with an excess of ascorbic acid. The reaction mixture was then mixed with ethyl acetate and water. The organic layer was washed several times with 0.2 M phosphate buffer (pH 7.8) until DDQ and its hydroquinone were completely removed. The metallo-Pphes and metallo-Ppheides were purified by column chromatography (see above). The following metallo-Pphes and metallo-Ppheides were prepared from the corresponding metallo-Phes and metallo-Pheides, respectively:

Zinc Protopheophorbide *a*/*a*′ (Zn Ppheide *a*/*a*′, Racemate) 3a,b. UV/vis: λ_{max} (nm) (relative absorbance) = 428 (1.00), 526 (0.03), 543 (0.04), 564 (0.05), 613 (0.14). LSIMS: molecular ion cluster at *m*/*z* (% found; % calcd for $C_{35}H_{32}N_4O_5Zn$) 651 (11; 0), 652 (100; 100), 653 (82; 41), 654 (83; 66), 655 (67; 33), 656 (58; 47), 657 (35; 17), 658 (13; 5), 8% $[M - H]^+$, 68% $M^{\bullet+}$, 24% $[M + H]^+$ ions, fragment cluster at *m*/*z* 593 (41%).

Zinc 13^2(*R*)-Protopheophytin *a* (Zn 13^2(*R*)-Pphe *a*) 4a. UV/vis: λ_{max} (nm) (relative absorbance) = 429 (1.00), 527 (0.02), 544 (0.03), 564 (0.04), 614 (0.14). LSIMS: molecular ion cluster at *m*/*z* (% found; % calcd for $C_{55}H_{70}N_4O_5Zn$) 929 (15; 0), 930 (100; 100), 931 (85; 64), 932 (86; 78), 933 (61; 50), 934 (58; 56), 935 (33; 29), 936 (13; 10), 9% $[M - H]^+$, 61% $M^{\bullet+}$, 30% $[M + H]^+$ ions; fragment cluster at *m*/*z* 653 (36%) and 593 (46%).

Zinc 13^2(*S*,*R*)-Hydroxyprotopheophorbide *a* (Zn 13^2-OH-Ppheide *a*′/*a*, Racemate) 5a,b. UV/vis: λ_{max} (nm) (relative absorbance) = 428 (1.00), 527 (0.03), 540 (0.04), 565 (0.05), 613 (0.14). ^{1}H-NMR: δ 10.44, 10.43, 10.26 (3 × s, 5-, 10-, 20-H), 8.55 (dd, 3^1-H_x, J_{BX} = 18 Hz, J_{AX} = 11 Hz), 6.47 (d, 3^2-H_B, J_{BX} = 18 Hz), 6.20 (d, 3^2-H_A, J_{AX} = 11 Hz), 3.98 (q, $H_2C[8^1]$, J = 7.5 Hz), 3.91, 3.68 (2 × s, $H_3C[12^1]$, $H_3C[18^1]$), 3.61, 3.60 (2 × s, $H_3C[2^1]$, 13^2-CO_2CH_3), 3.50 (s, $H_3C[7^1]$) 3.79, 3.33 (2 × m, $H_2C[17^1]$), 2.08, 1.66 (2 × m, $H_2C[17^2]$), 1.78 (t, $H_3C[8^2]$, J = 7.5 Hz). LSIMS: molecular ion cluster at *m*/*z* (% found; % calcd for $C_{35}H_{32}N_4O_6Zn$) 667 (17; 0), 668 (100; 100), 669 (78; 41), 670 (79; 66), 671 (61; 33), 672 (57; 47), 673 (33; 17), 674 (13; 5), 12% $[M - H]^+$, 68% $M^{\bullet+}$, 20% $[M + H]^+$ ions; fragment clusters at *m*/*z* 651 (65%), 639 (52%), 593 (50%), 576 (45%).

Zinc 13^2(*S*)- and 13^2(*R*)-Methoxyprotopheophorbide *a* (Zn 13^2-(*S*)-OMe-Ppheide a) 6a and (Zn 13^2(*R*)-OMe-Ppheide *a*) 6b. UV/vis and MS spectra are identical for both enantiomers within the margins of error. UV/vis: λ_{max} (nm) (relative absorbance) = 429 (1.00), 526 (0.03), 541 (0.04), 564 (0.05), 613 (0.15). LSIMS: molecule-peak cluster at *m*/*z* (% found; % calcd for $C_{36}H_{34}N_4O_6Zn$) 681 (10; 0), 682 (100%; 100), 683 (79; 43), 684 (91; 67), 685 (57; 34), 686 (53; 48), 687 (32; 18), 8% $[M - H]^+$, 69% $M^{\bullet+}$, 23% $[M + 1]^+$ ions; fragment clusters at *m*/*z* 651 (75%) and 623 (50%). CD: **6a** λ_m (nm) ($\Delta A \times 10^{-5}$): 429 (+2.6), 442 (−9.8), 597 (−1.2), 623 (+0.6). CD: **6b** λ_m (nm) ($\Delta A \times 10^{-5}$): 429 (−2.6), 442 (+9.3), 596 (+1.5), 623 (−0.6).

Zinc Pyroprotopheophorbide *a* (Zn pyro-Ppheide *a*) 7. UV/vis: λ_{max} (nm) (relative absorbance) = 429 (1.00), 525 (0.03), 563 (0.05), 611 (0.11). LSIMS: molecular ion cluster at *m*/*z* (% found; % calcd for $C_{33}H_{32}N_4O_3Zn$) 593 (54; 0), 594 100; (100), 595 (98; 39), 596 (87; 65), 597 (59; 32), 598 (50; 46), 599 (3; 16) 600 (13; 4), 33% $[M - H]^+$, 48% $M^{\bullet+}$, 19% $[M + H]^+$ ions; fragment clusters are not detectable.

Zinc 13^2(*R*)-Methoxypyroprotopheophorbide *a* (Zn 13^2(*R*)-OMe-pyro-Ppheide *a*) 8a. UV/vis: λ_{max} (nm) (relative absorbance) = 426 (1.00), 538 (0.05), 562 (0.06), 611 (0.12). ^{1}H-NMR: δ 10.50, 10.44, 10.30 (3 × s, 5-, 10-, 20-H), 8.55 (dd, 3^1-H_X, J_{BX} = 18 Hz, J_{AX} = 11 Hz), 7.84 (s, 13^2-H), 6.46 (d, 3^2-H_B, J_{BX} = 18 Hz), 6.18 (d, 3^2-H_A, J_{AX} = 11 Hz), 4.29 (s, 13^2-OCH_3), 4.03 (q, $H_2C[8^1]$), 3.97, 3.71, 3.63, 3.50 (4 × s, $H_3C[12^1, 18^1, 2^1, 7^1]$), 4.02, 3.53 (2 × m, $H_2C[17^1]$)), 1.81 (t, $H_3C[8^2]$), J = 7.5 Hz), 1.61 (m, $H_2C[17^2]$). LSIMS: molecular ion cluster at *m*/*z* (% found; % calcd for $C_{34}H_{32}N_4O_4Zn$) 623 (9; 0), 624 (100; 100), 625 (64; 40), 626 (74; 66), 627 (50; 33), 628 (52; 64), 629 (26; 17), 8% $[M - H]^+$, 79% $M^{\bullet+}$, 13% $[M + H]^+$ ions; fragment cluster at *m*/*z* 593 (64%). CD: λ_m (nm) ($\Delta A \times 10^{-5}$): 432 (+21.0), 588 (−0.8), 617 (+1.6).

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Table 1. Comparison of the Optical Activity of Natural and Synthetic Pigments Related to Protochlorophyll[a]

	λ_{max}	**2a**[b]	**2a**[c]	**1b**[d]	**1a**[e]	**1a**[f]	**1a**[g]	λ_{max}	**4a**[h]	λ_{max}	**3a**[i]	**3a**[j]
B_y	431−432	+13.74	+15.3	−15.2	+9.5	−[k]	+7.7[l]	429	+11.8	432	+22.5	+22.9
B_x	440−441	−4.15	+4.7	+4.6	−3.2	−	−1.6	441	−2.1	443	−6.2	−5.4
Q_x	603	−2.06	+2.2	+2.2	−1.3	−2.1[m]	−1.0[m]	591	−2.4	594	−1.7	−1.8
Q_y	626−627	+1.65	+1.7	−1.7	+1.0	+2.1[m]	+1.2[m]	619	+2.3	618	+1.9	+2.0

[a] All data are $\Delta A \times 10^{-5}$, taken from CD-spectra normalized to $A_{soret} = 1$. λ_{max} values are given in nm. [b] Pchl a, values from ref 11. [c] Pchl a prepared from Chl *a*. [d] Pchlide *a*′ prepared from Chlide *a*′. [e] Pchlide *a*, isolated from leaves, purified by chromatography. [f] Pchlide *a*, partially purified, from leaves infiltrated with buffer. [g] Pchlide *a*, partially purified, from leaves infiltrated with 5-aminolevulinate [h] Zn Pphe *a*, prepared from Zn Phe *a*. [i] Zn Ppheide *a*, calculated by addition of CD spectra of **8a** + **6a**. [j] Zn Ppheide *a*, calculated by subtraction of CD spectra of **8a** − **6b**. [k] CD bands from 400 to 500 nm not detectable due to excess of carotenoids. [l] Values of the B-bands not reliable due to carotenoids in the sample. [m] The maxima shifted 2−4 nm to longer wavelengths.

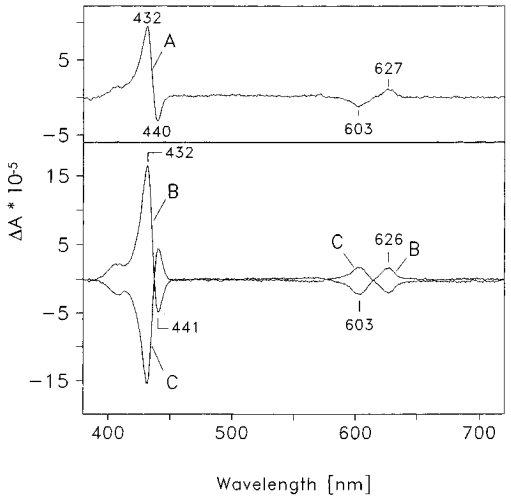

Figure 1. Circular dichroism spectra of Pchlide *a* (**1a**) isolated from dark-grown barley seedlings (spectrum A), Pchl *a* (**2a**) prepared from Chl *a* by dehydrogenation with DDQ (spectrum B), and Pchlide *a*′ (**1b**) prepared correspondingly from Chlide *a*′ (spectrum C). All spectra are measured in diethyl ether.

Results

1. CD Spectra. The CD signals of Pchlide *a* to be measured for determination of the absolute configuration at C-13^2 are expected to be very small: amplitudes of CD bands are usually much smaller for monomeric pigments than for their aggregates. Formation of aggregates has to be avoided in our case because their CD spectra cannot easily be interpreted. Therefore, a dilute solution of Pchlide *a* in diethyl ether (10^{-6}−10^{-7} M) was used. In the resulting range ($\Delta\epsilon/\epsilon \approx 10^{-5}$), erroneous "CD" bands can be observed at the absorption bands even of optically inactive pigments depending on the position of the photocell in the dichrograph. We corrected our spectra by subtracting such "ghost bands" from the actual spectrum. The amplitude of "ghost bands" was determined with racemic pigments or with pigments without asymmetric center (e.g., Zn pyro-Ppheide *a*, **7**).

Pchlide *a* was extracted from dark-grown barley seedlings and purified by column chromatography. The CD spectrum (Figure 1, spectrum A) indicates that natural Pchlide *a* is optically active. The correlation with the known absolute configuration at C-13^2 of Chl *a* was achieved by dehydrogenation of Chl *a* at C-17/C-18 with DDQ. The resulting Pchl *a* (**2a**) has the same signs of all CD bands (Figure 1, spectrum B) as natural Pchlide *a*. For control, Chlide *a*′ (13^2(*S*)-Chlide *a*) was also treated with DDQ. The CD spectrum of the resulting Pchlide *a*′ (**1b**) (Figure 1, spectrum C) is the mirror image of those of Pchl *a* and Pchlide *a*. *These results prove that natural Pchlide a has the same 13^2(R) configuration as Chl a*. The signs of CD bands are also identical with the signs determined for *Cucurbita* Pchl *a*:[11] positive for B_y and Q_y and negative for B_x and Q_x. Thus the 13^2(*R*) configuration was found not only for Pchlide *a* from dark-grown oat seedlings but also for esterified Pchl *a* from the seed coats of *Cucurbita*.

2. Racemization. The amplitudes of CD bands vary with the various Pchl and Pchlide preparations (Table 1). The highest amplitudes were found for Pchl *a* (**2a**) and Pchlide *a*′ (**1b**) that had been prepared by dehydrogenation of Chl *a* and Chlide *a*′, respectively. Pchl *a* isolated by Houssier and Sauer[11] from *Cucurbita* shows CD bands with somewhat reduced amplitudes. Extensive purification of Pchlide *a* from dark-grown barley leaves resulted in significantly reduced amplitudes of all CD bands due to the β-keto ester structure of the isocyclic ring. We conclude that partial racemization took place during purification. A known method to increase the amount of Pchlide *a* consists in incubating dark-grown plants with 5-aminolevulinate.[13] We obtained a 7-fold yield of Pchlide *a* compared to the amount of pigment extracted from the control plants, which were incubated with buffer. In the CD spectrum of partially purified Pchlide *a* from control plants (not shown), the Q_x and Q_y bands had amplitudes comparable to those of the chemically synthesized pigments (Table 1). The B_x and B_y bands were not measured due to the excess of xanthophylls absorbing in this wavelength region. On the other hand, Pchlide partially purified from the 5-aminolevulinate-treated plants showed about one-half of the optical activity of the controls (Table 1). This indicates partial racemization already during accumulation of Pchlide *a* within the plants.

We also determined the CD spectra of several Zn 13^2-methoxy-Ppheide derivatives of known absolute configuration. The methoxy group prevents racemization under the conditions of preparation or measurement. Assuming that each substituent at the asymmetric center contributes by a defined increment to the total optical activity, the addition of the spectra of **6a** and **8a** should eliminate the increment of the methoxy group, resulting in the CD spectrum of Zn 13^2(*R*)-Ppheide *a* (**3a**) (Figure 2, spectrum D). We could not compare this calculated spectrum with that of the authentic compound because dehydrogenation of Zn Pheide *a* with DDQ resulted only in the racemate **3a,b**. The same racemate was obtained starting with Zn Pheide *a*′. We succeeded, however, in obtaining optically active Zn Pphe *a* (**4a**). The experimental CD spectrum (Figure 2, spectrum C) corresponds within the limits of error to the calculated CD spectrum. The amplitudes of the B_x and B_y bands are smaller (about 50%) than those of the calculated CD bands, but the amplitudes of the Q_x and Q_y bands correspond more closely to those of the calculated CD bands (Table 1). We also determined the CD spectrum of Zn 13^2(*R*)-OMe-Ppheide *a* (**6b**) (not shown), which proved to be the exact mirror image of the 13^2(*S*) compound. Subtraction of this spectrum from the CD spectrum of the pyro compound **8a** resulted in an independently calculated CD spectrum for Zn 13^2(*R*)-Ppheide *a* (**3a**) (Table 1).

The described results indicated that the zinc compounds racemized more readily than the magnesium compounds. This

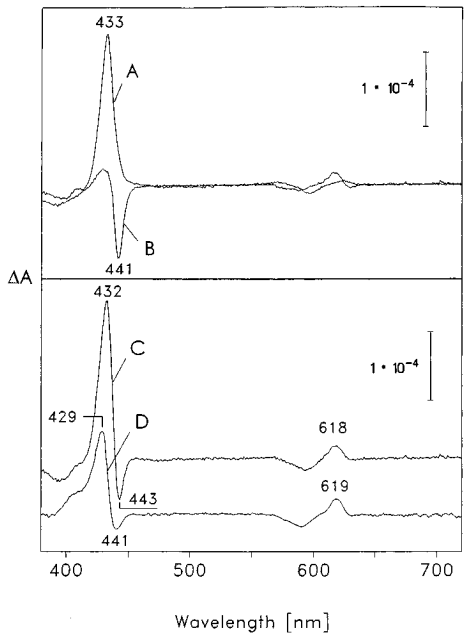

Figure 2. Circular dichroism spectra in diethyl ether of Zn 13²(*R*)-OMe-pyro-Ppheide *a* (**8a**, spectrum A), Zn 13²(*S*)-OMe-Ppheide *a* (**6a**, spectrum B), and Zn 13²(*R*)-Pphe *a* (**4a**, spectrum C). The CD spectrum of Zn 13²(*R*)-Ppheide *a* (**3a**) was calculated by addition of the spectra of **6a** and **8a** (spectrum D).

Table 2. Kinetics of Isomerization at C-13² of Chlorophyll Derivatives in Diethyl Ether Containing 100 mM Triethylamine at 298 K

compd	type	$k \times 10^5$ (s^{-1})
chlorophyll *a*	epimerization	ca. 3.5[a]
protochlorophyll *a*	racemization	3.9
zinc protopheophytin *a*	racemization	9.2

[a] Mean value $(k + k')/2$ for epimerization starting from Chl *a* and Chl *a*′, calculated from the data from Reference 16.

was confirmed by determination of the racemization rate (Table 2). The epimerization rate at C-13² of Chl derivatives was recorded by HPLC.[15,16] We had to use the decrease of optical activity to evaluate the racemization rate of Pchlide derivatives. Therefore CD spectra in the region of the Soret band were measured at different times. Zn Pphe *a* (**4a**) racemized more than twice as fast as Pchl *a* (**2a**). The racemization rate of **2a** was comparable to the epimerization rate of Chl *a*. However, we did not include the *k* value for Pchlide *a* ($k = 0.6 \times 10^{-5} s^{-1}$) in Table 2. After addition of triethylamine to the etheral pigment solution, a CD signal of 7−8-fold magnitude was observed. This strong signal can be due to aggregation or distortion of substituents in the vicinity of the C-13². Therefore the *k* values of Pchl *a* and Pchlide *a* are not comparable.

3. Enzymatic Reaction. The substrate specificity of POR was tested with isolated PLBs from dark-grown wheat seedlings.[9] The PLBs were preirradiated to transform the endogenous Pchlide *a* into Chlide *a*. The enzyme preparation depleted in this way from its natural substrate was solubilized and then used to bind and photoreduce exogenous substrate (e.g., Zn Ppheide *a*) in the presence of excess NADPH. The progress of this reaction can be detected directly in the difference absorption spectra of the solubilized enzyme preparation (spectrum after irradiation minus spectrum before irradation). The negative peak at 628 nm corresponds to disappearance of

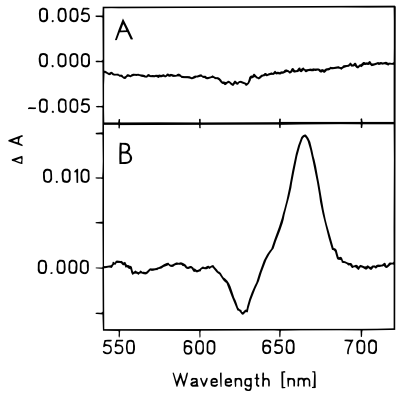

Figure 3. Difference absorption spectra of solubilized PLBs with Zn 13²-OH-Ppheide *a* (**5a,b**, spectrum A) and after subsequent addition of Zn Ppheide *a* (**3a,b**, spectrum B). The difference is always the spectrum after irradiation minus the spectrum before irradiation.

Zn Ppheide *a*, the positive peak at about 665 nm to formation of the product of hydrogenation (here, Zn Pheide *a*). Due to a larger value of the absorption coefficient, the increase at 665 nm is higher than the decrease at 628 nm.

The stereospecificity of chlorophyll synthase had been tested with 13²-substituted Zn Pheide *a* derivatives as potential substrates.[10,14] We prepared the corresponding Zn Ppheides (**5a,b; 6a; 6b; 7; 8a**) and tested whether they are accepted as substrates by POR. As shown in the difference absorption spectrum for **5a,b** (Figure 3, spectrum A), no reaction took place. The same result was obtained for the compounds **6a,b, 7**, and **8a** (not shown). Prolonged or strong irradiation has to be avoided in these experiments. This would lead to photooxidation of pigments detectable as negative peaks at both maxima in the difference absorption spectrum. We tested also the possibility that one of the compounds could be a competitive inhibitor or interfere otherwise with the enzyme reaction. The substrate **3a** was therefore added later. Subsequent irradiation resulted in normal phototransformation of **3a** even in the presence of the nontransformable compounds **5a,b** (Figure 3, spectrum B), **6a,b, 7**, and **8a** (not shown).

Zn Ppheide *a* used in these experiments was a racemate of the 13²-enantiomers **3a**,**b**. To deduce the stereospecifity of POR, we had to elucidate the configuration of possible remaining educts (**3a** or **3b**) and of the products. This was achieved by extracting the pigments into ethyl acetate immediately after irradiation. The procedure was performed as rapidly as possible to reduce the degree of epimerization of the product(s). In contrast to the enantiomers **3a**,**b**, the 13²-diastereomers of Zn Pheide a can be well separated by HPLC (Figure 4, trace C). To detect only the enzymatic hydrogenation products (Zn Pheides and Chlides), the detection wavelength was set at 658 nm. The endogenous pigment had been transformed into Chlide *a* by preirradiation (Figure 4, trace A). After the reaction, the only new peak belongs to Zn Pheide *a* (Figure 4, trace B). Only traces of Zn Pheide *a*′ are detectable (about 2% based on the *a* epimer); this amount is probably formed by epimerization of the *a* epimer in the extract. The question whether both or only one of the Pchlide *a* enantiomers is reduced by the enzyme had to be answered in another experiment. A corresponding enzyme assay was performed with racemic Pchlide *a* (**1a,b**). After a 5 min illumination as compared to 15 min in all other cases, the pigments were rapidly extracted into diethyl ether and directly measured in the CD spectrometer. The CD spectrum (Figure 5, spectrum A) is composed of signals of 13²(*R*)-Chlide *a* (e.g. at 662 nm) and of 13²(*S*)-Pchlide *a*, **1b** (at 432, 441, 605, and 625 nm). This can be shown more clearly by subtraction of the CD spectrum of pure Chlide *a* (Figure 5, spectrum B),

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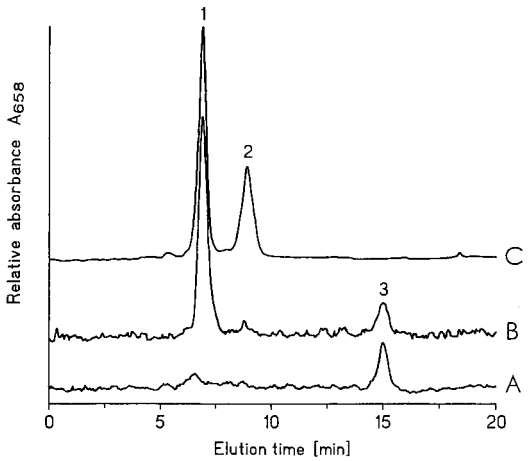

Figure 4. HPLC analysis of products extracted after the enzyme reaction: (A) pigments extracted from preirradiated PLBs (Chlide *a*, peak 3); (B) pigments extracted after subsequent addition of Zn Ppheide *a*/*a*′ (racemate) and another irradiation (Zn Pheide *a*, peak 1 is formed); (C) authentic Zn Pheides *a* and *a*′ (peaks 1 and 2, respectively).

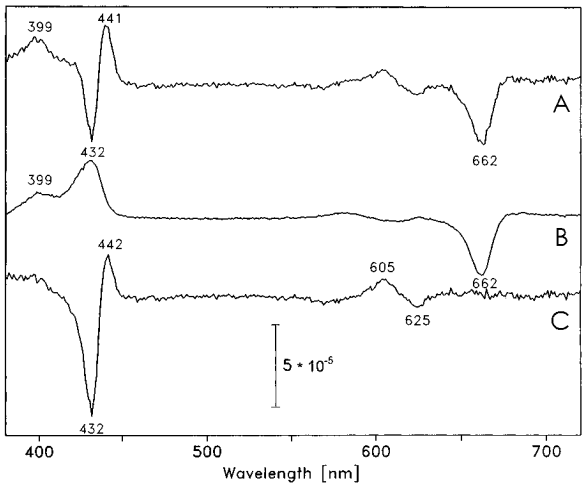

Figure 5. Circular dichroism spectra of the pigments extracted from PLBs after incubation with Pchlide *a*/*a*′ (**1a,b**, racemate) and irradiation: (spectrum A) total pigments extracted and transferred into diethyl ether; (spectrum B) isolated 13^2(*R*)-Chlide *a*; (spectrum C) difference spectrum A minus B = remaining Pchlide *a*′ (**1b**).

resulting in the correct spectrum of Pchlide *a*′ (Figure 5, spectrum C; compare with Figure 1, spectrum C). The signs are opposite to the corresponding CD bands of Pchlide *a* isolated from dark-grown seedlings (see Figure 1, spectrum A).

Discussion

Configuration of Natural Pchlide *a*. The present paper describes for the first time the CD spectrum of monomeric Pchlide *a* (**1a**,**b**). Brouers[17] determined the CD spectrum of aggregated Pchlide *a* and stated that upon disaggregation "the CD bands disappeared almost completely. Small single positive CD bands centered at the absorption maxima were observed". Brouers must have overlooked the negative CD bands, bands that can clearly be seen in our CD spectrum. The CD signals of Pchl and Pchlide aggregates are much more intense than those of the monomers but highly variable and hence not suitable for determination of the configuration at asymmetric centers. Brouers found a negative CD band at 654 nm and a positive band at 640 nm for aggregated Pchlide in benzene. A similar, but blue-shifted CD spectrum was found for aggregated Pchl in CCl_4 with a negative band at 628 nm and a positive band at 622 nm.[11] However, in a solid film of Pchl produced by evaporation of a diethyl ether solution, a negative CD band at the red absorption maximum (640 nm) was found only occasionally.[18] In most cases, the film had an absorption maximum at 635 nm and no CD signal was detectable at all unless the film was treated with NH_3 or acetone vapor. In this case a positive CD band appeared at the red maximum (644 or 654 nm, respectively). The shape of the CD spectrum of Pchl in micelles of Triton X-100 strongly depended on the pigment concentration.[19] Even preparations of the enzyme−substrate complex have been described with either a negative CD band at 647 nm and a positive CD band at 637 nm,[20] a positive CD band at 652 nm and a negative CD band at 638 nm,[3] or two negative CD bands at 635 and 615 nm.[21]

The CD spectrum of monomeric Pchlide *a* determined in this paper resembles the CD spectrum of monomeric Pchl *a* isolated from pumpkin seed coats.[11] Houssier and Sauer[11] stated that "the asymmetric carbons in the phytol chain are expected to be too far from the π electron system of the porphyrin ring to induce any optical activity in these porphyrin electronic transitions". The only asymmetric carbon in the porphyrin part of Pchl *a* and Pchlide *a* is C-13^2; the resemblance of the CD spectra indicates that both pigments have the same configuration at C-13^2.

The correlation with the *R*-configuration at C-13^2 of Chl *a* was achieved in two ways: (1) The dehydrogenation of Chl *a* with DDQ removes the asymmetric centers at C-17 and C-18 and leaves only the asymmetric center at C-13^2. This reaction produced Pchl *a*, which had the same CD spectrum as Pchlide *a* isolated from dark-grown barley seedlings and Pchl *a* isolated from pumpkin seed coats. The result confirmed the assumption that the phytol side chain has no influence on the CD spectrum in the visible range. (2) Even if it was unlikely that the reaction with DDQ turns around the configuration at C-13^2, a second, independent approach was performed. It has been found that each of the asymmetric centers in the series of Chl pigments acts independently on the transition moments.[11] This property has been applied to configurational assignments at 13^2-substituted methyl-Pheides by means of increment calculations.[22] We used the same principle to substract the contribution of the methoxy group from the CD spectrum of Zn 13^2-OMe-Ppheide *a*. The CD spectrum calculated in this way is qualitatively identical with the experimental CD spectrum of Zn-13^2(*R*)-Pphe *a* and, with respect to the wavelength shift caused by the magnesium complexes, of natural Pchlide *a*. The amplitudes of the CD signals correspond rather well for the Q_x and Q_y bands but are smaller in the experimental spectrum of Zn Pphe *a* than in the calculated spectrum for the B_x and B_y bands. Smaller amplitudes are expected if a part of the compound was racemic. We expect partial racemization due to the more rapid reaction of zinc complexes in comparison with magnesium complexes (Table 2). The data do not permit, however, a precise calculation of the degree of racemization.

Substrate Specificity of POR Compared with That of Chlorophyll Synthase. POR and chlorophyll synthase have the same requirement for the *R*-configuration at C-13^2 of their respective substrates. The corresponding 13^2(*S*) compounds are

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neither substrates nor competitive inhibitors. In the present paper, we showed this with racemic Pchlide *a* (**1a,b**) in vitro. The 13^2(*R*)-Pchlide *a* (**1a**) was photoreduced to Chlide *a*, whereas the 13^2(*S*)-Pchlide *a* (**1b**) remained unchanged in the reaction mixture. No Chlide *a*′ (= 13^2(*S*)-Chlide *a*) was formed. This selectivity of POR, together with the fact that Pchlide *a* accumulated in PLBs of dark-grown plants is exclusively the 13^2(*R*) compound (Table 1), seems to exclude, in the normal biosynthetic pathway, the accumulation of porphyrins or chlorins that cannot be transformed into chlorophyll. Incubation of plants with 5-aminolevulinate leads to accumulation of excess Pchlide *a* that is probably not enzyme-bound and becomes partially racemized in plants with time (Table 1). Whereas the 13^2(*R*)-Pchlide *a* can bind to the POR apoprotein after depletion of its substrate by a first irradiation and can then be phototransformed to Chlide *a*, the 13^2(*S*)-Pchlide *a* remains after irradiation and can possibly induce photodynamic damage. Severe damage caused by the "photodynamic herbizide" 5-aminolevulinate if applied together with metal-complexing agents[23] has been attributed to protoporphyrin and its Mg complex as the photodynamic pigments.[24] Accumulation of these pigments cannot be detected or is negligible after application of 5-aminolevulinate alone.[25,26] Damage occurring after 5-aminolevulinate application, e.g. impairment of greening of dark-grown seedlings,[27,28] most probably has to be traced back to excess Pchlide which is not phototransformed even after repeated flash illumination or illumination of the plants with continuous light for 1−2 h.[24,27,29] According to the present results, this is most probably 13^2(*S*)-Pchlide *a*. It is not known, however, whether the nontransformable Pchlide that has been detected occurring naturally in stems[30,31] might be 13^2(*S*)-Pchlide *a*.

In spite of the similar requirement for the *R*-configuration at C-13^2, substrate binding must be different in POR and chlorophyll synthase. Steric hindrance has been postulated for exclusion of compounds by chlorophyll synthase.[10] In 13^2(*R*)-Chlide *a*, the 13^2-hydrogen can be substituted by small functional groups (OH, OMe) without loss of acceptance by the enzyme. Larger groups (OEt) lead to reduction of product formation[14] The 13^2-methoxycarbonyl group itself is not absolutely necessary for the reaction; pyro-Chlide *a* is still esterified about one-half as well as Chlide *a*. On the other hand, POR requires the methoxycarbonyl group in any case, since neither Zn pyro-Ppheide *a* (**7**) nor Zn 13^2-OMe-pyro-Ppheide *a* (**8a**) is photoreduced by the enzyme. Substitution of the 13^2-H in Pchlide by any functional group (OH, OMe) leads to complete loss of substrate property. This means also loss of binding because none of the modified compounds is an inhibitor. So far, investigation of the enzyme mechanism of photoreduction has been concentrated upon C-17 and C-18.[32,33] Further elucidation of the enzyme mechanism of POR has to take into account also the properties of the isocyclic ring. Substituents at the isocyclic ring of Pchlide have also to be considered for substrate binding which is essential for transport of the POR-apoprotein into plastids[34] and for stabilization of the ternary Pchlide-NADPH-POR complex, which occurs in aggregated form in the PLBs of etioplasts.[35]

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