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Biochimica et Biophysica Acta 1605 (2003) 97–103

BIOCHIMICA ET BIOPHYSICA ACTA

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Chlorophylls of the *c* family: absolute configuration and inhibition of NADPH:protochlorophyllide oxidoreductase

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Received 11 December 2002; received in revised form 19 June 2003; accepted 20 June 2003

Abstract

Using circular dichroism (CD) spectroscopy, the stereochemistry at C-13^2 of members of the chlorophyll (Chl) *c* family, namely Chls c_1, c_2, c_3 and [8-vinyl]-protochlorophyllide *a* (Pchlide *a*) was determined. By comparison with spectra of known enantiomers, all Chl *c* members turned out to have the (*R*) configuration, which is in agreement with considerations drawn from chlorophyll biosynthesis. Except for a double bond in the side chain at C-17, the chemical structure of Chl c_1 is identical with Pchlide *a*, the natural substrate of the light-dependent NADPH:protochlorophyllide oxidoreductase (POR). Thus, lack of binding to the active site due to the wrong configuration at C-13^2, which had been proposed previously, cannot be an explanation for inactivity of Chl *c* in this enzymic reaction. Our results show rather that Chl c_1 is a competitive inhibitor for this enzyme, tested with Pchlide *a* and Zn-protopheophorbide *a* (Zn-Ppheide *a*) as substrates.

Keywords: Circular dichroism; Enzyme competitive inhibition; *Emiliana huxleyi*; *Endarachne binghamiae*

1. Introduction

In organisms capable of oxygenic photosynthesis, the reaction center contains chlorophyll (Chl) *a* with one exception (Chl *d* in *Acaryochloris marina* [1]) while the antenna complexes show some variation in the Chl content [2]: besides Chl *a*, it can be Chl *b* (e.g. in green algae and vascular plants), Chl *c* (e.g. in chromophyte algae), Chls *b* and *c* (e.g. in Prochlorophyta and Prasinophyceae), or Chl *d* [3]. The number of representatives of the Chl *c* family has grown from Chl c_1 and c_2 in the early 1970s to five structurally known and a series of partially characterized members [4–6]. The participation of Chls *c* in light harvesting has been shown in several algae, i.e. in *Mantoniella* and *Prochloron* [7–9]. The Chl *c* group differs from the other Chls in several points (Scheme 1): Chls *c* are porphyrins with an acrylic acid side chain at C-17 while the other Chls are chlorins with a propionic acid side chain esterified with phytol; in most members of the Chl *c* family, the side chain at C-17 is not esterified, and recently described nonpolar pigments of the Chl *c*-type do not, in contrast to previous reports [10,11], contain phytol but monogalactosyl diacylglycerol instead [6]. All elucidated Chl *c* structures have in common that the authors either did not state the absolute configuration at C-13^2 or just assumed it as (*R*) due to the configuration in Chl *a*. The knowledge of the absolute configuration will also answer a question concerning the different pathways of the precursor of Chls *c*: the Chl *c* structure resembles that of the Chl precursor protochlorophyllide *a* (Pchlide *a*), and it can be hypothesized that Pchlide *a* yields Chl c_1 by dehydrogenation of the side chain at C-17 [4]. On the other hand, hydrogenation of Pchlide *a* at ring D yields chlorophyllide *a*. This key step in the biosynthesis of Chls *a* and *b* is catalyzed in angiosperms by the light-dependent NADPH:protochlorophyllide oxidoreductase (POR). We also know that the natural Pchlide *a* has the 13^2(*R*) configuration and the 13^2(*S*) enantiomer is not accepted by POR [12]. It has been reported that Chls c_1 and c_2 are not substrates for this enzyme [13]. We confirmed this report and asked two questions: (i) What is the absolute configuration of the *c*-type Chls at C-13^2? The "wrong"

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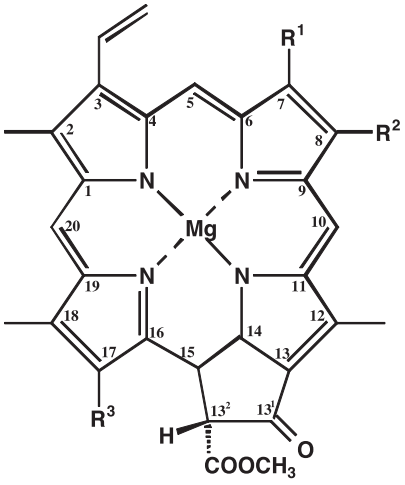
doi:10.1016/S0005-2728(03)00081-1



Pigment	R^1	R^2	R^3
Chl c_1	CH_3	CH_2CH_3	acrylic acid
Chl c_2	CH_3	$CH{=}CH_2$	acrylic acid
Chl c_3	$COOCH_3$	$CH{=}CH_2$	acrylic acid
MV-Pchlide *a*	CH_3	CH_2CH_3	propionic acid
DV-Pchlide *a*	CH_3	$CH{=}CH_2$	propionic acid

Scheme 1. Structure of presently known Chl *c* members: Chls c_1, c_2, c_3, and DV-Pchlide *a* showing the IUPAC/IUB numbering system.

configuration would explain the lack of acceptance by POR. The absolute configuration of *c*-type Chls has not yet been determined in the earlier studies. (ii) Is the missing reaction of Chls *c* caused by lack of binding to POR? We approach the second question here by means of enzyme kinetics.

2. Material and methods

2.1. Extraction and purification of optically active pigments

Chls c_1 and c_2 were extracted from freeze-dried *Endarachne binghamiae*, a brown alga that was collected at Clovelly Beach, Sydney, Australia. The algae were ground with sand and liquid nitrogen in a mortar. The powder from 1 g algae was extracted by grinding it several times with small volumes of 80% acetone/20% 250 mM aqueous ammonium acetate (NH_4OAc) until the extract was only light green. The acetone extracts were quickly transferred in a separatory funnel containing diethyl ether and saline water. The organic layer was washed at least three times with saline water, shortly dried over Na_2SO_4 and then concentrated in a rotary evaporator. Remaining water was removed by azeotropic distillation with acetone. The pigments were redissolved in 5 ml of 90% acetone/10% 25mM aqueous NH_4OAc and separated by HPLC on a self-packed preparative polyethylene column [14,15] using the following program: solvent A, 50% acetone/50% 25 mM NH_4OAc; solvent B, 100% acetone; flow rate, 10 ml/min; time in minutes (% solvent B) = 0(20), 15(30), 20(30), 25(40), 35(40), 40(50), 60(50), 65(60), 70(60), 80(100); retention times, Chl c_1 = 47 min, Chl c_2 = 65 min. Whereas Chl c_2 eluted without visible impurities, the Chl c_1 fraction contained small impurities of a carotenoid and a pheophorbide derivative. After elution, the Chl *c* fractions were quickly extracted into diethyl ether. The ether layer of Chl c_2 was prepared for circular dichroism (CD) spectroscopy by reducing the solvent in a rotary evaporator to a volume of approx. 6 ml (without prior drying over Na_2SO_4). The remaining water was frozen out of the ether by liquid nitrogen. Finally, 1% pyridine (v/v) was added to the ether solution. The Chl c_1 fraction was rechromatographed on a reversed-phase column [14] by isocratic elution with 60% acetone/40% 25 mM ammonium acetate. Isolation and preparation for CD measurement was done as with Chl c_2.

[8-vinyl]-Pchlide *a*, commonly named divinyl(DV)-Pchlide *a*, was prepared from freeze-dried *Prochloron* sp., which was harvested from its ascidian *Lissoclinum patella* [9]. The extraction procedure was basically identical to that of the Chls *c*, except that 70% acetone was used in the presence of solid $MgCO_3{\cdot}Mg(OH)_2$ for extraction.

Chl c_3 was accordingly isolated from *Emiliania huxleyi*, which was grown at 15 °C in F/2 medium of sea water from the North Sea (pH 8.0) at the Alfred-Wegener-Institut, Bremenhaven, Germany. The algae were harvested by filtration through a GF filter and frozen. The algae containing filters were extracted in the presence of small amounts of $MgCO_3{\cdot}Mg(OH)_2$ step by step with firstly 100%, secondly 90% and than twice with 80% acetone. Further extraction of the pigment extracts was done as described above. Using the same RP-HPLC system and elution program as for the second purification of Chl c_1, the Chl c_3 fraction eluted between 10 and 13 min at a flow rate of 3.0 ml/min.

2.2. Spectroscopy

2.2.1. Solutions of optically active pigment

The concentration of the freshly prepared Chl *c* solutions (see above) was adapted to an OD of approx. 0.9 (maximum of Soret band) by dilution with ether/pyridine (99:1, v/v). A Perkin Elmer Lambda 2 spectrophotometer was used for the measurements. λ_{max} (Soret) for Chl c_1 and DV-Pchlide *a*: 436 nm; λ_{max} for Chl c_2: 440 nm; λ_{max} for Chl c_3: 447 nm.

2.2.2. Solutions of optically inactive pigments (racemates) for background measurements

The dry pigments were predissolved in pyridine and diluted with 99% (v/v) diethyl ether. The pigment concentration was adjusted to the same OD as the optically active pigments.

CD spectra were measured on a Dichrograph CD6 (Jobin Yvon) at 277 K using a round, 2 cm pathway cuvette. A

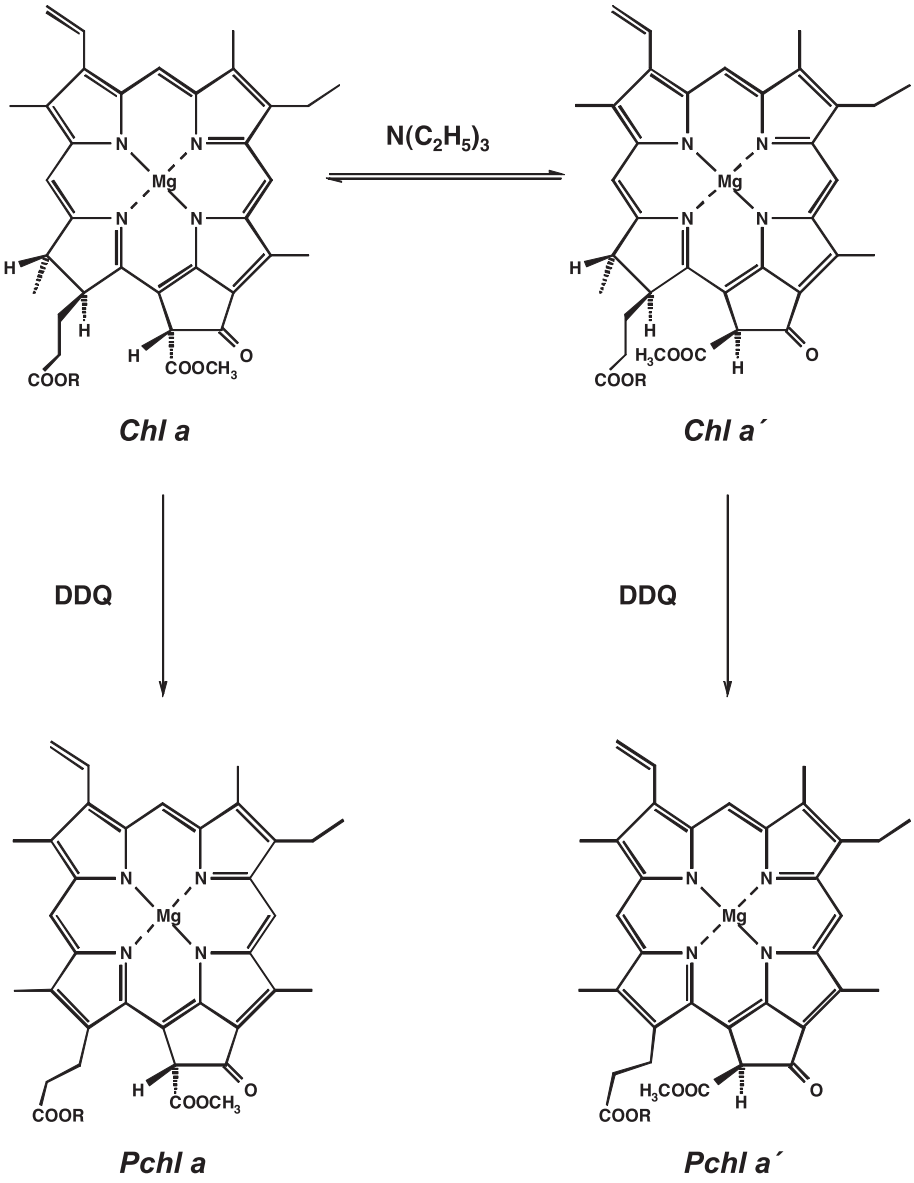
series of approx. 10–15 spectra were recorded and averaged. Prior or after measurement of the optically active compounds, a similar number of baseline spectra were recorded with the corresponding racemates.

2.3. Preparation of POR

For isolation of the recombinant POR protein, the plasmid pMAL-POR, kindly provided by Dr. Michael Timko, was transformed into *E. coli* ER2508 and the transformed cells were grown in rich medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, and 2 g glucose per liter) containing 100 mg/l ampicillin and 15 mg/l tetracycline as described by Martin et al. [17]. These cells contained the coding sequence of the bacterial maltose binding protein and the wild-type pea POR sequence. Induction of MBP-POR by IPTG, extraction of cells and affinity purification of MBP-POR on an amylose column were performed as previously described [17]. Pigment-free POR was isolated from oat etioplasts as described by Klement et al. [15]. Substrates Pchlide *a* and Zn-protopheophorbide *a* (Zn-Ppheide *a*) were prepared as described in Ref. [12].

2.4. Enzyme assay

The standard enzyme assay was performed in reaction buffer (50 mM Tris/HCl, pH 7.2; containing 1 mM NADPH, 1 mM EDTA, 1 mM DTT, 0.1% Triton X-100 and 30% glycerol) with either 0.75 μM pigment-free POR from oat or 1.3 μM MBP-POR. Various amounts of either



Scheme 2. Preparation of Pchl(ide) *a* and Pchl(ide) *a*′. Epimerization of Chl *a* to Chl *a*′ is catalyzed by triethylamine; dehydrogenation of Chl to Pchl is achieved by 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) without change of the configuration at C-13^2. R = phytyl for Chl and Pchl; R = H for Chlide and Pchlide.

Pchlide *a* (0.2–0.55 μM) or Zn-Ppheide *a* (1–6 μM) were added as substrates and the mixture was incubated for 5 min in the dark. The reaction was started by illumination (33 μmol m^{-2} s^{-1}) and absorption spectra were recorded at 5, 10, 15, 20, 30, 45 and 60 s. The initial reaction rate was plotted against substrate concentration (see Figs. 5 and 6).

For the test of enzyme inhibition, POR or MBP-POR in the reaction buffer was pre-incubated with various concentrations of Chl c_1 for 5 min at 4 °C before the substrate pigment was added. Otherwise, the assay was performed as described above. All pigments were added from stock solutions in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the enzyme assay did not exceed 0.5%, a concentration that has been shown not to inhibit the reaction.

3. Results and discussion

3.1. Circular dichroism

CD is a suitable method for determination of the absolute configuration of pigments related to Pchlide provided that authentic pigments with known absolute configuration are available. Starting from Chl *a* and Chlide *a* that have the (*R*)-configuration at C-13^2, we prepared Pchl *a* and Pchlide *a* by dehydrogenation with DDQ [12]; the (*R*)-configuration at C-13^2 is preserved under these conditions (Scheme 2, left part).

Epimerization of Chl *a* and Chlide *a* yielded Chl *a*′ and Chlide *a*′, respectively, that have the (*S*) configuration at C-13^2 [16], and the analogous dehydrogenation resulted in the formation of Pchl *a*′ and Pchlide *a*′ with the (*S*) configuration at C-13^2 (Scheme 2, right part). As expected, the CD spectra of 13^2(*R*) and 13^2(*S*) Pchl(ide) are image and mirror image of each other (Fig. 1); decisive for the 13^2(*R*) configuration is the positive sign of the B_y band at 432 nm and the negative sign of the B_x band at 441 nm (Fig. 1A). Vice versa, 13^2(*S*)-Pchlide shows a negative B_y band and a positive B_x band (Fig. 1B).

The physicochemical properties of the Chl *c* group renders the determination of their CD extremely difficult. A preferred solvent for most Chls is diethyl ether in which the pigments are relatively stable in terms of demetalation and racemization at C-13^2. However, the purified Chls of the *c* group are barely soluble in this and other nonpolar solvents; evaporating diethyl ether solutions to the concentration needed for CD measurements (OD = 0.9 at the Soret band) causes pigment aggregation. A very good solvent for Chls *c* is pyridine, which, however, causes rapid racemization of the pigments. If a solution of a Chl *c*-type pigment is evaporated to dryness and then redissolved in pyridine, only the racemate is obtained. We found the following procedure suitable for CD measurements of *c*-type Chls: the first step is repeated extraction of the freeze-dried algae with 80% acetone, buffered with acetate. The acetate buffer is necessary to avoid demetalation by acid components. This step is followed by transfer of the pigments to diethyl ether and evaporation to dryness. Due to the presence of by products such as lipids, carotenoids and esterified chlorophylls, which act as solution mediators, the pigments can be easily redissolved at this stage in a mixture of acetone and buffer suitable for purification by HPLC. The collected Chl *c* fraction is then transferred to diethyl ether, concentrated to the desired optical density (without evaporating to dryness) and aggregation is avoided by immediate addition of pyridine to a final concentration of 1%. Racemization is very slow in this solvent mixture; however, some racemization cannot be avoided until the CD measurement starts and we could not quantify the degree of racemization that had already occurred during extraction and purification. Therefore, the CD values shown in this paper must be taken qualitatively; the ΔA values of the pure enantiomers are probably higher than the values indicated here. It would have been interesting to determine the velocity rates of

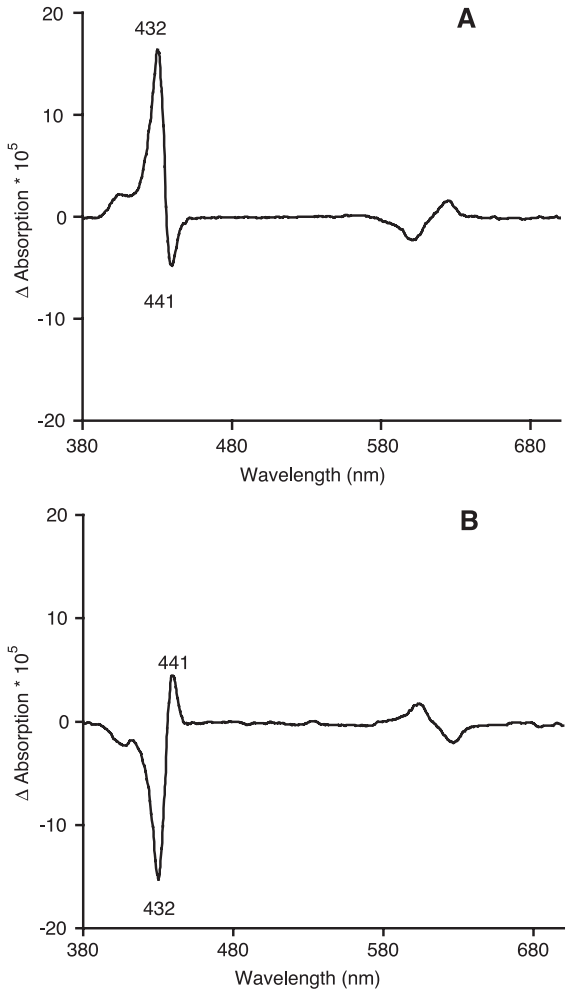


Fig. 1. CD spectra of authentic pigments in diethyl ether. (A) Protochlorophyll *a* (= 13^2 (*R*)-protochlorophyll *a*) prepared by dehydrogenation of Chl *a*. (B) Pchlide *a*′ (= 13^2(*S*)-Pchlide *a*) prepared by dehydrogenation of chlorophyllide *a*′. The maximum of the B_x band is at 441 nm and that of the B_y band at 432 nm.

racemization for the different Chls *c* in this solvent mixture by evaluating the single CD effects of a series taken over several hours. However, it would have been an extremely difficult task without having equal conditions of the pigment solutions such as the content of remaining water and the concentration of optically active pigment at t_0.

As discussed earlier [12], the expected ΔA values of the CD spectra are very small for the pigment monomers due to only one asymmetry centre at $C13^2$; the spectra shown here are corrected by subtracting the background spectra obtained from an optically inactive pigment solution after complete racemization. This procedure eliminates "ghost" bands caused by the geometry of the dichrograph. An absorption band of a UV/Vis spectrum is often composed of different, superposed bands of electronic transitions. These single transitions can be more clearly resolved in the corresponding CD spectrum due to positive and negative signs of the bands. Consequently, the influence of different auxochromic groups can be studied more precisely. Comparing the CD spectra of all measured pigments (Figs. 2–4; Table 1) in a qualitative manner, they all consist of a positive B_x, a negative B_y and a negative Q band between 606 and 618 nm. The signs of these CD spectra resemble that of natural monovinyl (MV)-Pchlide *a* [12], which has been shown to have the $13^2(R)$ configuration; the CD spectrum of $13^2(S)$-Pchlide *a* is the mirror image with the

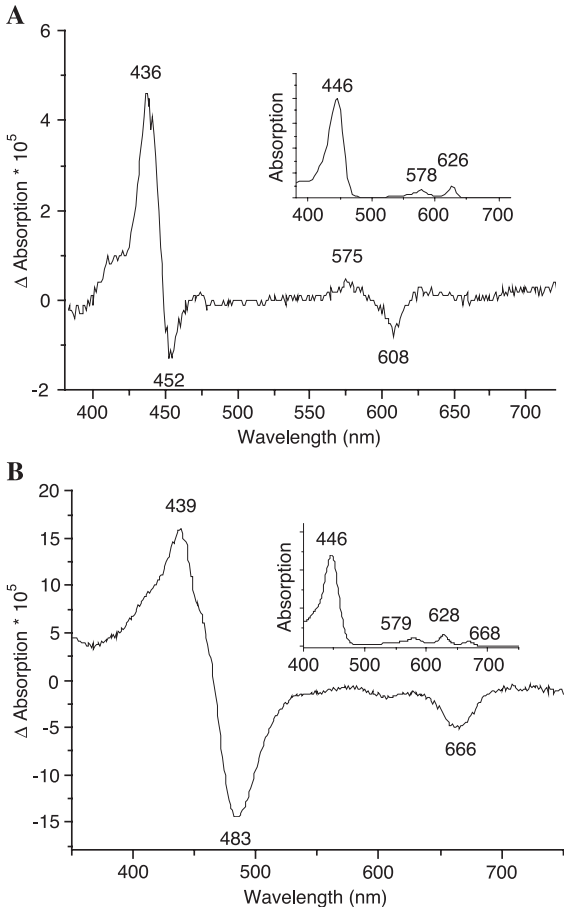


Fig. 2. CD spectrum (main spectrum) and UV/Vis absorption spectrum (insert) of Chl c_1. (A) Monomeric pigment in diethyl ether/1% pyridine. (B) Partially aggregated pigment in pure diethyl ether. The numbers are the peak positions in nm. All spectra were normalized to an optical density of $A = 1.0$ at the Soret band.

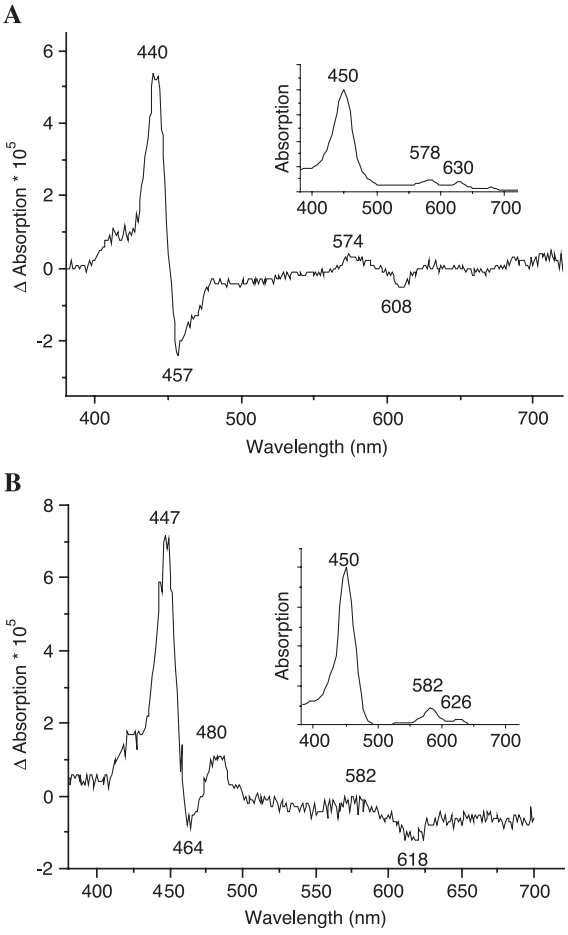


Fig. 3. CD spectrum (main spectrum) and UV/Vis absorption spectrum (insert) of monomeric Chl c_2 (A) and Chl c_3 (B) in diethyl ether/1% pyridine. For details, see legend of Fig. 2.

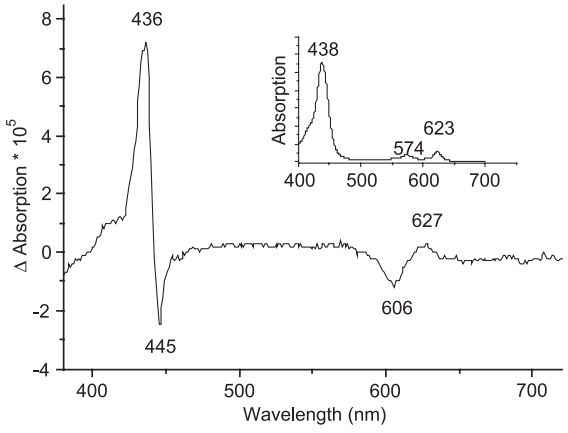


Fig. 4. CD spectrum (main spectrum) and UV/Vis absorption spectrum (insert) of DV-Pchlide *a* in diethyl ether/1% pyridine. For details, see legend of Fig. 2.

Table 1
Absorption maxima and minima [nm] of the CD spectra from different Chls *c*

Pigment	B_y	B_x	Q_x	Q	Q_y
$13^2(R)$-Pchl(ide) *a*	432 (+)	441 (−)	[540–580] (+)	603 (−)	626 (+)
$13^2(S)$-Pchl(ide) *a*	432 (−)	441 (+)	[540–580] (−)	603 (+)	626 (−)
Chl c_1	436 (+)	452 (−)	575 (+)	608 (−)	[620–650] (+)
Chl c_2	440 (+)	457 (−)	574 (+)	608 (−)	[620–650] (+)
Chl c_3	447 (+)	464 (−)	582 (+)	618 (−)	?
DV-Pchlide *a*	436 (+)	445 (−)	[550–590] (+)	606 (−)	627 (+)
MV-Pchlide *a*	432 (+)	440 (−)	[550–585] (+)	603 (−)	626 (+)

The signs in ( ) indicate the sign of the CD bands.
The values in [ ] belong to broad CD bands of extremely low amplitude.

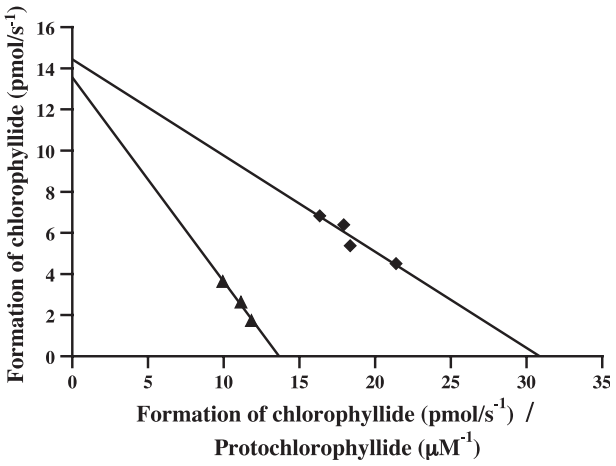


Fig. 5. Eadie–Hofstee plot of the photoreaction of Pchlide *a* with POR, isolated from etiolated oat seedlings. Black line and squares: control; gray line and triangles: after pre-incubation of the enzyme with 0.65 μM Chl c_1.

opposite sign of all bands (see Fig. 1). Whereas the maxima and minima of DV-Pchlide *a* are just shifted by 1–5 nm compared to the MV-Pchlide *a*, the CD spectra of the Chls *c* differ in some aspects to those of the Pchlides: Most obvious for the Chls *c* is a band between 574 and 582 nm (Table 1), which corresponds to the Q_x band in the UV spectra (see inserts of Figs. 2 and 3) but is of very low intensity in the CD spectra of Pchlides. Vice versa, the Q_y band around 626 nm is only clearly present as a (positive) band in the CD spectra of the Pchlides. The most prominent band in this region of the Chl *c* spectra is the negative band at approx. 608 nm, which has no obvious counterpart in the Vis spectrum; we consider it as either composite of vibrational overtones of the Q_y transition or as indication for traces of aggregates that are formed even in the presence of pyridine. The substitution of the propionic group by an acrylic group has a strong impact on the intensity of the Q-transitions in the CD bands. Besides, this substitution is accompanied by a bathochromic shift of 4 nm for the B_y band and 12 nm for the B_x band; the shift in the Q band is less pronounced (compare MV-Pchlide *a* vs. Chl c_1 and DV-Pchlide *a* vs. Chl c_2). Finally, the substitution of the 7-methyl group in Chl c_2 by a methoxycarbonyl group in Chl c_3 is accompanied by a long wavelength shift of approx. 10 nm for all bands. We also tried to synthesize for Chl c_3 a reference pigment with $13^2(R)$ configuration by oxidation of $13^2(R)$-Chl *b* with DDQ to $13^2(R)$-protochlorophyll *b*; however, we only obtained a racemate.

As outlined above, the high-aggregation tendency of Chls of the *c* group required 1% pyridine in diethyl ether to keep the monomeric state, while Helfrich et al. [12] were able to determine the CD spectrum of monomeric Pchlide *a* in pure diethyl ether. The tendency for aggregation of Chl c_1 in diethyl ether could be detected more readily in the CD spectrum (Fig. 2B, main curve) than in the absorption spectrum (Fig. 2B, insert); it was characterized by strong negative CD bands at 483 and 666 nm. The bands at these wavelengths suggest an excitonic coupling between monomers as a result of partly overlapping pigments forming aggregates.

In summary, all investigated Chls of the *c*-type have the same $13^2(R)$configuration as the natural Pchlide *a* isolated from etioplasts of higher plants; thus, lack of binding to POR caused by the "wrong" configuration at C-13^2 cannot be the reason for missing suitability as substrates.

3.2. Binding to POR

With the same configuration of C-13^2 as Pchlide *a*, the lack of reaction of Chl c_1 with POR raises the question whether the introduction of the double bond in the side chain at C-17 leads to steric constraints interfering with binding to the enzyme as discussed by Griffiths [13] or to

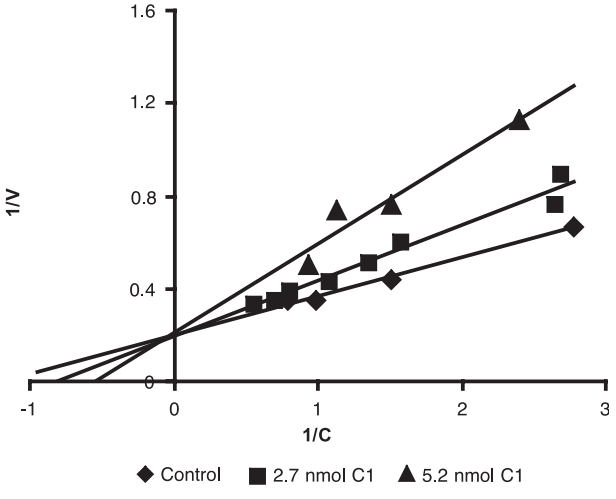


Fig. 6. Lineweaver–Burk plot of the photoreaction of Zn-Ppheide *a* with recombinant MBP–POR after pre-incubation with Chl c_1. The reaction volume was 1 ml. The final concentration of Chl c_1 was 2.7 and 5.2 μM.

disturbance of the photoreaction in spite of binding to the enzyme. We investigated this problem by determination of the kinetics of the reaction with Pchlide *a* and Zn-Ppheide *a* in the presence of Chl c_1. In preliminary experiments, we found 40% inhibition of the photoreaction when the enzyme was pre-incubated with Chl c_1 at equal concentrations to the substrate. This indicated some kind of interference with the enzyme but did not necessarily mean binding to the active center.

In a more detailed investigation, two series of experiments were carried out. In the first series, we used pigment-free POR, isolated from etiolated oat seedlings [15]. The enzyme was pre-incubated with 0.65 μM Chl c_1 and the reaction performed with various concentrations of Pchlide *a*. The Eadie–Hofstee plot (Fig. 5) shows the inhibition of the reaction by Chl c_1, an apparent K_M value of 0.99 μM in the presence of Chl c_1 versus the K_M value of 0.47 μM for the control can be deduced from the slopes of the trend lines. For a competitive inhibition, the trend lines should have the same intercept on the ordinate. This is only approximately the case, probably because of instability of the substrate. In the second series, recombinant pea POR was used in form of the MBP–POR fusion protein. Pre-incubation was performed with 2.7 and 5.2 nmol Chl c_1 and the reaction carried out with the more stable substrate Zn-Ppheide *a*. The Lineweaver–Burk plot (Fig. 6) shows trend lines with the identical intercept on the ordinate for control and both inhibitor concentrations. This clearly demonstrates competitive inhibition of the POR reaction by Chl c_1 and consequently binding of Chl c_1 to the active center of POR. Chl c_1 is the first competitive inhibitor so far for this important plant photoreceptor. Thus, the lack of reaction of Chl c_1 with POR is not lack of binding caused by steric constraints [13] but rather lack of catalytic reactivity at the active site despite binding. Since the double bond in the side chain at C-17 is in conjugation with the π-system of the porphyrin macrocycle, it can be hypothesized that the electronic structure of the S1 state or a photoproduct derived there from is changed by the additional double bond in such a way that the hydride transfer from NADPH is not possible any more. The results will be important for future work on the crystallization of POR since details of substrate binding can be deduced from the X-ray structure after binding of the competitive inhibitor.

Acknowledgements

We thank Dr. Michael Timko for the plasmid pMAL-POR, Dr. Siegrid Schoch for help with Figs. 2–4, and Christian Wiencke and Christina Langreder for growing *E. huxleyi*. The work was supported by the Deutsche Forschungsgemeinschaft, Bonn, and the Fonds der Chemischen Industrie, Frankfurt.

References

[1] Q. Hu, H. Miyashita, I. Iwasaki, N. Kurano, S. Miyachi, M. Iwaki, S. Itoh, A photosystem I reaction center driven by chlorophyll *d* in oxygenic photosynthesis, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 13319–13323.
[2] H. Scheer, Structure and occurrence of chlorophylls, in: H. Scheer (Ed.), Chlorophylls, CRC Press, Boca Raton, 1991, pp. 3–30.
[3] H. Miyashita, H. Ikemoto, N. Kurano, K. Adachi, M. Chilara, S. Miyachi, Chlorophyll *d* as a major pigment, Nature 383 (1996) 402.
[4] R.J. Porra, Recent progress in porphyrin and chlorophyll biosynthesis, Photochem. Photobiol. 65 (1997) 492–516.
[5] S.W. Jeffrey, Structural relationships between algal chlorophylls, in: S.W. Jeffrey, R.F.C. Mantoura, S.W. Wright (Eds.), Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods, UNESCO, Paris, 1997, pp. 566–571.
[6] J.L. Garrido, J. Otero, M.A. Maestro, M. Zapata, The main nonpolar chlorophyll *c* from *Emiliania huxleyi* (Prymnesiophyceae) is a chlorophyll *c*2-monogalactosyldiacylglyceride ester: a mass spectrometry study, J. Phycol. 36 (2000) 497–505.
[7] C. Wilhelm, The existence of chlorophyll *c* in the Chl b-containing, light-harvesting complex of the green alga *Mantoniella squamata* (Prasinophyceae), Bot. Acta 101 (1988) 14–17.
[8] M.W. Fawley, A new form of chlorophyll *c* involved in light harvesting, Plant Physiol. 91 (1989) 723–727.
[9] A.W.D. Larkum, C. Scaramuzzi, G.C. Cox, R.G. Hiller, A.G. Turner, Light-harvesting chlorophyll *c*-like pigment in *Prochloron*, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 679–683.
[10] J.R. Nelson, S.G. Wakeham, A phytol-substituted chlorophyll *c* from *Emiliania huxleyi* (Prymnesiophyceae), J. Phycol. 25 (1989) 761–766.
[11] M. Zapata, J.L. Garrido, Occurrence of phytylated chlorophyll *c* in *Isochrysis galbana* and *Isochrysis* sp. (clone T-ISO) (Prymnesiophyceae), J. Phycol. 33 (1997) 209–214.
[12] M. Helfrich, S. Schoch, W. Schäfer, M. Ryberg, W. Rüdiger, Absolute configuration of protochlorophyllide *a* and substrate specificity of NADPH–protochlorophyllide oxidoreductase, J. Am. Chem. Soc. 118 (1996) 2606–2611.
[13] W.T. Griffiths, Protochlorophyllide photoreduction, in: H. Scheer (Ed.), Chlorophylls, CRC Press, Boca Raton, 1991, pp. 433–449.
[14] M. Helfrich, A. Ross, G.C. King, A.G. Turner, A.W.D. Larkum, Identification of [8-vinyl]-protochlorophyllide a in phototrophic prochlorophytes and algae: chemical and spectroscopic properties, Biochim. Biophys. Acta 1410 (1999) 262–272.
[15] H. Klement, M. Helfrich, U. Oster, S. Schoch, W. Rüdiger, Pigment-free protochlorophyllide oxidoreductase from *Avena sativa* L. Purification and substrate specificity, Eur. J. Biochem. 265 (1999) 862–874.
[16] M. Helfrich, S. Schoch, U. Lempert, E. Cmiel, W. Rüdiger, Chlorophyll synthase cannot synthesize chlorophyll *a*′, Eur. J. Biochem. 219 (1994) 267–275.
[17] G.E.M. Martin, M.P. Timko, H.M. Wilks, Purification and kinetic analysis of pea (*Pisum sativum* L.) NADPH:protochlorophyllide oxidoreductase expressed as a fusion with maltose-binding protein in *Escherichia coli*, Biochem. J. 325 (1997) 139–145.