Chlorophylls and Bacteriochlorophylls: Biochemistry, Biophysics, Functions and Applications
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Supplement to chapter 14
Biosynthesis of Chlorophylls a and b: the Last Steps (W. Rüdiger)

I. Substrate specificity of POR

Several modified Pchlid derivatives were tested for substrate activity with POR (Griffiths, 1991; Helfrich, 1995; Helfrich et al., 1996; Schoch et al., 1995; Klement et al., 1999). The central Mg can be substituted by Zn while the metal-free Pchlid and the Ni, Co, and Cu complex are inactive. Modifications of the side chains at rings A and B are throughout tolerated without essential loss of activity (Table S1). Starting from Pchlid *b*, the 7^1-phenylamino derivative was prepared via the Schiff base. This turned out to be a good substrate indicating free space around ring B when the substrate is bound to the active center of POR. By contrast, only very limited space around ring E is apparently left after substrate binding: only compounds with the natural 13^2(*R*)-configuration (e.g. Pchlid *a*) are substrates while the 13^2 (*S*)-compounds (Pchlid *a'*) are not, and the activity is almost lost by exchange of the natural 13^3-methoxy compound by the 13^3-ethoxy compound (Table S2). The activity is completely lost, in contrast to the properties of Chl synthase (see section on Chl synthase), by exchange of the proton at C-13^2 against a hydroxy- or methoxy-group.

The free propionic acid side chain is essential for activity, neither the methyl ester nor the phytyl ester are accepted as substrates (Griffiths, 1991; Klement et al,. 1999). Previous reports on the phototransformation of esterified Pchl *in vivo* must be corrected: a certain part of Chlid (about 10 – 15 % of Chlid produced by saturating photoconversion of Pchlid) is esterified within several seconds (Adra and Rebeiz, 1998; Domanskii and Rüdiger, 2001; Domanskii et al., 2002), and this rapidly appearing esterified Chl was erroneously assumed by some authors to be formed from esterified Pchl. A careful analysis revealed that the source of the rapidly formed Chl is not the esterified Pchl (Domanskii et al., 2002).

Table S1. Enzymatic photoconversion of Pchlid derivatives modified at rings A and B; the positions of the substituents are indicated in the head of the Table. (data from Klement et al., 1999 and unpublished data by S. Schoch and W. Rüdiger)

	M	C-3	C-7	C-8	V_{max}* [%]
Pchlid *a*	Mg	$CH=CH_2$	CH_3	C_2H_5	100
8[vinyl]Pchlid *a*	Mg	$CH=CH_2$	CH_3	$CH=CH_2$	58
Pchlid *b*	Mg	$CH=CH_2$	CHO	C_2H_5	86
Zn Ppheid *a*	Zn	$CH=CH_2$	CH_3	C_2H_5	61
Zn Ppheid *b*	Zn	$CH=CH_2$	CHO	C_2H_5	35
Zn[3-acetyl]Ppheid *a*	Zn	$COCH_3$	CH_3	C_2H_5	60
Zn[3-formyl]Ppheid *a*	Zn	CHO	CH_3	C_2H_5	26
Zn 7^1-hydroxy-Ppheid *a*	Zn	$CH=CH_2$	CH_2OH	C_2H_5	26
Zn 7^1-phenylamino-Ppheid *a*	Zn	$CH=CH_2$	$CH_2NHC_6H_5$	C_2H_5	27
Zn 3^1-phenylamino-Ppheid *a*	Zn	$CH_2NHC_6H_5$	CH_3	C_2H_5	7

Table S2. Modification of side chains at rings D and E of Pchlid that leads to complete loss of substrate activity of all listed compounds except for the ethoxycarbonyl compound which shows about 5% of the activity of the methoxycarbonyl compound, listed in Table 1; the positions of the substituents are indicated in the head of the Table (data from Griffiths, 1991; Helfrich, 1995; Klement et al., 1999).

	M	**C-17**	**(*S*)C-13^2**	**(*R*)C-13^2**
Zn[13^2(*R*)ethoxycarbonyl]Ppheid *a*	Zn	$CH_2CH_2CO_2H$	H	$CO_2C_2H_5$
Chl c_1	Mg	$CH{=}CH\text{-}CO_2H$	H	CO_2CH_3
Pchl *a*	Mg	$CH_2CH_2CO_2C_{20}H_{39}$	H	CO_2CH_3
Pchlid *a* methylester	Mg	$CH_2CH_2CO_2CH_3$	H	CO_2CH_3
Zn-Ppheid *a* methylester	Zn	$CH_2CH_2CO_2CH_3$	H	CO_2CH_3
13^2(*S*) Pchlid *a* (=Pchlid *a*´)	Mg	$CH_2CH_2CO_2H$	CO_2CH_3	H
Zn-13^2(*S*) Ppheid *a*	Zn	$CH_2CH_2CO_2H$	CO_2CH_3	H
Zn-13^2(*R,S*)-hydroxy-Ppheid *a*	Zn	$CH_2CH_2CO_2H$	OH,	CO_2CH_3
Zn-13^2(*S*)-methoxy-Ppheid *a*	Zn	$CH_2CH_2CO_2H$	OCH_3	CO_2CH_3
Zn-13^2(*R*)-methoxy-Ppheid *a*	Zn	$CH_2CH_2CO_2H$	CO_2CH_3	OCH_3
Zn-13$^{2\text{-}}$pyroPpheid *a*	Zn	$CH_2CH_2CO_2H$	H	H
Zn-13^2(*R*)-methoxy-pyroPpheid *a*	Zn	$CH_2CH_2CO_2H$	H	OCH_3

II. Substrate specificity of Chl synthase

Modification of the Chlid substrate structure gave results with Chl synthase comparable to those with POR after modification of the Pchlid structure. Modification of the substituents at rings A and B are accepted by the enzyme (Table S3). This modification includes the transformation of the 7-methyl group to the 7-formyl group: Chlids *a* and *b* are equally well accepted (Oster and Rüdiger, 1997; Schmid et al., 2002). Even bulky substituents are tolerated. Steric hindrance around ring E after substrate binding to Chl synthase was deduced from the reactivity after modification at this ring (Table S4): Chlid *a´* was not accepted as a substrate while the substitution of the proton at C-13^2 of Chlid *a* by a hydroxy- or a methoxy-group was tolerated (Helfrich et al., 1994); the ethoxy-group at this position resulted in siginificant loss of activity (Helfrich, 1995). By contrast to the siuation with POR, the pyro compound is accepted by Chl synthase.

Table S3*:* Enzymatic esterification of ZnPheids with modified structures at ring A, ring B or C-20. The activity was determined at substrate saturation with excess geranylgeranyl diphosphate as the second substrate. The standard substrate ZnPheid *a* was tested under identical conditions; the positions of the substituents are indicated in the head of the Table (after Helfrich, 1995).

Compound Zinc complex of	**C-7**	**C-20**	**C-13^2**	**Yield of product** pmol•min^{-1}	**Relative yield** (%)
Pheid *a*	CH_3	H	H	13.3	100.0
Pheid *b*	CHO	H	H	15.3	115.0
7^1-OH-Pheid *a*	CH_2OH	H	H	15.0	112.8
7^1-(4-azidophenyl)-imino-Pheid *b*	$CH{=}NC_6H_4N_3$	H	H	4.3	32.3
7^1-(4-azidophenyl)-amino-Pheid *a*	$CH{=}NC_6H_4N_3$	H	H	9.0	67.7
20-Cl-Pheid *a*	CH_3	Cl	H	12.7	95.5
20-Br-13^2-OH-Pheid *a*	CH_3	Br	OH	0.5	3.8

Table S4. Enzymatic esterification of ZnPheids containing modified structures at ring E with geranygeranyl diphosphate added in excess. All ZnPheids were applied at substrate saturation. The yield of each product is compared with that of the standard substrate ZnPheid *a*, which yields 13.3 pmol product/min (= 100 %)under the applied conditions (after Helfrich, 1995).

Compound	**Yield of product**	**Relative yield**
Zinc complex of	pmol • min^{-1}	[%]
Pheid *a*	13.3	100
Pheid *a´*	0*	0*
13^2(R,S)-OH-Pheid *a***	7.7	57.9
13^2(S)-OMe-Pheid *a*	9.0	67.7
13^2(R)-OMe-Pheid *a*	0	0
13^2(S)-OEt-Pheid *a*	1.8	13.5
pyro-Pheid *a*	7.0	52.6
13^2(R)-OMe-pyro-Pheid *a*	5.6	42.1
Chlorin e_6 -13^1,15^2 –Me_2	5.9	44.4
Chlorin e_6 -13^1-benzamido-15^2 Me	0	0

** ratio (R):(S) compound = 1:3

III. Substrate specificity of Bchl synthases

Expression of the *bchG* gene from *R. capsulatus* in *E. coli* yielded active Bchl synthase that accepted Bchlid *a* but not Chlid *a* (Table S5, Oster et al., 1997). The two *bchG* genes from *Chloroflexus aurantiacus* yielded two Bchl synthases with different substrate specificities (Table S5, Schoch et al., 1999): bchG required the 3-acetyl group of the tetrapyrrole, the best substrate was Bchlid *a* followed by 3[acetyl]Chlid *a* (tested as its Zn analogue) while modification of the substituent at C-3 to the hydroxymethyl or to the vinyl group resulted in complete loss of activity. The second enzyme of *C. aurantiacus*, bchG2, required the hydroxymethyl group at C-3, a proton instead of the methoxycarbonyl substituent at C-13^2 (i.e. the so-called pyro-structure), and the unsaturated ring B; equally good substrates were Bchlids *c*, *e* and *d* (tested as their Zn analogues), neither Bchlid *a* nor Chlid *a* were accepted as substrates. In summary, the substrate specificity indicates that modification of the side chains of the tetrapyrrole have to be completed before the esterification occurs. Another difference exists in the specificity of the second substrate: bchG prefers geranylgeranyl diphosphate over all other potential substrates (phytyl diphosphate, farnesyl diphosphate), bchG2 has a much reduced specificity and accepts even the non-isoprenoid cetyl diphosphate to a certain extent (Schoch et al., 1999). This property corresponds to the finding of aliphatic alcohols in Bchl *c* when *C. aurantiacus* is grown on appropriate substrates (Larsen et al., 1995; Steensgaard et al., 1996).

Table S5*:* Synthase reaction with BChG and BChG2 using modified Bchlid substrates; the positions of the substituents and the presence of a single bond (-) or double bond (=) between C-7 and C-8 are indicated in the Table, + signifies reaction, - signifies no reaction (after Oster et al., 1997; Schoch et al., 1999).

M	C-3	C-7	C7-C8	C-13^2	C-20	BChG	BChG2
Mg	$COCH_3$	CH_3	–	CO_2CH_3	H	+	–
Zn	$COCH_3$	CH_3	=	CO_2CH_3	H	+	–
Zn	$CHOHCH_3$	CH_3	–	CO_2CH_3	H	–	–
Mg	$CH=CH_2$	CH_3	=	CO_2CH_3	H	–	–
Zn	$CHOHCH_3$	CH_3	=	H	CH_3	–	+
Zn	$CHOHCH_3$	CHO	=	H	CH_3	–	+
Zn	$CHOHCH_3$	CH_3	=	H	H	–	+
Zn	$CHOHCH_3$	CH_3	–	H	H	–	–
Mg	$CH=CH_2$	CH_3	=	H	H	–	–
Zn	$CHOHCH_3$	CH_3	=	CO_2CH_3	H	–	–

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