Biochem. J. (1988) 255, 685–692 (Printed in Great Britain)

The magnesium-protoporphyrin IX (oxidative) cyclase system

Studies on the mechanism and specificity of the reaction sequence

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Mg-protoporphyrin IX monomethyl ester cyclase activity was assayed in isolated developing cucumber (*Cucumis sativus* L. var. Beit Alpha) chloroplasts [Chereskin, Wong & Castelfranco (1982) Plant Physiol. **70**, 987–993]. The presence of both 6- and 7-methyl esterase activities was detected, which permitted the use of diester porphyrins in a substrate-specificity study. It was found that: (1) the 6-methyl acrylate derivative of Mg-protoporphyrin monomethyl ester was inactive as a substrate for cyclization; (2) only one of the two enantiomers of 6-β-hydroxy-Mg-protoporphyrin dimethyl ester had detectable activity as a substrate for the cyclase; (3) the 2-vinyl-4-ethyl-6-β-oxopropionate derivatives of Mg-protoporphyrin mono- or di-methyl ester were approx. 4 times more active as substrates for cyclization than the corresponding divinyl forms; (4) at the level of Mg-protoporphyrin there was no difference in cyclase activity between the 4-vinyl and 4-ethyl substrates; (5) reduction of the side chain of Mg-protoporphyrin in the 2-position from a vinyl to an ethyl resulted in a partial loss of cyclase activity. This work suggests that the original scheme for cyclization proposed by Granick [(1950) Harvey Lect. **44**, 220–245] should now be modified by the omission of the 6-methyl acrylate derivative of Mg-protoporphyrin monomethyl ester and the introduction of stereospecificity at the level of the hydroxylated intermediate.

INTRODUCTION

MgProto IX monomethyl ester (oxidative) cyclase is a complex of enzymes that catalyse the formation of the chlorophyll isocyclic ring. The mechanism of this reaction sequence (shown in Scheme 1) has been suggested to involve the β-oxidation of the 6-methylpropionate side chain of MgProtoMe to a methyl-β-oxo('keto')propionate group (Granick, 1950). The active methylene carbon atom, situated between the carboxylate ester and the β-carbonyl, then becomes bonded to the bridge carbon in an oxidizing reaction during which two hydrogen atoms are lost. The feasibility of isocyclic ring formation from β-oxoester derivatives of (Mg) protoporphyrin has already been demonstrated by using chemical-synthetic approaches *in vitro* (Cox *et al.*, 1969, 1974; Kenner *et al.*, 1974). In the present study the postulated intermediates in the biological cyclization process were tested with an isolated organelle preparation. The structure and nomenclature for our synthetic substrates are shown in Fig. 1.

In our laboratory, the activity of the cyclase enzyme system has been studied in isolated plastids and, more recently, in a reconstituted system obtained from developing cucumber (*Cucumis sativus*) chloroplasts (Wong & Castelfranco, 1985). A requirement for molecular O_2 and NADPH has been shown (Chereskin *et al.*, 1982; Wong & Castelfranco, 1984). Cyclization is inhibited by artificial electron carriers such as Methylene Blue (Chereskin *et al.*, 1982) and by thiol-group-complexing reagents such as *N*-ethylmaleimide (Wong & Castelfranco, 1985). The cyclase system in wheat (*Triticum aestivum*) etioplasts has also been investigated (Nasrulhaq-Boyce *et al.*, 1987) and appears to be similar to that in developing cucumber chloroplasts. In that study, a continuous spectroscopic assay system was used. It was reported that cyclization could be inhibited by mycobactin, suggesting the involvement of iron ions in the reaction.

Granick's (1950) scheme for cyclization has been partially confirmed by Wong *et al.* (1985) who showed that the synthetic β-oxo intermediate could be converted into Pchlide in the cyclase system. Moreover, the β-hydroxy ester (β-OH) was found to accumulate as an intermediate in the cyclization *in vitro*. The β-OH ester was isolated from reaction mixtures and subsequently converted into Pchlide. We have extended these studies, examining the specificity of the cyclase system at the level of the β-oxo and β-OH intermediates. We have also tested an organically synthesized 6-methylacrylate derivative of MgProto which is reputed to be an intermediate according to Granick's (1950) hypothetical scheme.

MATERIALS AND METHODS

Materials

Hepes, Tes, NADPH (tetrasodium salt; type II; enzymically reduced), SAM (chloride salt), cysteine hydrochloride, EDTA, sorbitol and $ProtoMe_2$ were

Abbreviations used: BSA, bovine serum albumin; Chlide, chlorophyllide; DMSO, dimethyl sulphoxide; MgProto, Mg-protoporphyrin IX; MgProtoMe, Mg-protoporphyrin IX monomethyl ester; $MgProtoMe_2$, Mg-protoporphyrin IX dimethyl ester; MV, 2-vinyl-4-ethyl; DV, 2,4-divinyl; MgDVP, Mg-2,4-divinyl pheoporphyrin a_5; $Mg\text{-}AcMe_2$, 6-acrylate derivative of $MgProtoMe_2$; $Mg\text{-}HPMe_2$, 6β-hydroxy(OH) derivative of $MgProtoMe_2$; $Mg\text{-}KPMe_2$, 6β-oxo ('keto') derivative of $MgProto_2$; Pchlide, protochlorophyllide (this abbreviation is used generically to denote the pheoporphyrin product obtained in *in vitro* cyclization reactions without regard to the oxidation state of the 2 and 4 substituents); SAM, *S*-adenosylmethionine; p.i.c, paired-ion chromatography; PAR, photosynthetically active radiation.

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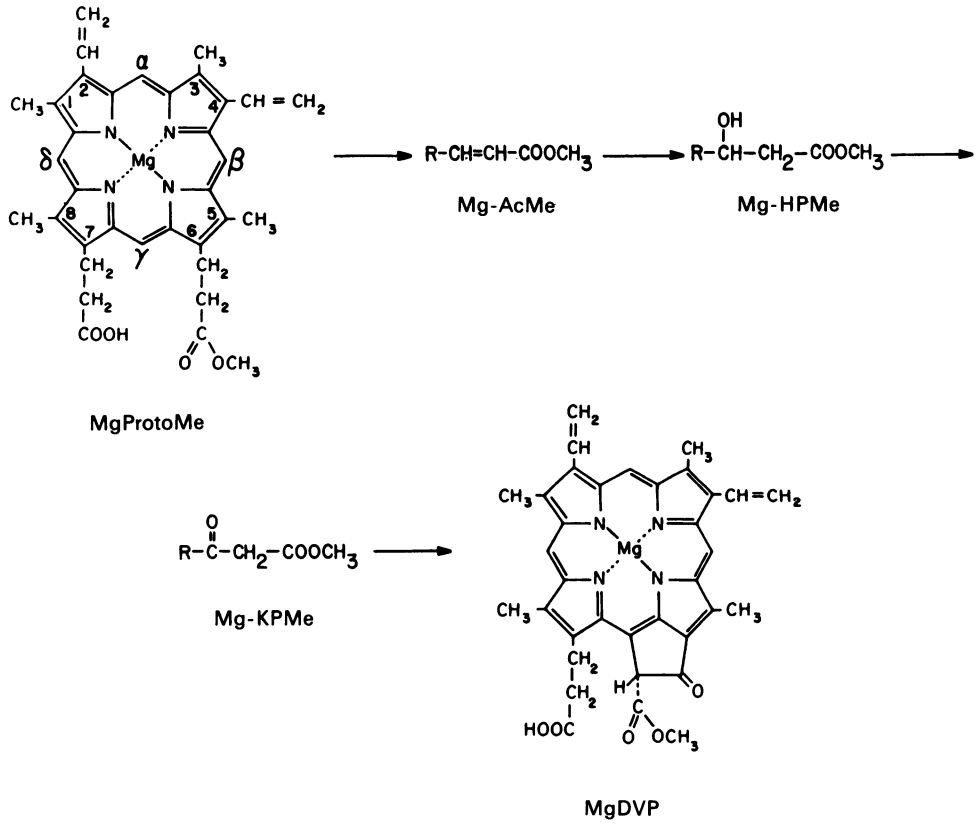


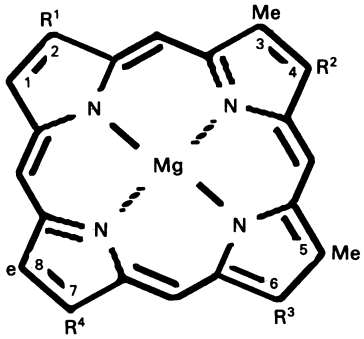
Scheme 1. Conversion of Mg-ProtoMe into Pchlide [reproduced from Wong *et al.* (1985) with permission from the publishers of Plant Physiology]

obtained from Sigma. BSA (fatty-acid-poor) was obtained from Calbiochem. $MgCl_2$ was obtained from Baker. Methylene Blue and DMSO (reagent grade) were purchased from Matheson, Coleman and Bell. Percoll was obtained from Pharmacia. Acetone, hexanes and diethyl ether (for anaesthesia) were obtained from Mallinckrodt. H.p.l.c.-grade acetone, methanol and water were purchased from Fisher Scientific. P.i.c. reagent A was obtained from Waters Associates and diluted according to the direction of the manufacturer. Cucumber (*Cucumis sativus* L. cv. Beit Alpha) seeds were a gift from Harris Moran Seeds, Salinas, CA, U.S.A.

Methods

Plant Tissue. Cucumber seeds were germinated in the dark at 30 °C for 5–6 days (Hardy *et al.*, 1970). Etiolated seedlings were exposed to white light (60–80 $\mu Em^{-2} \cdot s^{-1}$ PAR at 30 °C) for 20 h.

Mg-porphyrin substrates. MeProto, MgProtoMe and $MgProtoMe_2$ were prepared as previously described (Wong & Castelfranco, 1985). Mg-$HPMe_2$ enantiomers were prepared by $NaBH_4$ reduction (Griffiths *et al.*, 1976) of the α-carbonyl functionality of the 6β-oxoester chlorin (the synthesis of which was described by Smith &



Substrates	R^1	R^2	R^3	R^4
MgProto	$-CH=CH_2$	$-CH=CH_2$	$-CH_2CH_2COOH$	$-CH_2CH_2COOH$
MgProtoMe	$-CH=CH_2$	$-CH=CH_2$	$-CH_2CH_2COOCH_3$	$-CH_2CH_2COOH$
$MgProtoMe_2$	$-CH=CH_2$	$-CH=CH_2$	$-CH_2CH_2COOCH_3$	$-CH_2CH_2COOCH_3$
MV Mg-ACMe	$-CH=CH_2$	$-CH_2-CH_3$	$-CH=CHCOOCH_3$	$-CH_2CH_2COOH$
MV Mg-$AcMe_2$	$-CH=CH_2$	$-CH_2-CH_3$	$-CH=CHCOOCH_3$	$-CH_2-CH_2-COOCl$
DV MgAcMe	$-CH=CH_2$	$-CH=CH_2$	$-CH=CHCOOCH_3$	$-CH_2CH_2COOH$
DV Mg-$AcMe_2$	$-CH=CH_2$	$-CH=CH_2$	$-CH=CHCOOCH_3$	$-CH_2CH_2COOCH_3$
Mg-$HPMe_2$	$-CH=CH_2$	$-CH_2-CH_3$	$-CHOHCH_2COOCH_3$	$-CH_2CH_2COOCH_3$
MV Mg-KPMe	$-CH=CH_2$	$-CH_2-CH_3$	$-COCH_2COOCH_3$	$-CH_2CH_2COOH$
MV Mg-$KPMe_2$	$-CH=CH_2$	$-CH_2-CH_3$	$-COCH_2COOCH_3$	$-CH_2CH_2COOCH_3$
DV MgKPMe	$-CH=CH_2$	$-CH=CH_2$	$-COCH_2COOCH_3$	$-CH_2CH_2COOH$
DV Mg-$KPMe_2$	$-CH=CH_2$	$-CH=CH_2$	$-COCH_2COOCH_3$	$-CH_2CH_2COOCH_3$

Fig. 1. Structure of the Mg-porphyrin substrates used in the present study

The tetrapyrrole is drawn with the number designations for the substituent positions.

Lewis, 1981) to form two diastereomers which were then separated by normal-phase h.p.l.c. (Waters Dynamax silica column with a mobile phase of propan-2-ol/cyclohexane, 3:197, v/v). Oxidation with dicyanodichlorobenzoquinone (Kenner *et al.*, 1973) and magnesium insertion (Smith, 1975) yielded the two desired enantiomers. Final purification of Mg-HPMe$_2$ was achieved by using a Waters C_{18} reverse-phase column with an acetone/water (3:1, v/v) mobile phase as described by Wong *et al.* (1985). All 6-β-oxopropionate derivatives were prepared as previously described (Smith & Goff, 1986). The various 2,4-substituted deuteroporphyrins were synthesized by the counter-clockwise attachment of the four appropriate pyrroles to form the desired porphyrin [methods were described by Smith (1975)], followed by 'magnesiation'. The authenticity of these compounds was established by comparison with the data of Miller & Rapoport (1977). The DV Mg-Ac substrates were prepared by a method essentially similar to that described by Griffiths *et al.* (1976); the porphyrin was constructed as detailed by de Almeida *et al.* (1976). Further synthetic and structural details of the β-OH and acrylate substrates may be obtained from K.M.S. on request. MgDVP standard was a gift from Dr. T. Fan, NMR Facility, University of California, Davis, CA 95616, U.S.A. All Mg-porphyrin substrates were dissolved in DMSO at a concentration of 500 μM before being added to plastid incubations.

Plastid isolation. Developing chloroplasts were isolated from greening cucumber cotyledons by Percoll centrifugation as described previously (Wong & Castelfranco, 1984).

Incubation conditions. Routine incubations were carried out in 1 ml of buffer containing 0.5 M-sorbitol, 10 mM-Hepes, 20 mM-Tes, 1 mM-$MgCl_2$, 0.5 mg of BSA and 1 mM-EDTA, pH 7.7. Substrates and cofactors appropriate to the reaction along with 2–6 mg of plastid protein were included. Incubations were carried out in the dark to prevent phototransformation or photodestruction of newly synthesized Pchlide. All incubations were carried out at 30 °C for 1 h in a metabolic shaker at 60–65 double strokes/min and terminated by freezing at −15 °C. In time-course studies, samples were frozen more rapidly in a solid-CO_2/acetone bath.

Pchlide extraction and determination. Pchlide formed during incubation was extracted into diethyl ether by a previously described procedure (Castelfranco *et al.*, 1979) and was determined by using a Perkin–Elmer MPF 44-A fluorescence spectrophotometer as described by Chereskin *et al.* (1982). To analyse reaction products by reverse-phase h.p.l.c., diethyl ether extracts of incubation mixtures were washed with water, stored overnight at −15 °C to remove dissolved water and then evaporated to dryness under N_2. The residue was then dissolved in a small volume of methanol before injection.

H.p.l.c. analysis of porphyrins. Extracts of incubation mixtures containing mono- and di-carboxylic magnesium-porphyrins were analysed by reverse-phase h.p.l.c. as described by Fuesler *et al.* (1982).

Protein determinations. Plastid protein concentrations were determined by the method of Bradford (1976), with BSA as a standard.

RESULTS

7-methylesterase activity

Wong *et al.* (1985) reported that MgProtoMe$_2$ could act as a substrate for the formation of Pchlide in a reconstituted cyclase system. This observation pointed to the presence of 7-methylesterase activity in greening cucumber plastids.

The data in Table 1 confirmed that MgProtoMe$_2$ is a substrate for cyclase activity. The activity of the physiological substrate, MgProto, was 2-fold greater than that of the diester. In this and in subsequent Tables, and in Fig. 6, the nature of the 2- and 4-substituents in the cyclized product was not determined. The heading 'Pchlide' used in these cases is generic and does not imply either mono- or di-vinyl substitution. Table 1 also shows the requirement of these two substrates for the biological methyl donor SAM. With the diacid substrate, approx. 86% of the cyclase activity was lost when SAM was omitted, whereas with the diester substrate, only a 40% loss of activity was observed. The activity of the diester substrates was lower and more variable between plastid preparations than the activity of the diacid substrates. The data shown in Table 1 are typical.

The 7-methylesterase activity was demonstrated more directly by observing the fate of MgProtoMe$_2$ upon incubation with cucumber plastids. Fig. 2 shows a series of h.p.l.c. tracings highlighting the cofactor requirement for the cyclization of MgProtoMe$_2$. A 405 nm absorbance detector was used, which failed to detect the Pchlide that was formed in the completely uninhibited incubation mixture (Fig. 2*a*), but detected MgProto and MgProtoMe with high sensitivity. The spectrofluorimetric values of MgProto(Me) and Pchlide are given in the legend. In the presence of SAM and NADPH (Fig. 2*a*), the substrate MgProtoMe$_2$ was converted into both MgProtoMe and Pchlide. If both methyltransferase and cyclase activities were inhibited by the omission of SAM and the addition of Methylene Blue, respectively (Fig. 2*b*), MgProto was the only porphyrin accumulated to any significant extent. In the control sample (Fig. 2*c*), in which the plastids were

Table 1. SAM requirement for cyclase activity with MgProto diacid and diester substrates

Component concentrations were: porphyrin substrate, 10 μM; NADPH, 5 mM; SAM, 1 mM (included as indicated). Other incubation conditions were as described in the Materials and methods section. In all Tables, entries represent the mean and the range of duplicates, and the term 'Pchlide' is used for the '*in vitro*' cyclization product without regard to the oxidation state of the 2 and 4 substituents.

Incubation conditions		Pchlide synthesized
Porphyrin	SAM	(pmol/h per mg of protein)
MgProto diacid	+	243±5
	−	33±2
MgProto diester	+	137±15
	−	84±3
*MgProto	+	Not detectable
None	+	Trace

* Incubated 0 min.

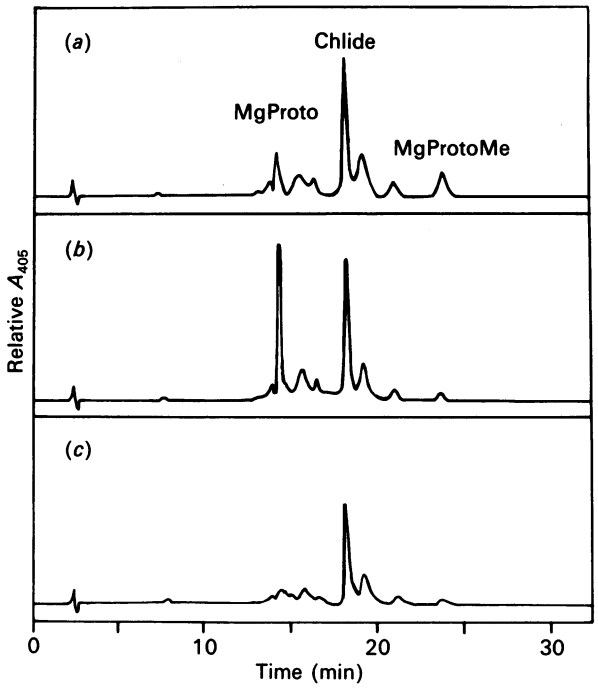


Fig. 2. Demethylation of $MgProtoMe_2$

Plastids (2.0 mg of protein) were incubated with the following substrates in a total volume of 1 ml: (*a*) $MgProtoMe_2$, 10 μM; NADPH, 5 mM; SAM, 1 mM; (*b*) $MgProtoMe_2$, 10 μM; Methylene Blue, 20 μM; (*c*) NADPH, 5 mM; SAM, 1 mM. MgProto(Me) and Pchlide in each incubation were quantified fluorimetrically: (*a*) MgProto(Me), 123 ± 23 pmol; Pchlide, 351 ± 15 pmol; (*b*) MgProto(Me), 364 ± 23 pmol; Pchlide, trace; (*c*) MgProto(Me), 50 ± 17 pmol; Pchlide, trace. Samples of extracted porphyrins were also analysed by h.p.l.c. and identified by comparison with authentic standards (Fuesler *et al.*, 1982). Procedures and other conditions were as described in the Materials and methods section.

incubated with the cofactors SAM and NADPH without porphyrin substrate, no accumulation of MgProto(Me) or Pchlide was detected. The Chlide peak, which is prominent in this tracing (Fig. 2*c*) and present also in Figs. 2(*a*) and 2(*b*), is due to the hydrolysis of the endogenous chlorophyll during the incubation by the enzyme chlorophyllase (Holden, 1961; Klein & Vishniac, 1961), which is generally present in our incubation mixtures. The Chlide peak, which fluoresces at 670 nm, is also prominent in Fig. 3 (below). It was apparent from these data that both 6- and 7-methyl ester groups were being hydrolysed in these incubations. The h.p.l.c. analysis shown in Fig. 2 does not distinguish between the chemically similar 6- and 7-MgProto methylpropionate esters; hence, resolution of these two esterase activities was not possible with currently available techniques.

Substituted deuteroporphyrins

The activities of various 2,4-substituted deuteroporphyrins as substrates in the synthesis of Pchlide (and corresponding Pchlide analogues) were investigated. For each substrate, activity was tested by its inclusion in a standard cyclase assay and was compared with the activity of control Mg-2,4-divinyldeuteroporphyrin (MgProto) or Mg-2,4-divinyldeuteroporphyrin dimethyl ester ($MgProtoMe_2$). For all substrates, both diacids and diesters were tested. Table 2 shows the activity with these substrates, expressed as percentage of controls. It should be emphasized that, although the data shown in Table 2 and subsequent Tables represent the results from a single experiment, the data were selected as being typical; in all cases the trends in cyclase activity observed with various Mg-porphyrin substrates were consistently reproducible. In both diacid and diester substrates, Mg-2-vinyl-4-ethyldeuteroporphyrin activity was almost exactly equal to that of the control. However, in the substrates with a 2-ethyl group, this activity decreased to less than 50% of the control for the diacids and even lower for the diesters (25%). There appeared to be little difference between the activities of 2-ethyl-4-vinyl- and 2,4-diethyldeuteroporphyrins. The cyclization products of Mg-2-ethyl-4-vinyldeuteroporphyrin and Mg-2,4-diethyldeuteroporphyrin appeared to be photoconvertable (C. J. Walker & P. A. Castelfranco, unpublished work).

Table 2. Synthesis of Pchlide and Pchlide analogues from different 2,4-substituted Mg-deuteroporphyrins

Incubation conditions were as described in the Materials and methods section. All samples contained 1 mM-SAM, 5 mM-NADPH and 10 μM-porphyrin. The activity of the Mg-2,4-divinyldeuteroporphyrin (MgProto) control was 250 pmol of Pchlide/h per mg of protein and the activity of the Mg-2,4-divinyldeuteroporphyrin dimethyl ester ($MgProtoMe_2$) control was 90 pmol of Pchlide/h per mg of protein. It was assumed that specific emission at the fluorescence maximum was the same for all the Pchlide analogues. See also the legend to Table 1.

	Pchlide synthesis (% of control)	
Substituted Mg-deuteroporphyrin	Diacid substrate	Diester substrate
2,4-Divinyl (control)	100	100
2-Ethyl-4-vinyl	49 ± 13	26 ± 17
2-Vinyl-4-ethyl	96 ± 4	99 ± 10
2-Ethyl-4-ethyl	39 ± 3	13 ± 5

Intermediates in the formation of the chlorophyll isocyclic ring

Granick (1950) proposed a series of intermediates in the conversion of the 6-methylpropionate side chain into the chlorophyll isocyclic ring (Wong *et al.*, 1985; see Scheme 1). The 6-methylacrylate derivative of MgProtoMe was the first of these postulated intermediates. Chemically synthesized MV Mg-AcMe, MV $Mg\text{-}AcMe_2$, DV Mg-AcMe, and DV $Mg\text{-}AcMe_2$ were found to be inactive as substrates for cyclase activity.

Fig. 3 shows the fluorescence spectra of porphyrin extract from a plastid incubation mixture with DV Mg-AcMe as substrate. The emission peaks at about 618 and 670 nm correspond to the acrylate substrate and Chlide respectively. At both zero time and 60 min, only the acrylate and Chlide were detectable. The presence of Pchlide, which would be characterized by an emission peak at about 630 nm, was not seen, even after 60 min. To test possible inhibitory effects of acrylate substrates, MgProto was incubated with plastids in the presence and absence of DV $Mg\text{-}AcMe_2$. Table 3 shows that the rate

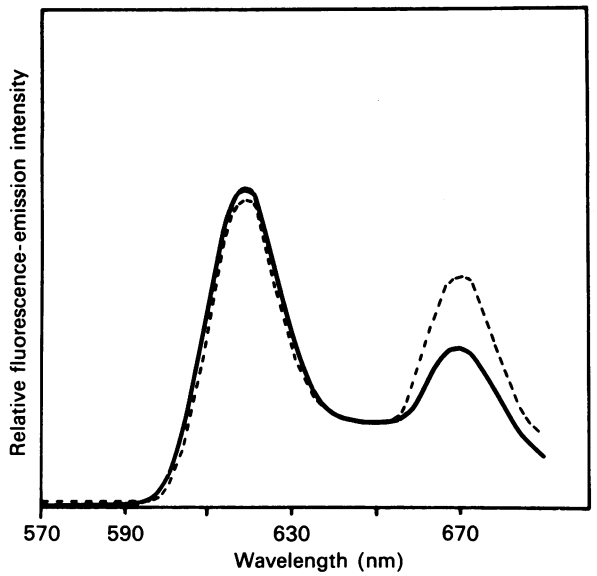


Fig. 3. Activity of DV Mg-AcMe as a substrate for cyclization

Plastids were incubated in the presence of 10 μM-DV Mg-AcMe, 5 mM-NADPH and 1 mM-SAM. The Figure shows fluorescence spectra (uncorrected) of a porphyrin extract of samples incubated for 0 (——) and 60 (----) min. Fluorimetry and plastid incubations were carried out as described in the Materials and methods section.

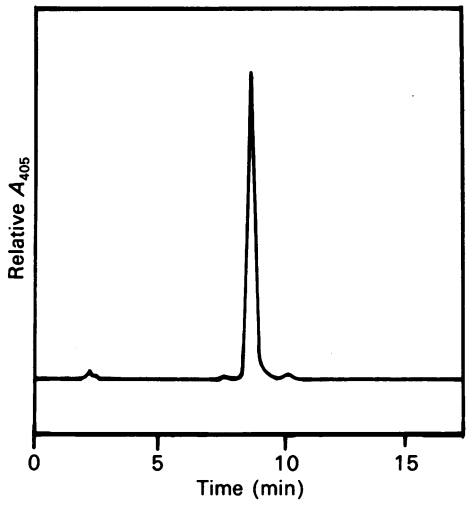


Fig. 4. H.p.l.c. profiles of Mg-HPME$_2$ preparations

Porphyrin samples were chromatographed in acetone/water (3:1, v/v) (see under 'Methods') and detected by absorbance at 405 nm. The trace shows a co-injection of h.p.l.c.-purified band 1 and 2 enantiomers.

of accumulation of Pchlide in the presence of the acrylate (99 ± 5 pmol of Pchlide/h per mg of protein) was the same as in the absence (103 ± 1 pmol of Pchlide/h per mg of protein), suggesting that the acrylate was not inhibitory.

During the course of a study of the formation of the isocyclic ring in isolated developing plastids, Wong *et al.* (1985) observed the accumulation of a porphyrin which displayed the kinetic behaviour of an intermediate; this was subsequently identified as Mg-HPME. Since the β carbon atom of this intermediate is asymmetric, we decided to determine the stereoselectivity at this point in the formation of the isocyclic ring. The starting material for this asymmetric organic synthesis was the free chlorin, which gave rise to two diastereomers when the 6β-OH-methylpropionate side chain was introduced into the molecule (further details from K.M.S. on request). The two diastereomers were resolved by normal-phase h.p.l.c. (see under 'Methods'). After dehydrogenation at the 7–8 position and magnesiation, the desired 6β-OH-propionate derivatives of MgProto dimethyl ester were obtained: these were designated as 'band 1' and 'band 2' according to the chromatographic elution profile of the β-OH diastereomer from which they had been derived. The two enantiomers were purified by the method of Wong *et al.* (1985) using as the standard authentic DV Mg-HPME$_2$ synthesized and characterized by Smith & Goff (1986). When these purified Mg-porphyrins were co-injected into the h.p.l.c. system (see under 'Methods'), they moved as a single peak that was eluted at about 8.6 min (Fig. 4). They also had identical absorption spectra, with absorption maxima at 412 (soret), 548 and 590 nm (Fig. 5). However, only band 1 had any detectable activity as a substrate for the cyclase (171 ± 9 pmol of Pchlide/h per mg of protein Table 4). Determining the configuration of the two enantiomers, to see which of them is biologically active, has yet to be completed.

Since, according to Granick's (1950) scheme, the oxoester represents the intermediate immediately before the cyclization proper, it is predicted that it should be a more active substrate than those further removed from Pchlide in the biosynthetic pathway. Table 5(*a*) shows the relative activities of MgProto, (105 ± 5 pmol of Pchlide/h per mg of protein), its 6-methylated product MgProtoMe (249 ± 12 pmol of Pchlide/h per mg of protein) and MV Mg-KPMe (498 ± 18 pmol of Pchlide/h per mg of protein). The oxoester was 5.7 times more active than MgProto and 2.2 times more active than MgProtoMe.

Several 6β-oxomethylpropionate derivatives were tested, and all of them were found to be active. Table 5(*b*) shows that the 4-ethyl substrates in both diacid (260 ± 57 pmol of Pchlide/h per mg of protein) and diester (230 ± 19 pmol of Pchlide/h per mg of protein) forms showed much higher activity than the corresponding 4-vinyl derivative (45 ± 8 pmol of Pchlide/h per mg of protein).

A study was carried out to ascertain whether this

Table 3. Effect of DV Mg-AcMe$_2$ on Pchlide synthesis from MgProto

Plastids were incubated for 60 min with 5 mM-NADPH, 1 mM-SAM and porphyrin as indicated in the Table. See also the legend to Table 1.

Porphyrin substrate	Pchlide synthesis (pmol/h per mg of protein)
MgProto (5 μM)	103 ± 1
MgProto (5 μM) + DV Mg-AcMe$_2$ (5 μM)	99 ± 5

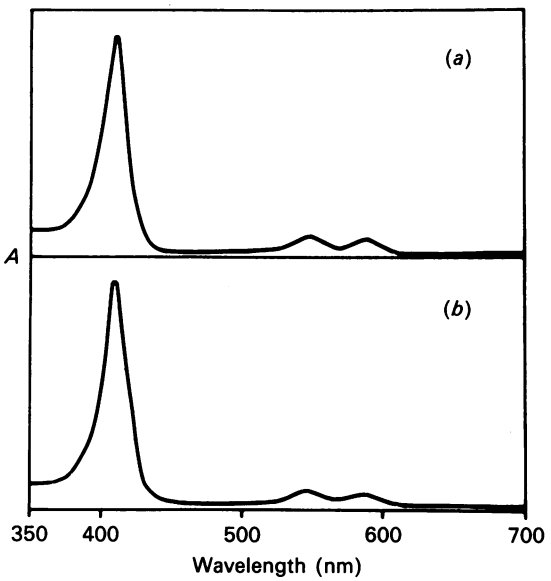


Fig. 5. Visible spectra of the h.p.l.c.-purified Mg-HPMe$_2$ enantiomers

Spectra (*a*) and (*b*) (in diethyl ether) are of band 1 and 2 enantiomers respectively. In both spectra absorption maxima are at 412 (soret), 548 and 590 nm.

Table 4. Cyclase activity of the two enantiomers of Mg-HP Me$_2$

All samples were incubated with 5 mM-NADPH and porphyrin concentrations as indicated. Band 1 and 2 refer to the two enantiomers of Mg-HPMe$_2$ (see the text for explanation). See also the legend to Table 1.

Porphyrin substrate	Pchlide synthesis (pmol/h per mg of protein)
Band 1 (5 μM)	171 ± 9
Band 2 (5 μM)	0

Table 5. Synthesis of Pchlide from the 6-oxoester derivatives of MgProtoMe and MgProtoMe$_2$

All samples contained 1 mM-SAM, 5 mM-NADPH and 10 μM-porphyrin substrate. Other incubation conditions were as in the Materials and methods section. See also the legend to Table 1.

	Porphyrin substrate	Pchlide synthesis (pmol/h per mg of protein)
(*a*)	MgProto	105 ± 5
	MgProtoMe	249 ± 12
	MV Mg-KPMe	498 ± 18
(*b*)	MV Mg-KPMe	260 ± 57
	MV Mg-KPMe$_2$	230 ± 19
	DV Mg-KPMe$_2$	45 ± 8

difference between 4-ethyl and 4-vinyl substrates was consistent as a function of time. Fig. 6 shows that, for both substrates, the time course of product accumulation was qualitatively similar, continuing at a linear rate for about 60 min, after which cyclase activity diminished. However, the extent of Pchlide accumulation was clearly quite different; after 120 min, plastids incubated with the 4-ethyl substrate had accumulated about 1000 pmol of Pchlide, but those incubated with the 4-vinyl substrate had formed only 300 pmol of cyclized product.

DISCUSSION

We have previously reported on esterases in developing cucumber plastids which attack Mg-protoporphyrin methyl esters. Chereskin *et al.* (1982) demonstrated the presence of an esterase which hydrolysed the 6-methyl group of MgProtoMe. Also, Wong *et al.* (1985) reported that MgProtoMe$_2$ could be cyclized to Pchlide, suggesting the presence of 7-methylesterase activity in the plastids. We observed (Fig. 2) that MgProto accumulates when cucumber plastids are incubated with MgProtoMe$_2$ and

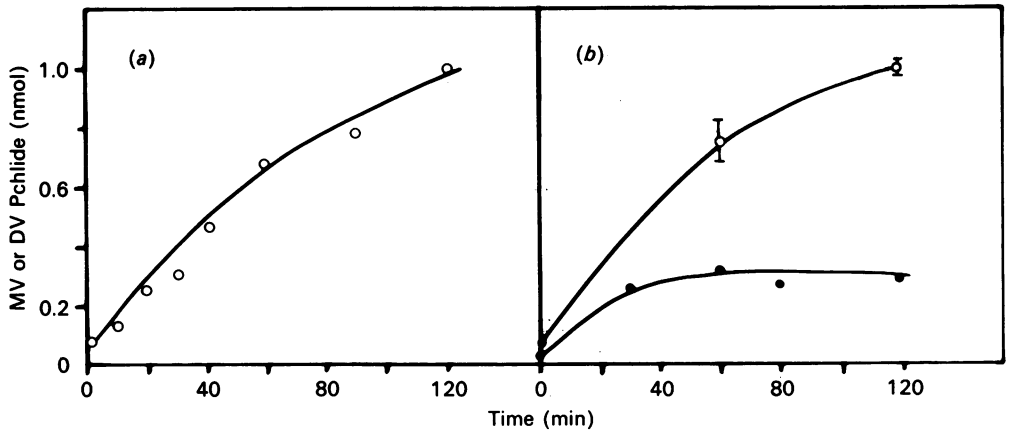


Fig. 6. Time course of Pchlide synthesis from MV Mg-KPMe and DV Mg-KPMe substrates

Plastids were incubated with 5 mM-NADPH, 1 mM-SAM and either mono- (○) or di-vinyl (●) Mg-KPMe. Samples were frozen at time intervals in an acetone/solid-CO_2 mixture. The extracted porphyrins were analysed by h.p.l.c., and the Pchlide was quantified by peak area in comparison with a Pchlide standard. (*a*) and (*b*) represent two individual experiments with plastid protein concentrations of 2.1 (*a*) and 3.6 (*b*) mg/ml.

cyclization is inhibited by the presence of Methylene Blue. Therefore both 6- and 7-methylesterase activities seem to be present. The stimulation of cyclization by SAM observed with the $MgProtoMe_2$ substrate (Table 1) can therefore be attributed to the remethylation of MgProto formed during the incubation. Nasrulhaq-Boyce *et al.* (1987) found that $MgProtoMe_2$ was inactive as a substrate for cyclase in their system. This discrepancy may reflect a difference in the biological materials used (wheat etioplasts as against cucumber chloroplasts) or a difference in the assay methodology (a rapid method relying mainly on the spectrophotometric measurement of Chlide as against a slower assay relying on the fluorimetric measurement of the cyclization product itself, namely Pchlide).

The occurrence of these esterases was useful for our experiments, since during the organic synthesis the porphyrin substrates were prepared as dimethyl esters. Although both β-oxo and acrylate compounds (which have a double bond α–β to the carboxymethyl ester) can be selectively hydrolysed to give the 6-monoester, the specific removal of the 7-methyl ester group of the β-OH compounds is not possible by chemical means. Thus in Table 4 diester substrates were used.

Three acrylate substrates were tested for activity in our cyclase assay (Fig. 3), but none were found to be active. However, we cannot completely dismiss the acrylate as an intermediate in isocyclic-ring formation, since we have synthesized and tested only the *trans* isomer. A *cis*-acrylate would be unstable, short-lived in solution and difficult to prepare, but it could conceivably exist as an enzyme-stabilized intermediate. An example of such an intermediate is 11-*cis*-retinal in the visual rhodopsin system (Hubbard & Wald, 1952). It is also possible that an acrylate active site in the cyclase enzyme system may not be accessible to exogenous substrate, although this is not the case for the β-OH or β-oxo intermediates.

Our data confirm the earlier work of Wong *et al.* (1985), who demonstrated the involvement of a β-OH intermediate in the cyclization sequence (Table 3). Interestingly, it appears that only one of the two enantiomers of this compound is active in our cyclase system. More information needs to be obtained on the apparent stereoselective hydroxylation of the 6-methyl-propionate side chain, but this will have to await the determination of the configuration of the active enantiomer.

We have also extended the studies of Wong *et al.* (1985) on the β-oxo intermediate in the formation of the isocyclic ring. When MV and DV β-oxoesters were compared for cyclase activity, both were active, but the MV substrate was approx. 4 times more active than the DV one (Table 5*b* and Fig. 6). These results suggest that the enzyme catalysing ring closure has a preference for a reduced substituent in position 4. By contrast, the data in Table 2 show that, at the level of MgProto, reduction of the 4-vinyl side chain has no significant effect on the rate of Pchlide accumulation. The point in the chlorophyll biosynthetic pathway at which 4-vinyl-side-chain reduction occurs is a matter of controversy that has been carefully reviewed (Beale, 1984), but is still far from settled and continues to elicit considerable interest from some workers in the chlorophyll-biosynthesis field (see, e.g., Carey & Rebeiz, 1985; Carey *et al.*, 1985; Rebeiz *et al.*, 1986; Richards *et al.*, 1987; Tripathy & Rebeiz, 1986). Our main purpose in the present study was not the question of when the 4-vinyl substituent gets reduced, but rather the mechanism of the formation of the isocyclic ring. However, our data (Tables 2 and 5 and Fig. 6) would favour the hypothesis that the 4-vinyl reduction takes place between MgProto and the formation of the β-oxo intermediate.

The present paper raises the question of how the oxygen function is introduced into the isocyclic ring, since our data argue against the involvement of an acrylate intermediate, but confirm the involvement of both β-OH and β-oxo intermediates. If an acrylate is not involved in the cyclization sequence, the introduction of the hydroxy function into the side chain at position 6 must involve a stereospecific hydroxylation. This is particularly interesting from an enzymological point of view. The requirement of the cyclization for the two cofactors NADPH and O_2 also suggests the participation of a mixed-function oxidase in the formation of the β-OH intermediate.

This research was supported by National Science Foundation grant DMB 85-06587.

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Received 2 March 1988/17 May 1988; accepted 31 May 1988