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Synthesis of divinyl protochlorophyllide

Enzymological properties of the Mg-protoporphyrin IX monomethyl ester oxidative cyclase system

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The resolution and reconstitution of the Mg-protoporphyrin IX monomethyl ester oxidative cyclase system into a supernatant and a pellet fraction was accomplished by a procedure involving salt treatment followed by osmotic shock. Recombination of pellet and supernatant fractions was required for cyclase activity. This restoration effect could be demonstrated using either Mg-protoporphyrin IX or Mg-protoporphyrin IX monomethyl ester as the cyclase substrate in the presence or absence of *S*-adenosylmethionine. Pretreatment of the pellet fraction with either 8-hydroxyquinoline or desferal mesylate inhibited cyclase activity, indicating that there is a heavy-metal-ion requirement in this fraction. The cyclase supernatant protein(s) was not internalized by Sephadex G-50 and did not bind to Blue Sepharose, suggesting that it has a molecular mass of over 30 kDa and that it does not bind the cofactor NADPH. The cyclase supernatant protein did bind to $MgProtoMe_2$-bound Sepharose and could be eluted by raising the pH to 9.7 in the presence of 4 mM-n-octyl glucoside. The pH optimum of the cyclase was 9.0. About a 40-fold purification of the cyclase supernatant protein was achieved by a combination of $(NH_4)_2SO_4$ fractionation and phenyl-Sepharose chromatography.

INTRODUCTION

The Mg-protoporphyrin IX monomethyl ester (MgProtoMe) oxidative cyclase system (or cyclase) catalyses the conversion of MgProtoMe to protochlorophyllide (Pchlide) in the chlorophyll (Chl) synthesis pathway. Granick (1950) proposed that isocyclic-ring synthesis involved the β-oxidation of the 6-methylpropionate side chain of MgProtoMe to a methyl-β-oxopropionate group. The modification of the 6-methylpropionate side chain was thought to go through 6-acrylate and 6-β-hydroxypropionate intermediates by analogy to the β-oxidation of fatty acids.

Recent work in this laboratory has both confirmed and modified this scheme. The chemically synthesized 6-acrylate derivative of MgProtoMe proved to be an inactive substrate for the cyclase (Walker *et al.*, 1988), whereas both the 6-β-hydroxypropionate and 6-β-oxopropionate derivatives could be converted into Pchlide (Wong & Castelfranco 1984, 1985; Wong *et al.*, 1985; Walker *et al.*, 1988). These findings suggested a hydroxylation reaction involving atmospheric oxygen. This model was subsequently confirmed by the observation of the direct incorporation of molecular oxygen into the isocyclic ring (Walker *et al.*, 1989).

Although our understanding of the mechanism of the cyclase has improved, there is still little information on the individual enzymes. Since several intermediates are involved in the reaction, the cyclase must be a collection or a complex of several enzymes. Previous work has shown that it is possible to resolve the cyclase into membrane-bound and soluble fractions by the sonication of intact plastids and ultracentrifugation (Wong & Castelfranco, 1984). Recombination of the pellet and supernatant fractions was required to restore cyclase activity. Neither fraction alone was sufficient to catalyse the conversion of chemically synthesized intermediates to Pchlide, for which the two cofactors, NADPH and O_2, were likewise required (Wong *et al.*, 1985). The product of the cyclization reaction *in vitro* was shown to be divinyl-Pchlide (2,4-divinylpheoporphyrin a_5) (Chereskin *et al.*, 1983).

In the present study we have continued to investigate the enzymology of the cyclase using a reconstituted system derived from salt-shocked and osmotically lysed, rather than sonicated, plastids. The membrane-bound component exhibits a heavy-metal requirement, which agrees with the results of previous studies carried out with whole plastids (Chereskin & Castelfranco, 1982; Nasrulhaq-Boyce *et al.*, 1987). Evidence is presented that the soluble fraction binds the porphyrin, but not the other substrate for the cyclase, NADPH. Partial purification of the supernatant protein is described.

MATERIALS AND METHODS

Materials

General. Hepes, Tes, NADPH (tetrasodium salt, chemically reduced), *S*-adenosylmethionine (SAM; chloride salt), cysteine hydrochloride, EDTA, sorbitol, SDS, Trizma, glycine, bis-acrylamide, $(NH_4)_2SO_4$, Coomassie Brilliant Blue G-250, 2-mercaptoethanol, ammonium persulphate and Sepharose 4B (CNBr-activated) were purchased from Sigma. NaCl, trichloroacetic acid and all organic solvents were obtained from Fisher. Spectrapor dialysis tubing was obtained from American Scientific Products; *NNN′N′*-tetramethylethylenediamine (TEMED) and acrylamide were purchased from Bio-Rad. $MgCl_2$ was obtained from Mallinckrodt. Centricon 10 microconcentrators were purchased from Amicon. BSA (fraction V, fatty-acid-poor) was from Calbiochem. Sephadex G-50 was purchased from Pharmacia. Cucumber (*Cucumis sativus* L., cv. Beit Alpha) seeds were a gift from Harris Moran Seeds, Salinas, CA, U.S.A. Desferal mesylate was a gift from CIBA Pharmaceutical Co., Summit, NJ 07901, U.S.A.

Abbreviations used: Chl, chlorophyll; CSP, cyclase supernatant protein(s); DMSO, dimethyl sulphoxide; MgProto, Mg-protoporphyrin IX; MgProtoMe, Mg-protoporphyrin IX monomethyl ester; $MgProtoMe_2$, Mg-protoporphyrin IX dimethyl ester; ME, MgProtoMe esterase(s); MT, MgProto methyltransferase (EC 2.1.1.11); PAR, photosynthetically active radiation; Pchlide, protochlorophyllide (this abbreviation is used generically to denote the product of the '*in vitro*' cyclization reaction without regard to the oxidation state of the 2 and 4 substituents); SAM, *S*-adenosylmethionine; TEMED, *NNN′N′*-tetramethylethylenediamine.

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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 $\mu E \cdot m^{-2} \cdot s^{-1}$ PAR at 30 °C) for 20 h.

Mg-protoporphyrin IX (MgProto). This was prepared by alkaline hydrolysis of Mg-protoporphyrin IX dimethyl ester ($MgProtoMe_2$) (Chereskin & Castelfranco, 1982); the latter was obtained from Porphyrin Products, Logan, UT 84321, U.S.A. MgProtoMe was made biologically as described previously (Chereskin *et al.*, 1982). All porphyrin substrates were dissolved in dimethyl sulphoxide (DMSO) at a concentration of 500 μM before addition to cyclase assays.

Methods

Plastid isolation and resolution of the cyclase. Developing chloroplasts were isolated from greening cucumber cytoledons as described by Fuesler *et al.* (1984), except that 'pellet 2' was resuspended in 5 ml (per pellet) of Buffer A (0.5 M-sorbitol/1 mM $MgCl_2$/1 mM-EDTA/20 mM-Tes/10 mM-Hepes, pH 7.7). The pellet suspension was then illuminated for 5 min at 0 °C to remove remaining traces of Pchlide (Chereskin *et al.*, 1982) before dilution with 35 ml of Buffer C (0.2 M-NaCl/1 mM-$MgCl_2$/1 mM-EDTA/20 mM-Tes/10 mM-Hepes, pH 7.7), which causes the plastids to loose some integrity (Chereskin *et al.*, 1982). After 10 min at 0 °C the suspension was centrifuged at 500 ***g*** for 10 min. The supernatant was discarded and the pellet vigorously resuspended in 3.4 ml of hypo-osmotic Buffer B (10 mM-Hepes/10 mM-Tes/1 mM-EDTA/1 mM-$MgCl_2$, pH 7.7) and lysed for 5 min at 0 °C, then centrifuged at 12000 ***g*** for 10 min. The supernatant was kept and the pellet washed by resuspension in 35 ml of Buffer B and centrifugation at 12000 ***g*** for 10 min. Recombination of the pellet and supernatant was necessary to restore cyclase activity.

$(NH_4)_2SO_4$ precipitation and dialysis. In some experiments the supernatant was adjusted to 60%-satd. $(NH_4)_2SO_4$ and, after 10 min at 0 °C, centrifuged at 12 000 ***g*** for 10 min. The pellet was discarded and the supernatant was adjusted to 85% $(NH_4)_2SO_4$ saturation. After 30 min at 0 °C the precipitated proteins were pelleted by centrifugation at 12000 ***g*** for 10 min. Pellets were resuspended in a minimal volume of Buffer B and dialysed against 500 ml of the same buffer for 1 h at 4 °C. Saturated $(NH_4)_2SO_4$ solutions were adjusted to pH 7.7 with concentrated aq. NH_3 before use.

Column chromatography. Typically, chromatography was carried out with 1–2 ml of gel in 0.8 cm-diameter columns. Flow was by gravity (rates were approx. 1 ml/min), and samples and buffers were applied manually. Fractions (2–3 ml) were collected on ice and concentrated to about 50 μl by using microconcentrators. If $(NH_4)_2SO_4$ was included in the running buffers, fractions were dialysed (as described above) before concentration.

Preparation of $MgProtoMe_2$ affinity column. CNBr-activated Sepharose 4B was coupled to $MgProtoMe_2$ by the method of Richards *et al.* (1987). An absorption spectrum of the gel in glycerol/methanol (1:1, v/v) showed peaks at 557 and 595 nm.

Incubation conditions. Routine incubations with the reconstituted system were carried out in a total volume of 1 ml in Buffer B. Substrates and cofactors were 10 μM-MgProto or MgProtoMe, 1 mM-SAM and 5 mM-NADPH; the samples were incubated in darkness to prevent phototransformation or photodestruction of newly formed Pchlide. 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.

Pchlide extraction and determination. Pchlide formed during the incubation was extracted into diethyl ether as described by Castelfranco *et al.* (1979) and was determined by using a Perkin–Elmer MP 44-A fluorescence spectrophotometer as described by Chereskin *et al.* (1982).

Protein determinations. Protein was determined by the method of Bradford (1976), with BSA as a standard.

H.p.l.c. Pigments were separated by the h.p.l.c. system previously described by Fuesler *et al.* (1982).

Quantitative expression of the cyclase assay. Cyclase activity is expressed in pmol of Pchlide/h per mg of protein. The legend to each table indicates whether this activity is expressed with respect to pellet, cyclase supernatant protein (CSP) or total protein. All other departures are likewise stated. Incubations were carried out for 1 h in order to increase the yield of product. It was established that cyclase activity was directly proportional to the amount of plastid protein added. Hence all assay values are comparable, even though Pchlide formation is not linear with time (Walker *et al.*, 1988).

SDS/PAGE. SDS/PAGE analysis of protein fractions were carried out by the method of Laemmli (1970). All analyses were done using 12%-(w/v)-acrylamide separating gels. Standard proteins (α-lactalbumin, 14.2 kDa; trypsin inhibitor, 21.1 kDa; carbonic anhydrase, 29.0 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36.0 kDa; egg albumin, 45.0 kDa; bovine serum albumin, 66.0 kDa) were co-electrophoresed for molecular-mass estimations.

RESULTS

Comparison of sonication and osmotic-shock methods for the resolution of the cyclase.

Table 1 compares typical reconstitution experiments according to the method described by Wong & Castelfranco (1984) and the osmotic-lysis method used in all the experiments reported here. In both cases, the pellet or supernatant alone had little cyclase activity, but recombination of these fractions restored considerable activity. In these experiments the small activity measured in the 'pellet alone' controls was subtracted from the activity of the other samples. Pellet fractions could be stored in Buffer B/glycerol (1:1, v/v) at −80 °C; supernatant fractions were stable at −80 °C in Buffer B.

Table 1. Resolution and reconstitution of the cyclase

Resolution and reconstitution of the cyclase was performed as described by Wong & Castelfranco (1984) (Method 1) or as described under 'Methods' (Method 2), except that the pellet was not washed, in order to allow direct comparison of the two techniques. Component concentrations were: MgProto, 10 μM; NADPH, 5 mM; SAM, 1 mM. The protein values used to calculate the cyclase activities represent the total protein in each incubation. Other incubation conditions were as described under 'Methods'. In all Tables, where shown, entries represent the mean and 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 (Fischer formula).

Method and fraction	Cyclase activity (pmol of Pchlide/h per mg of protein)	Cyclase activity (% of that of intact plastids)
Method 1: sonication		
Intact plastids	350 ± 11	100
Pellet	21	6
Supernatant	25	7
Pellet + supernatant	140 ± 10	40
Method 2: osmotic lysis		
Intact plastids	208 ± 12	100
Pellet	24 ± 9	12
Supernatant	0	0
Pellet + supernatant	109 ± 2	52

Table 2. Activity of the reconstituted cyclase with different substrates

The reconstituted cyclase was tested in four separate experiments with four substrate conditions: 10 μM-MgProto or 10 μM-MgProtoMe, with or without 1 mM-SAM. The protein values used to calculate the cyclase activities in lysed chloroplast preparations were for CSP only, but in the unresolved chloroplast controls they represented total protein. During the resolution the protein of the original chloroplast is divided up as follows: CSP, 36%; pellet, 35%; lost, 29%. Washed pellets alone showed no activity. Other incubation conditions were as described in Table 1 and under 'Methods'.

Substrates	Enzyme system	Cyclase activity (pmol of Pchlide/ h per mg of protein)
MgProto	CSP	0
	CSP+pellet	8±0
	Control	38
MgProto+SAM	CSP	0
	CSP+pellet	61±1
	Control	168
MgProtoMe	CSP	0
	CSP+pellet	35±7
	Control	63
MgProtoMe+SAM	CSP	0
	CSP+pellet	111±9
	Control	199

Comparison of MgProto and MgProtoMe as substrates for the reconstituted cyclase system.

The reconstitution of the cyclase activity was tested using two Mg-porphyrin substrates, MgProto and MgProtoMe, with or without SAM (Table 2). In all cases the pellet and the supernatant alone had no activity, but the recombined samples had significant activity. The activities of the recombined samples are expressed on a CSP basis, but the activities of the chloroplasts before salt treatment (controls) are expressed in terms of total plastid protein. In order to compare the activity of the initial pellet with that of the reconstituted system, one must keep in mind that, during the resolution, 35% of the protein remains in the pellet, 36% ends up in the supernatant and 29% is lost.

The protein that was washed away in the salt treatment was recovered by $(NH_4)_2SO_4$ precipitation and dialysed against buffer. This fraction was shown to contain no CSP activity and not to be inhibitory.

As expected, SAM strongly enhanced the cyclization of MgProto, but also stimulated the cyclization of MgProtoMe, because of the combined action of MgProtoMe esterase(s) (ME) and MgProto methyltransferase (MT) present in the chloroplasts (Chereskin *et al.*, 1982).

After the spectrofluorimetric determination of the reaction product, the porphyrin mixtures were analysed by h.p.l.c. After the salt-osmotic-shock treatment the ME activity was found in the CSP, and MT activity in the pellet, since CSP alone was able to convert MgProtoMe into MgProto, and pellet alone was able to convert MgProto into MgProtoMe in the presence of SAM. Hardly any esterase activity was found in the pellet, or any transferase activity in the CSP (results not shown).

In the subsequent experiments described in this paper, 10 μM-MgProto and 1 mM-SAM were used, endogenous MT activity being relied upon to produce the oxidative cyclization substrate, MgProtoMe, during the incubation. However, in the experiments on the pH-dependence of the cyclase reaction and on the stability of CSP, MgProtoMe was substituted for MgProto in order to ensure that the pH-dependence measured was, so far as possible, that of the cyclase itself.

The action of metal-ion chelators on the cyclase. Previous experiments have shown that the cyclase can be inhibited by metal-ion chelators (Nasrulhaq-Boyce *et al.*, 1987). Table 3 shows the effect of 8-hydroxyquinoline and desferal mesylate on the pellet fraction of the cyclase. In this experiment pellet fractions were exposed to the chelator for 10 min at 0 °C, then washed twice with Buffer B to remove the chelator before recombination with untreated supernatant fraction. With both chelators, cyclase activity was partially inhibited; 8-hydroxyquinoline (10 mM was required for 89% inhibition) was a stronger inhibitor than desferal mesylate (90 mM was required for 82% inhibition). To ascertain whether chelator remained in the pellet after the buffer wash, the chelator-treated and -untreated pellets were combined in a 1:1 ratio and incubated with the supernatant fraction. It was expected that any chelator trapped in the treated pellet would be free to inhibit the untreated pellet during the incubation. Table 3 shows that, with 8-hyroxyquinoline, the activity of the mixture was 38% of the untreated control, whereas the predicted activity was 55%, suggesting that some chelator may have been retained by the treated pellet. In contrast, at both 10 and 90 mM-desferal mesylate, the observed activities for the mixture were approximately those predicted. Cyclase

Table 3. Pretreatment of the pellet fraction with 8-hydroxyquinoline and desferal mesylate

Pellet fractions were exposed to the chelator concentration indicated at 0 °C for 10 min. Chelator was removed by diluting 1 ml of pellet suspension with 35 ml of Buffer B, centrifuging at 12000 *g* for 10 min, and finally washing the pellet once more in 35 ml of Buffer B. 'Control' samples were treated identically, except for the omission of chelator. Treated and untreated pellets were recombined with their respective CSP that had been stored on ice; pellet recovery was assumed to be 100%. In 'control+chelator-treated' samples, half the usual amount of pellet was taken from each sample. Values in parentheses are percentages of control values; emboldened values are the predicted activities for these samples. Total-protein values were used to calculate cyclase activities. Substrates and incubations were as described in the legend to Table 1.

	Cyclase activity (pmol of Pchlide/h per mg of protein)		
Chelator...	8-Hydroxyquinoline	Desferal mesylate	
Concn. (mM)...	10	90	10
Control	57±5 (100)	15±1 (100)	19±1 (100)
Chelator-treated	6±0 (11)	3±0 (18)	11±1 (57)
Control+ chelator-treated (1:1)	23±3 [38(**55**)]	8±0 [52(**59**)]	16±1 [85(**79**)]

Table 4. Chromatography of the supernatant fraction on Sephadex G-50 and Blue Sepharose

Columns were prepared and used as described under 'Methods'. For substrates and incubation conditions, see the legend to Table 1. In the bioassay, pellet protein for Sephadex G-50 was 1 mg/ml, and for Blue Sepharose, 1.3 mg/ml. Recovery of the supernatant protein from the columns was not determined; the amount of pellet was known to be saturating (see Fig. 4 and Table 5).

Column fraction	Cyclase activity (pmol of Pchlide/h)	
	Sephadex G-50	Blue Sepharose
Loaded sample	173	108
Void volume	0	0
Unabsorbed/excluded	160	116
Absorbed/internalized	0	0

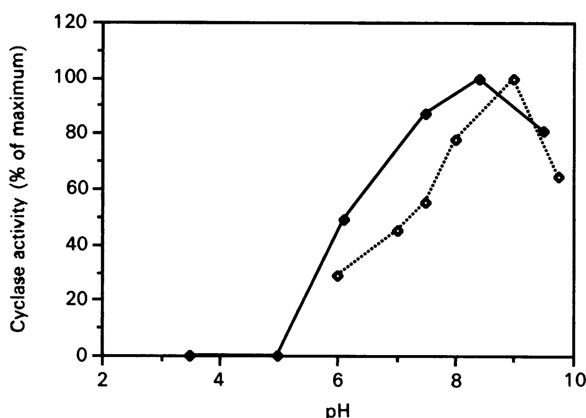

Fig. 1. pH-dependence of the cyclase reaction, and of CSP stability

For the pH-dependence of the cyclase reaction, the activity of the reconstituted system (◇) was assayed in a buffer consisting of (20 mM each) citric acid, Mes, Hepes, Tricine and glycine, plus 1 mM-EDTA and 1 mM-$MgCl_2$, adjusted to the appropriate pH with KOH or HCl; 100% activity, taken at the pH optimum, was 167 pmol of Pchlide/h per mg of protein. The stability of CSP (◆) was determined by incubating CSP at 0 °C for 2 h in the above buffers, followed by microconcentration to 50 μl and resuspension in 500 μl of Buffer B. CSP was bioassayed as described under 'Methods'. Substrates were 10 μM-MgProtoMe, 1 mM-SAM and 5 mM-NADPH; 100% activity was 143 pmol of Pchlide/h per mg of protein.

activities in this experiment were low because of the instability of the pellet under the treatment conditions. Complementary experiments pretreating the supernatant fraction with these metal-ion chelators were attempted, but in every case complete removal of the chelator by $(NH_4)_2SO_4$ precipitation and dialysis was not achieved.

Sephadex G-50 and Blue Sepharose chromatography. Attempts to solubilize the pellet fraction for further analysis were unsuccessful. However, it was found that the cyclase was stable to the detergent n-octyl glucoside and was slightly stimulated by it at the lower concentrations (4–6 mM; results not shown). Subsequent experiments were therefore confined to the supernatant fraction.

Table 4 shows the behaviour of the supernatant fraction on two columns. Column fractions in these and all further experiments were bioassayed for cyclase activity by combining with pellet fraction (0.5–1.5 mg of protein per incubation) and incubating with the appropriate substrates as described under

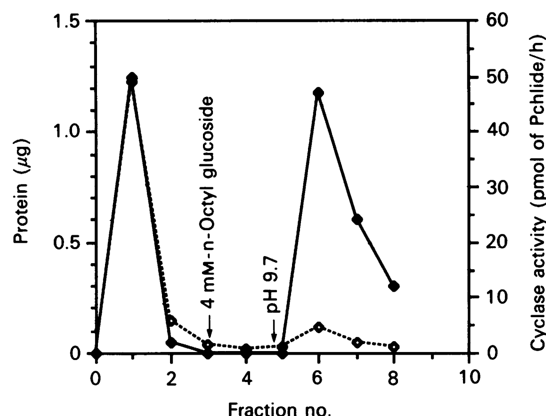

Fig. 2. Affinity chromatography of CSP on MgProtoMe$_2$-bound Sepharose

Column chromatography and fraction bioassay was as described under 'Methods'. The protein (2.8 mg) was loaded in 500 μl of Buffer B and eluted at pH 9.7 in the presence of 4 mM-n-octyl glucoside. Cofactors and substrates were 10 μM-MgProto, 5 mM-NADPH and 1 mM-SAM. Pellet protein per bioassay was 1.0 mg. The addition of 4 mM-n-octyl glucoside and the pH change to 9.7 are indicated in the Figure. ◇, Protein; ◆, cyclase activity.

'Methods' and in the Tables and Figure legends. The data in Table 4 show that CSP was not internalized by Sephadex G-50, indicating a molecular mass in excess of 30 kDa. On Sephadex G-100, CSP activity was lost (results not shown). Presumably, CSP was internalized, but was broken down with attempts to elute it from the column.

Two of the substrates for the cyclase are NADPH and MgProtoMe. It was therefore of interest to establish whether the supernatant fraction could bind either of these two compounds. Table 4 shows that CSP did not bind to Blue Sepharose, a gel which retains many nucleotide-binding enzymes (Heyns & DeMoor, 1974). Washing with buffer containing NaCl (0.1–0.3 M) failed to remove any CSP activity from the column.

pH responses of the cyclase. Fig. 1 shows the pH-dependence of the stability of CSP and of the cyclase assay itself. After a 2 h preincubation at different pH values, all the CSP samples were adjusted to pH 7.7 and tested as described under 'Methods'. CSP was stable at alkaline pH, but was strongly inactivated below pH 7.0. The pH optimum of the cyclase assay was about pH 9.0.

MgProtoMe$_2$ affinity chromatography. The ability of CSP to bind porphyrin was tested by affinity chromatography on a Sepharose column with bound MgProtoMe$_2$ (see under 'Methods'). Elution from these types of columns often requires pH changes.

The chromatography of CSP on the MgProtoMe$_2$ affinity column is presented in Fig. 2. The protein profile shows that most of the recovered protein (88%) was not bound to this column. Some CSP was also present in this unabsorbed fraction (36% of recovered activity). The addition of 4 mM-n-octyl glucoside to the running buffer did not promote the dissociation of protein or of CSP from the column until the pH was raised to 9.7. A small amount of protein was eluted under these conditions that contained a significant amount of CSP (64% of recovered activity). Raising the pH in the absence of n-octyl glucoside was not effective in eluting CSP (results not shown). The binding of CSP to the column could be increased to virtually 100% by loading in the presence of 4 mM-n-octyl glucoside, but elution by raising the pH then did not occur (results not shown). Inclusion of MgProtoMe$_2$ in elution buffers was ineffective, probably owing to the absorption of this porphyrin on to the column itself (results not shown).

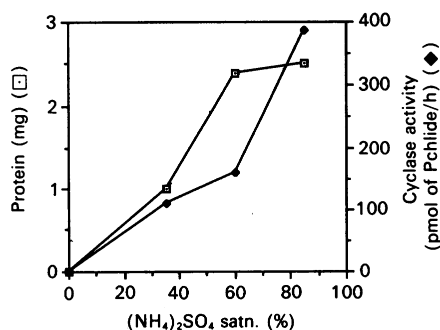

Fig. 3. $(NH_4)_2SO_4$ fractionation of the supernatant

The supernatant fraction was adjusted to either 35, 60 or 85% $(NH_4)_2SO_4$ [by the addition of the appropriate amount of saturated $(NH_4)_2SO_4$] and, after 15 min at 0 °C, insoluble proteins were collected by centrifugation at 12000 *g* for 10 min. Remaining $(NH_4)_2SO_4$ in the precipitated proteins was removed by resuspension in Buffer B and dialysis, as described under 'Methods'. Each sample was bioassayed for cyclase activity against 0.8 mg of pellet protein. The control sample was treated identically, with the exception that no $(NH_4)_2SO_4$ was added.

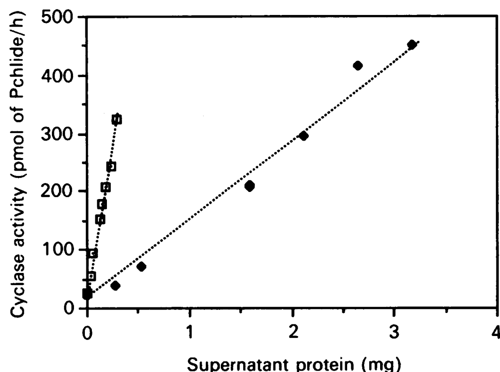

Fig. 4. Linearity of cyclase activity with supernatant protein in the bioassay procedure

Two preparations of CSP were bioassayed at different concentrations against a constant amount (0.6 mg) of pellet protein: ◆, unfractionated supernatant; □, 60–85%-satd.-$(NH_4)_2SO_4$ fraction. Incubation and bioassay conditions were as described in the legend of Fig. 1.

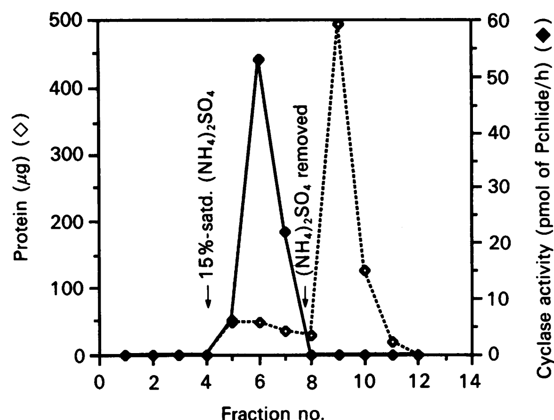

Fig. 5. Chromatography of CSP on phenyl-Sepharose

Column chromatography and fraction bioassay were as described under 'Methods'. A sample of the 60–85%-satd.-$(NH_4)_2SO_4$ fraction (0.8 mg of protein) was resuspended in 1.5 ml of Buffer B/100%-satd. $(NH_4)_2SO_4$ (3:1, v/v) and applied to the column, which had been equilibrated in the same mixture. CSP was eluted by reducing the concentration of $(NH_4)_2SO_4$ in the running buffer to 15% saturation. The amount of pellet protein per assay was 0.6 mg. Substrates and cofactors were as in Table 1. The reduction of $(NH_4)_2SO_4$ saturation to 15% and the omission of $(NH_4)_2SO_4$ from the running buffer are indicated on the Figure.

$(NH_4)_2SO_4$ fractionation of CSP. Fig. 3 shows the protein and CSP activity precipitated from the supernatant at three different $(NH_4)_2SO_4$ concentrations. Although some CSP was precipitated by 35 and 60% saturation of $(NH_4)_2SO_4$, 85%-satd. $(NH_4)_2SO_4$ gave the greatest yield of CSP. However, the protein precipitated by 60%-satd. $(NH_4)_2SO_4$ was comparable in amount with that precipitated at 85% (Fig. 3). Thus significant purification of CSP from the supernatant was achieved by removing those proteins which were insoluble at 60%-satd. $(NH_4)_2SO_4$, then collecting those precipitated by increasing the concentration to 85% saturation.

In order to quantify the purification of CSP in this and other steps, it was necessary to demonstrate that, for each bioassay, CSP was limiting and that the amount of Pchlide formed was proportional to the amount of CSP protein added. Fig. 4 shows a titration of CSP from both an unfractionated supernatant sample and a 60–85%-satd.- $(NH_4)_2SO_4$ fraction. Each sample was bioassayed with a constant amount of pellet, which was sufficient to saturate the assay in the presence of the highest CSP concentrations used as determined by a separate experiment. A linear relationship between μg of CSP protein added and cyclase activity was observed for both samples. As was expected, the more purified $(NH_4)_2SO_4$ fraction gave a line with a steeper slope than the uncut supernatant. An 8.5-fold purification for the $(NH_4)_2SO_4$-fractionation step was calculated from the ratio of the gradients of these two lines.

Hydrophobic-interaction chromatography. Further purification of CSP after the $(NH_4)_2SO_4$ fractionation was possible by using hydrophobic-interaction chromatography. The behaviour of CSP on a phenyl-Sepharose column is shown in Fig. 5. The sample was loaded in Buffer B, 25% saturated with respect to $(NH_4)_2SO_4$, to promote binding to the column; in the absence of $(NH_4)_2SO_4$ no CSP would bind (results not shown). Decreasing the saturation of $(NH_4)_2SO_4$ in the running buffer to 15% released some protein, along with the bound CSP. No additional CSP was eluted when $(NH_4)_2SO_4$ was totally removed from the running buffer, but most of the protein (80%) was eluted at that point. This column gave a 4.8-fold purification of CSP with a 35% recovery of activity.

Fig. 6 shows an SDS/PAGE analysis of the partial purification of CSP from unfractionated supernatant (track 1). Track 2 shows the supernatant after the 60–85%-satd.-$(NH_4)_2SO_4$ fractionation. Many of the supernatant proteins were removed by this step; the major protein band, of molecular mass 52 kDa (track 1), probably corresponds to the large subunit of ribulose bisphosphate carboxylase. Track 3 shows the 60–85%-satd.-$(NH_4)_2SO_4$ fraction after passing through a phenyl-Sepharose column. At this point, four major bands, or groups of bands, were visible on the gel: two single bands of 68 and 47 kDa; a group of four proteins ranging from 34 to 38 kDa; a doublet of 25 and 27 kDa. A few minor proteins were also visible. Although CSP did not bind to Blue Sepharose (Table 4), a small degree of purification was achieved by passing the purified fraction (track 3) through this column (track 4). The four proteins of 34–38 kDa were absorbed on to Blue Sepharose and could be eluted by 0.1–0.3 M-NaCl (results not shown).

Table 5 summarizes the purification data for the various steps described here. The data are expressed as 'relative specific activity' since the term 'specific activity' would be misleading in a bioassay system where the addition of another, as yet undefined,

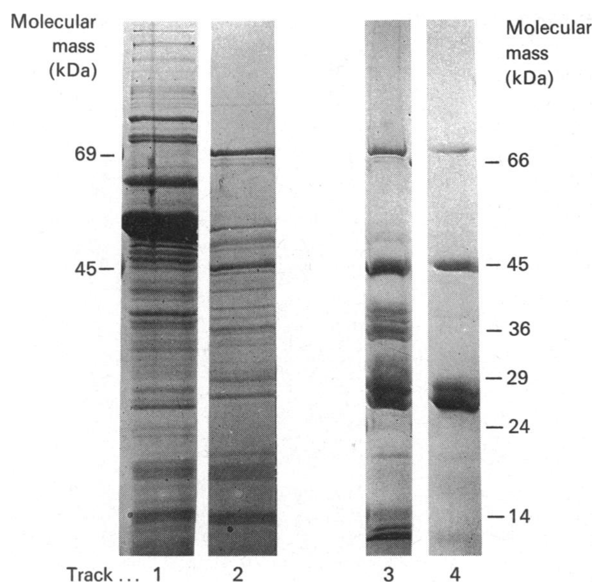

Fig. 6. SDS/PAGE analysis of $(NH_4)_2SO_4$-fractionated, phenyl-Sepharose-purified and Blue-Sepharose-purified supernatant fractions

The Figure shows SDS/PAGE analysis of the supernatant fraction (track 1) after sequential purification by 60–85% saturation with $(NH_4)_2SO_4$ (track 2), phenyl-Sepharose chromatography (track 3) and Blue Sepharose chromatography (track 4). The molecular-mass values shown are derived from the position of protein standards (see under 'Methods').

Table 5. Summary of the purification of CSP

Purification factors were calculated as described in the Results section. Fig. 4 displays the rationale of this calculation. $(NH_4)_2SO_4$ fractionation, phenyl-Sepharose and Blue Sepharose chromatography are shown in series starting from unfractionated supernatant (relative specific activity 1). Purification on $MgProtoMe_2$–Sepharose is shown as a separate experiment.

Purification step	Relative specific cyclase activity	Recovery of cyclase activity (%)
$(NH_4)_2SO_4$ fractionation		
Supernatant	1	100
60–85%-satd. $(NH_4)_2SO_4$	8.5	31
Phenyl-Sepharose		
15%-satd. $(NH_4)_2SO_4$ fraction	40.8	11
Blue Sepharose		
Unabsorbed fraction	43.4	5
$MgProtoMe_2$–Sepharose		
Supernatant	1	100
Unbound	0.5	21
pH 9.7-eluted	9	34

protein fraction is required to measure the enzymic activity. The Table shows that the purification of CSP from the original supernatant fraction achieved by combining $(NH_4)_2SO_4$ fractionation with phenyl-Sepharose and Blue Sepharose chromatography was 43.4-fold; the recovery of CSP was 5%. Clearly, the total purification from the intact plastid, before fractionation into pellet and supernatant, was greater. The affinity-chromatography step (Fig. 2), which by itself gave a 9-fold purification of CSP (Table 5) with a 34% recovery, is essentially an analytical tool which, at the present time, is hardly suitable for preparative use. Therefore it has not yet been combined with the other steps in an overall purification scheme.

DISCUSSION

Since Wong & Castelfranco (1984) reported the resolution of the cyclase into soluble and membrane-bound components, no attempt has been made to assign specific roles for these fractions in the cyclization process. Our recent findings (Walker *et al.*, 1988, 1989) require at least one oxygen-binding protein responsible for the stereospecific hydroxylation of the 6-methylpropionate side chain of MgProtoMe, followed by two enzyme components catalysing the formation of the 6-β-oxopropionate derivative and the completion of cyclization (C–C bond-formation). Therefore we would expect a minimum of three enzymes to be involved in this process.

In the method used here to resolve the cyclase, the use of lengthy ultracentrifugation to separate pellet and supernatant fractions is avoided; hence, the preparation time is greatly reduced compared with the previously used method (Wong & Castelfranco, 1984). This is an advantage, since the pellet has low stability at 4 °C.

It has been demonstrated that MT activity is contained mainly in the pellet, and negligible activity is associated with CSP. Since reconstitution of the cyclase system can be achieved with MgProtoMe even in the absence of SAM, the possibility of the reconstitution merely involving the addition of a solubilized MT component can be eliminated. H.p.l.c. analysis of the experiments summarized in Table 2 also revealed that CSP contained essentially all the ME activity. This activity has not yet been fully characterized.

At least one of the components of the cyclase has a heavy-metal requirement. Chereskin & Castelfranco (1982) were able to demonstrate an iron requirement *in vivo*, but saw only a slight inhibition by α,α'-dipyridyl on Pchlide synthesis *in vitro*. However, Nasrulhaq-Boyce *et al.* (1987) reported that, in intact wheat (*Triticum aestivum*) plastids, 8-hydroxyquinoline inhibited the cyclase, but the more water-soluble desferrioxamine was only slightly inhibitory; these authors suggested that the metal was located in a hydrophobic environment. Our own data (Table 3) confirm this pattern of inhibition. Pretreatment of the pellet fraction with chelator inhibited cyclization, suggesting that a heavy-metal-containing component is embedded in the membrane (Table 3). On the other hand, the metal-ion requirement of the CSP seems to be complex. Inhibition by 8-hydroxyquinoline and desferal mesylate could not be investigated, since removal of these chelators after the CSP treatment was unsuccessful. Using an alternative approach, we have recently demonstrated that the reconstituted cyclase system can be stimulated by Mg^{2+} ions and that this metal-ion requirement seems to be associated with the CSP (B. J. Whyte, P. Vijayan and P. A. Castelfranco, unpublished work). Other bivalent cations (Ca^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+} and Zn^{2+}) and La^{3+} are inhibitory, whereas Na^{+} at higher concentration can partially replace Mg^{2+}.

The inability of CSP to bind to Blue Sepharose (Table 4) suggests that CSP is not the NADPH-binding component of the cyclase; this raises the possibility that the NADPH-binding protein is located in the membrane. Conceivably, the metal-ion and NADPH-binding functions might both be involved in a membrane-bound oxygen-binding complex as seen, for example, in some mixed-function oxidases. However, to date there is no evidence to suggest the involvement of a haemoprotein in the cyclase.

The CSP had an affinity for $MgProtoMe_2$–Sepharose (Fig. 2). Although $MgProtoMe_2$ was the porphyrin initially coupled to

Sepharose, it should be noted that the extracts applied to the column contained ME activity (Chereskin *et al.* 1982; Wong *et al.*, 1985; Walker *et al.*, 1988); thus this column could have a mixture of MgProto, MgProtoMe and $MgProtoMe_2$ bound to the Sepharose lattice. Elution of CSP with porphyrin solutions was not possible, owing to the high affinity of this gel for such compounds (Richards *et al.*, 1987). The elution of porphyrin-binding proteins from such affinity columns by raising the pH has been previously reported (Hinchigeri *et al.*, 1981); this was also observed in the present study. Since NADPH was not required to elute CSP from the column, we surmise that NADPH is not needed to bind the porphyrin substrate to CSP. Moreover, the cyclase has a high pH optimum (pH 9.0, Fig. 1). If the release of the porphyrin from the CSP were the rate-limiting step in this catalysis [as it is with Pchlide reductase (Griffiths 1978)], there might be an interesting correlation between the release of CSP from the affinity column and the release of product from the enzyme in the catalytic sequence.

Reasonable purification of CSP was achieved with a combination of $(NH_4)_2SO_4$ fractionation, phenyl- and Blue Sepharose chromatography (Table 5). Although only four major bands were visible after this purification procedure (Fig. 6), it is still possible that CSP is a minor protein component.

In order to understand the mechanism of the cyclization process, we need to consider tentatively two hypothetical models. The first model would involve a membrane component consisting of an NADPH-dependent oxygen-binding hydroxylating enzyme with the CSP containing all the other proteins required for the cyclization. In the other model, the membrane component would contain all the enzymes necessary for cyclization, and the CSP would act as a Mg-porphyrin carrier. Such a putative porphyrin carrier has recently been proposed in *Rhodobacter capsulatus* (Bauer & Marrs, 1988; Bauer *et al.*, 1988).

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