Further observations on the Mg-protoporphyrin IX monomethyl ester (oxidative) cyclase system

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The Mg-protoporphyrin IX monomethyl ester (oxidative) cyclase was strongly inhibited by CN^- and N_3^- in a reconstituted system, but was inhibited slightly or not at all by the same reagents in intact developing chloroplasts. Known inhibitors of cytochrome *P*-450 processes showed no consistent effect. Benzoquinone and quinol, which can give rise to the same semiquinone by one-electron redox events, were strong inhibitors of the cyclase. It was previously shown that O_2 and a source of electrons are required in the cyclization process. The substrates for the dehydrogenases of the pentose phosphate pathway (glucose 6-phosphate and 6-phosphogluconate) were effective reductants in the reconstituted system with supernatant that had been dialysed

or passed through Sephadex G-50, in the absence of added NADP^+ . However, inhibitor studies suggested that the electrons from these sugar phosphates reached the cyclase system via NADPH . Therefore we infer the presence of protein-bound NADP^+ that can be reduced by glucose 6-phosphate and 6-phosphogluconate and donate reducing equivalents to the cyclase system. This bound NADPH pool may be particularly effective in the cyclization process, owing to channelling. These findings are discussed in relation to the results of a companion paper [Whyte and Castelfranco (1993) *Biochem. J.* **290**, 361–367] on the breakdown of chloroplast pigments in the same reconstituted system.

INTRODUCTION

The cyclization of Mg(II)-protoporphyrin IX monomethyl ester (MgProtoMe) to divinylprotochlorophyllide (DVPchl) is an essential step in the synthesis of known chlorophylls and bacteriochlorophylls (Beale and Weinstein, 1990; Scheer, 1991). This step has been studied in some detail in this and other laboratories. In developing cucumber (*Cucumis sativus*) chloroplasts, it has been established that MgProtoMe is converted into Pchl through the 6- β -hydroxy analogue and the 6- β -oxo analogue, and that the latter is then cyclized with the formation of a new carbon-carbon bond between the active methylene of the 6- β -oxo-methylpropionate side chain and the γ -meso carbon of the macrocycle. Overall, this process is a six-electron oxidation. Molecular oxygen is required as well as a reductant, which is most probably NADPH . Atmospheric oxygen is incorporated into the carbonyl functionality of the new cyclopentanone ring. The enzymes involved have not been purified to homogeneity, but the catalytic system of developing cucumber chloroplasts has been resolved into pellet and soluble components, and the latter has been purified about 40-fold (Walker et al., 1991; Vijayan et al., 1992, and references therein).

In the present paper we report on new inhibitor studies, on new studies bearing on the identity of the endogenous reductant, and on studies of the pellet and supernatant factors, including their interaction within the plastidic unit that catalyses the overall process.

MATERIALS AND METHODS

Plant material

Cucumber (*Cucumis sativus* L., cv. Beit Alpha MR) seeds were obtained from Sunseeds Co., Hollister, CA, U.S.A., and were germinated in the dark at 30 °C for 6 days. The etiolated seedlings were exposed to light [$52 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ photosynthetically active radiation (p.a.r.) at 28–30 °C] for 20 h.

Chemicals

Cysteine, EDTA, glucose 6-phosphate disodium salt (Glc-6-P), 6-phosphogluconate trisodium salt (6-P-gluconate), 3-phosphoglyceric acid disodium salt (3-PGA), glucose-6-phosphate dehydrogenase (Glc-6-PDH) type XII from *Torula* yeast (EC 1.1.1.49), Hepes, Tes, MgCl_2 , NADP^+ , 2'-phosphoadenosine 5'-diphosphoribose (PADPR), Percoll, NaN_3 and sorbitol were purchased from Sigma.

Amitrole (3-amino-1,2,4-triazole), a technical product obtained from Amchem, was crystallized from ethanol. Amino-benzotriazole was a gift of Dr. Paul Ortiz de Montillano, School of Pharmacy, Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94143, U.S.A. BAS 110, BAS 111, Tetcyclasis (BAS 106W) and LAB 150978 were all technical-grade chemicals (96–98 % pure) and were gifts of the BASF Corporation, Research Triangle Park, NC 27709, U.S.A. Imazalil was a gift from Dr. David B. Helmer, Janssen

Abbreviations used: ABT, 1-aminobenzotriazole; amitrole, 3-amino-1,2,4-triazole; BAS 110, 1-(2,4-dichlorophenyl)-2-methoxy-1-methyl-2-(1*H*-1,2,4-triazol-1-yl)ethanol; BAS 111, 1-phenoxy-3-(1*H*-1,2,4-triazol-1-yl)-4-hydroxy-5,5-dimethylhexane; DMSO, dimethyl sulfoxide; 1,3-diPGA, 1,3-diphosphoglyceric acid; DV, divinyl; Gal-3-PDH, glyceraldehyde-3-phosphate dehydrogenase; Glc-6-P, glucose 6-phosphate; Glc-6-PDH, glucose-6-phosphate dehydrogenase; imazalil, 1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl]-1*H*-imidazole; LAB 150978, 1-(trifluoromethyl)-2-(1,2,4-triazolyl)-(1)-3-(5-methyl-1,3-dioxan-5-yl)propen-3-ol; MgProtoMe, Mg(II)-protoporphyrin IX monomethyl ester; MgKPM, Mg(II)-7-(2-carboxyethyl)-4-ethyl-1,3,5,8-tetramethyl-6-[(1-oxo-2-methoxycarbonyl)ethyl]-2-vinylporphyrin, the 6-methyl β -oxopropionate analogue of MgProtoMe (2-vinyl-, 4-ethyl-substituted); PADPR, 2'-phosphoadenosine 5'-diphosphoribose; p.a.r., photosynthetically active radiation; Pchl, protochlorophyllide; 3-PGA, 3-phosphoglyceric acid; 6-P-gluconate, 6-phosphogluconate; 6-P-gluconicDH, 6-phosphogluconate dehydrogenase; BAS 106W, Tetcyclasis {1-(4-chlorophenyl)-3a,4,4a,6a,7,7a-hexahydro-4,7-methano-1*H*-[1,2]diazeto[3,4-f]benzotriazole}; SAM, S-adenosyl-L-methionine.

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Pharmaceutical, Piscataway, NJ 08854, U.S.A. *p*-Benzoquinone was from Eastman Kodak; quinol and Methylene Blue were from Matheson, Coleman and Bell. KCN was obtained from Mallinckrodt. Piperonyl butoxide (90% technical grade) was obtained from Aldrich. Sephadex G-50 was from Pharmacia Fine Chemicals.

Mg-porphyrin substrates

Synthetic MgProtoMe was prepared by the method of Shiau et al. (1991); Mg(II)-7-(2-carboxyethyl)-4-ethyl-1,3,5,8-tetramethyl-6-[(1-oxo-2-methoxycarbonyl)ethyl]-2-vinylporphyrin (MgKPME, the 6 β -oxopropionate analogue of MgProtoMe), was prepared as described by Cox et al. (1974). Stock solutions of Mg-porphyrin substrates were 333 μ M in dimethyl sulphoxide (DMSO).

Buffers

Plastids were isolated in hypertonic buffer A (10 mM Hepes/20 mM Tes/1 mM MgCl₂/2 mM sodium EDTA/0.5 M sorbitol, pH 7.7). Lysis was achieved using hypotonic buffer B (10 mM Hepes/20 mM Tes/1 mM MgCl₂/2 mM sodium EDTA, pH 7.7).

Plastid isolation and resolution of MgProtoMe (oxidative) cyclase

Intact plastids were isolated by the procedure of Fuesler et al. (1984), which involves passing the crude pellets through a 45%-(w/v)-Percoll cushion in buffer A. For the reconstituted chloroplast system, the intact chloroplasts were lysed in buffer B for 10 min at 0–4 °C. The lysate was centrifuged at 12000 *g* for 10 min. The resulting pellet was washed once in buffer B (Walker et al., 1991).

The 12000 *g* supernatant was cloudy, but the cloudiness did not interfere with the cyclase assay (see below). A perfectly clear, enzymically active, fluid was obtained by centrifuging the 12000 *g* supernatant for 30 min at 100000 *g*. This step was necessary prior to Sephadex G-50 gel filtration.

MgProtoMe (oxidative) cyclase assay

MgProtoMe (oxidative) cyclase was assayed spectrofluorimetrically as described by Walker et al. (1991). Incubations were for 60 min in the dark at 30 °C in 1.0 ml of total volume. Hypertonic buffer A was used in incubations with intact plastids; hypotonic buffer B was used in incubations with the reconstituted cyclase system. Synthetic MgProtoMe was used routinely as the substrate. The product, DVPchl_a, was extracted into diethyl ether (Castelfranco et al., 1979) and assayed by the method of Chereskin et al. (1982). NADPH was added as a regenerating system (6.5 mM Glc-6-P/0.8 mM NADP⁺/1.1 units of Glc-6-PDH) (Shiau et al., 1991).

Protein determinations

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

RESULTS

Effect of haematin-complexing agents and other redox inhibitors

The nature of MgProtoMe cyclization to DVPchl_a, a six-electron oxidation in which O₂ plus NADPH functions as the

oxidant and in which atmospheric oxygen is incorporated into the new isopentanone ring (Chereskin and Castelfranco, 1982; Wong and Castelfranco, 1984; Walker et al., 1989), prompted us to examine the effect of putative inhibitors of cytochrome *P*-450 processes (Halkier and Moller, 1991; Werck-Reichhart et al., 1990) (Table 1). Of these, only imazalil showed appreciable inhibition of the cyclase at 100 μ M concentration. The four BASF products (BAS 110, BAS 111, LAB 150978 and Tetcyclasis), which are particularly effective inhibitors of the cytochrome *P*-450 dependent ent-caurene oxidase (Rademacher et al., 1987), failed to inhibit MgProtoMe cyclase at the concentration used. 1-Aminobenzotriazole (ABT), a well-characterized cytochrome *P*-450 inhibitor (Werck-Reichhart et al., 1990) showed little effect at concentrations as high as 5 mM. A related compound, amitrole, was inhibitory at high concentration, but although this compound has well-established herbicidal properties, its mode of action is unclear. The effect of imazalil on the cyclase as well as on the breakdown of thylakoid pigments is detailed in a parallel study in the accompanying paper (Whyte and Castelfranco, 1993).

We also examined the effect of two classical haematin-complexing agents, CN[−] and N₃[−]. KCN was a strong inhibitor of the cyclase activity in the reconstituted system, and a much weaker inhibitor in intact chloroplasts (Figure 1). NaN₃ was a strong cyclase inhibitor in the reconstituted system, but was hardly inhibitory in intact chloroplasts (Figure 2).

Two compounds that react readily with superoxide ion (O₂^{•−}) are benzoquinone and quinol (Afanas'ev, 1989). The former undergoes a one-electron reduction, and the latter a one-electron oxidation to the same intermediate redox form, benzosemiquinone. Both benzoquinone and quinol strongly inhibited the cyclase (Figure 3).

Source of electrons for the oxidative cyclase

It is difficult to ascertain what is the endogenous source of electrons in intact chloroplasts. In our reconstituted system we observed that both NADH and NADPH were effective (Wong and Castelfranco, 1984), but that, at low concentration, NADPH

Table 1 Inhibition of reconstituted cyclase activity by putative cytochrome *P*-450 inhibitors

The Table was compiled from the results from a number of experiments. Pellet protein ranged from 1.4 to 2.2 mg; supernatant protein ranged from 2.2 to 3.9 mg. Incubations included 10 μ M MgProtoMe, an NADPH-regenerating system, 1 mM S-adenosyl-L-methionine (SAM), pellet and supernatant fractions (see the Materials and methods section). Pchl_a contents were corrected by subtracting the amounts initially present in the respective pellets. In this and subsequent Tables the incubation time was 60 min (see the Materials and methods section).

Compound	Cyclase activity (% of control)
Imazalil (0.1 mM)	58.0
BAS 110 (0.1 mM)	104.0
BAS 111 (0.1 mM)	106.0
LAB 150978 (0.1 mM)	108.0
Piperonyl butoxide (0.1 mM)	103.1
Tetcyclasis (0.1 mM)	99.0
ABT	
0.1 mM	92.0
1 mM	85.0
5 mM	97.8
Amitrole	
0.1 mM	83.3
1 mM	56.8

Figure 1 Inhibition of cyclase activity by KCN in intact (○) and reconstituted (●) chloroplasts

Incubations included 10 μ M MgProtoMe, 1 mM SAM, NADPH-regenerating system. Symbols: ○, 3.6 mg of chloroplast protein was added; ●, 1.6 mg of pellet protein and 2.9 mg of supernatant protein were added. KCN was added at the indicated concentrations. The zero-time control values were subtracted from the Pchlide readings prior to calculating the percentage inhibition. In this and subsequent Figures the incubation time was 60 min (see the Materials and methods section).

Figure 2 Inhibition of cyclase activity by NaN_3 in intact (○) and reconstituted (●) chloroplasts

Incubations included 10 μ M MgProtoMe, 1 mM SAM, NADPH-regenerating system. Symbols: ○, 3.8 mg of chloroplast protein was added; ●, incubations contained 2.1 mg of pellet protein and 3.7 mg of supernatant protein. NaN_3 was added at the indicated concentrations. The percentage inhibitions were calculated as in Figure 1.

Figure 3 Inhibition of reconstituted cyclase activity by benzoquinone (●) and quinol (○)

Incubations included 10 μ M MgProtoMe, 1 mM SAM, NADPH-regenerating system, 1.4 mg of pellet protein and 2.3 mg of supernatant protein. Inhibitors were added as indicated. The zero-time control values were subtracted from the Pchlide readings prior to calculating the percentage inhibition.

was the more effective of the two. Therefore, we surmised that this nucleotide is the physiological electron donor. If exogenous NADPH is used to obtain maximal cyclase activity in the

Figure 4 Effect of components of the NADPH-regenerating system on reconstituted cyclase activity

Incubations included 10 μ M MgProtoMe and 1 mM SAM. The components of the NADPH-regenerating system (6.5 mM Glc-6-P, 0.8 mM NADP^+ and 1.1 units of Glc-6-PDH/ml) were added as indicated. In Expt. 1, 1.8 mg of pellet protein and 3.3 mg of supernatant protein were used. In Expt. 2, 1.5 mg of pellet protein and 2.7 mg of supernatant protein were used. The zero-time control values were subtracted from the Pchlide readings.

Table 2 PADPR inhibition of cyclase activity mediated by Glc-6-P and 6-P-gluconate

Incubations included 10 μ M MgProtoMe, 1 mM SAM, 1.5 mg of pellet protein and 3.4 mg of supernatant protein. Glc-6-P and 6-P-gluconate concentrations were 7 mM; 0.5 mM PADPR was added where indicated. Control values (minus Glc-6-P or 6-P-gluconate) were subtracted from all activities prior to calculating the percentage inhibition.

Treatment	Cyclase activity (pmol of DVPchlide/h)	Inhibition (%)
Glc-6-P	595.2 $\pm$ 8	0
Glc-6-P + PADPR	112.8 $\pm$ 4	81.0
6-P-Gluconate	560.1 $\pm$ 20	0
6-P-Gluconate + PADPR	135.2 $\pm$ 19	75.9

reconstituted system, it must be added at fairly high concentration (approx. 5 mM). Recently (Shiau *et al.*, 1991) we have provided the NADPH to our incubations as a regenerating system consisting of NADP^+ , Glc-6-P and Glc-6-PDH. However, in analysing the requirement for each of these components, we observed that only Glc-6-P was actually required (Figure 4). Even if the supernatant was dialysed or passed through Sephadex G-50, no exogenous NADP^+ requirement for the cyclase assay was seen. Glucose-6-PDH activity could be demonstrated in the supernatant by monitoring the 340 nm absorbance rise in the presence of both exogenous NADP^+ and Glc-6-P (results not shown).

Like Glc-6-P, 6-P-gluconate was able to activate the cyclase reaction (Table 2), and in both cases the cyclase was inhibited by PADPR, an NADP(H) analogue (Ben Hayyim *et al.*, 1967).

The cyclase activated by exogenous Glc-6-P was also inhibited by an NADPH trap consisting of 3-PGA plus ATP (Table 3). In the presence of 3-PGA kinase and ATP, 3-PGA is converted into 1,3-diPGA, the substrate for Gal-3-PDH, which reduces it to triose phosphate at the expense of any NADPH that might be present in the incubation mixture (Fuesler *et al.*, 1984). The presence of 3-PGA kinase and Gal-3-PDH were previously investigated in isolated cucumber chloroplasts at this same stage

Table 3 Inhibition of Glc-6-P-mediated cyclization by an enzymic NADPH trap (3-PGA + ATP) and by Methylene Blue

Incubations included 10 μ M MgProtoMe, 1 mM SAM, 7 mM Glc-6-P, 2.1 mg of pellet protein and 3.3 mg of supernatant protein; 3-PGA and ATP were both 5 mM. Methylene Blue was 20 μ M.

Treatment	Cyclase activity (pmol of DVPchlide/h)	Inhibition (%)
Glc-6-P	234.7 $\pm$ 0	0
Glc-6-P + PGA + ATP	71.6 $\pm$ 26	69.5
Glc-6-P	146.4 $\pm$ 13	0
Glc-6-P + Methylene Blue	33.4 $\pm$ 1	77.2

Figure 5 Reconstituted cyclase activities for different pellet/supernatant ratios

Incubations included 10 μ M MgProtoMe, 1 mM SAM and NADPH-regenerating system. In (a) the supernatant was held constant at 2.7 mg of protein; in (b) the pellet was held constant at 1.9 mg of protein.

of development (Fuesler et al., 1984). Not only was it found that these enzymes were present, but this 3-PGA reductive system was actually used as a stromal marker, in full agreement with the previous findings of Heber et al. (1963). Being soluble stromal proteins, these enzymes are released into our supernatant during the resolution of the cyclase. Glc-6-P-mediated cyclization was also inhibited by Methylene Blue (Table 3), in agreement with earlier results obtained using a chloroplast preparation isolated in hypertonic buffered sucrose and containing both intact and broken organelles (Pardo et al., 1980), and exogenous NADPH as the required reductant (Chereskin et al., 1982).

Pellet-supernatant interactions

Figure 5 summarizes the activity of the cyclase in the reconstituted system when the concentration of the pellet (Figure 5a) and the concentration of the supernatant (Figure 5b) were varied. It can be seen that, within the limits of what is experimentally possible,

the supernatant did not become saturating, whereas the pellet became saturating at about 3–4 mg of protein/ml.

DISCUSSION

Kleinig (1989) has pointed out that the true cytochrome *P*-450 reactions would be expected to be localized in the endoplasmic reticulum rather than in the plastids. However, many plastid-localized reactions are known that require molecular oxygen, like the desaturation of stearyl-acyl-carrier protein to oleoyl-acyl-carrier protein (Stumpf, 1980). MgProtoMe cyclase is another plastidic O_2 -dependent oxidizing reaction. Our studies with inhibitors of cytochrome *P*-450 oxidases (Table 1) failed to establish or to rule out the cytochrome *P*-450-dependence of the cyclase.

In the early days of our cyclase research we isolated developing chloroplasts in buffered sucrose as a mixture of intact and broken organelles (Pardo et al., 1980). Moreover, the early experiments with a subplastidic reconstituted system utilized treatments that could easily shear extrinsic proteins: sonication (Wong and Castelfranco, 1984) or osmotic shock preceded by 0.2 M NaCl treatment (Walker et al., 1991). Recently, we have observed that these treatments are unnecessary and decrease the activity of the reconstituted cyclase. In the present study we isolated intact organelles through a Percoll cushion (Fuesler et al., 1984) and lysed them gently in hypertonic buffer.

Comparison of the CO-inhibition data (Chereskin et al., 1982) with the KCN and NaN_3 data reported here (Figures 1 and 2) is not altogether straightforward, because of the difference in the plastid material. Chereskin et al. (1982) failed to observe CO inhibition of the cyclase in isolated chloroplasts; later, J. D. Olechno, Y. S. Wong and P. A. Castelfranco (unpublished work) confirmed this finding using a reconstituted cyclase system prepared by sonication.

Therefore, the lack of inhibition by CO was observed with chloroplast and subplastidic preparations that were not identical with those used in the KCN- and NaN_3 -inhibition experiments (Figures 1 and 2). Nevertheless, the difference between CO and these two other haem ligands is striking. We speculate that it might stem from the fact that CO combined only with ferrous haems, whereas CN^- and N_3^- combine with both ferrous and ferric haems and possibly show a preference for the latter (Lemberg and Legge, 1949). We conclude that, most likely, the cyclase system involves a membrane-bound haemoprotein that is not accessible in intact chloroplasts and that contains trivalent iron, and which is, therefore, inhibited much more strongly by CN^- and N_3^- in the reconstituted system than in the intact chloroplasts (compare curves $\circ$ and $\bullet$ in Figures 1 and 2).

Benzoquinone and quinol are uncharged and hydrophobic enough to diffuse and interfere with oxidation occurring at a hydrophobic protein site. Both are powerful inhibitors of the cyclase (Figure 3). Like the three inhibitors that were discovered previously (Chereskin et al., 1982), they could undergo a one-electron redox change to generate a reactive free radical.

In our reconstituted system, the two substrates for the dehydrogenases of the pentose phosphate pathway are sufficient to provide the reducing equivalents needed for the cyclase (Figure 4; Table 2). The co-substrate $NADP^+$ does not have to be added. We postulate that a small amount of $NADP^+$ bound to either pellet or supernatant proteins (and which could not be detected spectrophotometrically by the Glc-6-PDH assay) was reduced by the pentose phosphate pathway enzymes to a small NADPH pool which functions effectively in the cyclase. Perhaps a channelling phenomenon makes this enzymically produced NADPH more efficient than the exogenous nucleotide added to

the reaction mixture. A bound NADP(H) pool was postulated by Stobart and Ameen-Bukhari (1986) to explain the regulation of 5-aminolaevulinic acid synthesis in etiochloroplasts by the Pchlide-Pchlide reductase-NADPH ternary complex.

It is well established that Glc-6-PDHs are oligomeric enzymes that are inactive in the subunit form (molecular mass about 50–60 kDa) (Levy, 1979). In most cases, the active species are dimers, tetramers or hexamers of this basic peptide. In the Glc-6-PDH from human erythrocytes (Bonsignore et al., 1969), brewer's yeast (Kuby et al., 1974; Yue et al., 1969), *Neurospora crassa* (Scott, 1971), and sweet-potato (*Ipomoea batatas*) root (Muto and Uritani, 1972), the active oligomers contain bound NADP⁺, which, at least in the case of the erythrocyte enzyme, is exchangeable with NADP⁺ in solution (Bonsignore et al., 1969). The binding ratio appears to be one NADP⁺ molecule per monomeric subunit (Yue et al., 1969). This bound NADP⁺, which is exchangeable with free NADP⁺, probably turns over during catalysis. Glucose-6-PDH from various photosynthetic tissues has been studied (Lendzian and Bassham, 1975; Levy, 1979; Lendzian, 1980; Srivastava and Anderson, 1983). However, so far as we know, bound NADP⁺ has not been demonstrated in plastidic Glc-6-PDH.

The lack of demonstrable NADP⁺ requirement for cyclase activity in the reconstituted system (Figure 4) could be explained if the Glc-6-PDH present in the cucumber chloroplast supernatant contained bound NADP⁺, which can be reduced by Glc-6-P and reoxidized by donating electrons to the cyclase system. At present, of course, this explanation is highly speculative, but it may be worthy of consideration.

The inhibition by PADPR and 3-PGA plus ATP when Glc-6-P or 6-P-gluconate are used to activate the cyclase without exogenous NADP⁺ (Tables 2 and 3) indicates that NADP(H) must be present at least as a transient component of the cyclase system. PADPR is a competitive inhibitor of many NADP⁺-dependent dehydrogenases (Ben Hayyim et al., 1967). With pig heart isocitrate dehydrogenase, PADPR not only behaves kinetically as a competitive inhibitor, but binds to the enzyme with the same stoichiometry as NADP⁺ (1.0 molecule of ligand per subunit), and the dissociation constant of the PADPR complex is about half as large as for the NADP⁺ complex (Mas and Coleman, 1985). We presume that this behaviour is typical of what can be expected with other NADP⁺-dependent systems.

Pellet-supernatant titration curves show that, under our experimental conditions, the pellet may become saturating, but the supernatant does not. This result is in agreement with a previous study on the purification of the supernatant factor in which we utilized pellet-saturating incubation conditions (Walker et al., 1991; Figure 4).

Theoretically, a plot of the supernatant/pellet ratio versus rate of enzymic cyclization could give us a measure of when the pellet becomes saturating. However, our system is probably not defined enough to allow this kind of analysis and we have therefore postponed this consideration until a later time. Most of the experiments presented here were not carried out under pellet-saturating conditions. Nevertheless, this fact does not alter their validity within their carefully specified contexts.

In our companion paper (Whyte and Castelfranco, 1993) we show that stromal proteins extracted from chloroplasts under conditions identical with those used for the cyclase supernatant, participate in a detergent-activated breakdown of chloroplast

pigments. O₂-requirement and inhibitor studies suggest a relationship between these two systems, which has been sketched in the Discussion of the companion paper (Whyte and Castelfranco, 1993). However, the degree of commonality between the MgProtoMe (oxidative) cyclase and the pigment breakdown systems can be elucidated only by the exhaustive purification of the supernatant factors, in order to ascertain whether some of the same proteins are involved in both cases.

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