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Intraplastidic Localization of the Enzymes that Convert δ-Aminolevulinic Acid to Protoporphyrin IX in Etiolated Cucumber Cotyledons[1]

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ABSTRACT

The intraplastidic localization of the enzymes that catalyze the conversion of δ-aminolevulinic acid (ALA) to protoporphyrin IX (Proto) is a controversial issue. While some researchers assign a stromal location for these enzymes, others favor a membrane-bound one. Etiochloroplasts were isolated from etiolated cucumber cotyledons (*Cucumis sativus*, L.) by differential centrifugation and were purified further by Percoll density gradient centrifugation. Purified plastids were highly intact, and contamination by other subcellular organelles was reduced five- to ninefold in comparison to crude plastid preparations. Most of the ALA to Proto conversion activity was found in the plastids. On a unit protein basis, the ALA to Proto conversion activity of isolated mitochondria was about 2% that of the purified plastids, and could be accounted for by contamination of the mitochondrial preparation by plastids. Lysis of the purified plastids by osmotic shock followed by high speed centrifugation, yielded two subplastidic fractions: a soluble clear stromal fraction and a pelleted yellowish one. The stromal fraction contained about 11% of the plastidic ALA to Proto conversion activity while the membrane fraction contained the remaining 89%. The stromal ALA to Proto conversion activity was in the range of stroma contamination by subplastidic membrane material. Complete solubilization of the ALA to Proto activity was achieved by high speed shearing and cavitation, in the absence of detergents. Solubilization of the ALA to Proto conversion activity was accompanied by release of about 30% of the membrane-bound protochlorophyllide. It is proposed that the enzymes that convert ALA to Proto are loosely associated with the plastid membranes and may be solubilized without the use of detergents. It is not clear at this stage whether the enzymes are associated with the outer or inner plastid membranes and whether they form a multienzyme complex or not.

It is presently acknowledged that the enzymes that convert ALA[3] to protochlorophyll(ides) and Chl are located in the plastids (5, 6, 20, 22, 23). The intraplastidic localization of these enzymes is more controversial. On the basis of osmotic lysis of crude etiochloroplast preparations accompanied by differential centrifugation, Smith and Rebeiz (27) proposed that the enzymes that catalyzed the conversion of ALA to Proto were localized in the stroma while enzymes that converted Proto to Pchlides were membrane-bound. This hypothesis was partially corroborated by Castelfranco *et al.* (4) who reported that PBGD (EC 4.3.1.8), which converts porphobilinogen to a linear tetramer in which four PBG units are condensed head to tail, is a stromal enzyme. Working with green pea leave chloroplasts, Smith (26) reached similar conclusions upon investigating the intracellular distribution of PBGD and ALA dehydratase (EC 4.2.1.24). The latter converts two molecules of ALA to PBG. Earlier work, however, by Carell and Kahn (3) had indicated that in *Euglena* chloroplasts purified by sucrose density gradient centrifugation, ALA dehydratase was membrane-bound. Likewise, Jacobs and Jacobs (13) reported that protoporphyrinogen oxidase, the enzyme that converts protoporphyrinogen IX to Proto was also membrane-bound. Finally Nasri *et al.* (17) reported that the intraplastidic distribution of ALA dehydratase in etiochloroplasts of radish cotyledons was heterogeneous. They concluded that about two-thirds of the activity was stromal while the remaining third was membrane-bound.

Thorough understanding of the regulation of Chl biosynthesis is intimately linked to an understanding of the supramolecular organization of the Chl biosynthetic pathway. That in turn depends on a thorough understanding of the intraplastidic localization of the enzymes that catalyze the conversion of ALA to Chl. In view of the confusion surrounding the intraplastidic localization of the early reactions of the Chl biosynthetic pathway, it has become important to reexamine this issue. In this work we demonstrate that in etiochloroplasts of cucumber cotyledons (*Cucumis sativus* L. cv Muncher) the enzymes that catalyze the conversion of ALA to Proto appear to be loosely bound to the plastid membranes.

MATERIALS AND METHODS

Plant Material

Etiolated cucumber (*Cucumis sativus* L. cv Muncher) seeds were grown in moist vermiculite in darkness at 28°C, for 4 d (21). Seeds were purchased from J. Mollema & Son, Inc., Grand Rapids, MI.

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[3] Abbreviations: ALA, δ-aminolevulinic acid; Proto, protoporphyrin IX; PBGD, porphobilinogen deaminase; G6PDH, gluconate-6-phosphate dehydrogenase.

Chemicals

ALA and kinetin were purchased from Sigma Chemical Co. and potassium gibberellate from Calbiochem, La Jolla, CA.

Pretreatment of Etiolated Seedlings

Four-day-old etiolated cucumber cotyledons were excised with hypocotyl hooks under subdued laboratory light (about 5 foot candles) (11). The cotyledons were incubated at 28°C for 20 h in darkness, in deep Petri dishes (80 × 100 mm), each containing 3 g of tissue and 9 mL of an aqueous solution composed of 2 mM potassium gibberellate and 0.5 mM kinetin (pH 4.3) (6, 23).

Isolation of Intact Purified Etiochloroplasts

All procedures were conducted under subdued laboratory light. After removal of the hypocotyl hooks, 20 g of pretreated cotyledons were hand-ground in a cold ceramic mortar containing 75 mL of homogenization medium which consisted of 500 mM sucrose, 15 mM Hepes, 30 mM Tes, 1 mM $MgCl_2$, 1 mM EDTA, 0.2% (w/v) BSA, and 5 mM cysteine at a room temperature pH of 7.7 (30). The homogenate was filtered through two layers of Miracloth (Calbiochem), and was centrifuged at 200*g* for 5 min in a Beckman JA-20 angle rotor at 1°C. The supernatant was decanted and centrifuged at 1500*g* for 20 min at 1°C. The pelleted etiochloroplasts were gently resuspended in 5 mL of homogenization medium using a small paintbrush. The resuspended plastids were further purified by layering over 35 mL of homogenization medium containing 45% Percoll, in a 50 mL centrifuge tube and centrifugation at 6000*g* for 5 min in a Beckman JS-13 swinging bucket rotor at 1°C (10). Intact etiochloroplasts were recovered as a pellet, whereas broken etiochloroplasts and other cell organelles formed a band at the top of the tube.

Measurement of Etiochloroplast Intactness

Measurement of etiochloroplast intactness was by a modification of the procedure of Journet and Douce (14) using G6PDG as a stromal marker. Etiochloroplasts were added to 1.0 mL reaction mixture containing 300 mM sucrose, 20 mM Tes-NaOH (pH 7.5), 10 mM $MgCl_2$, 0.17 mM $NADP^+$, and 0.2 mM gluconate-6-phosphate. The reaction was initiated by addition of purified etiochloroplasts resuspended in incubation medium, and the change in absorbance was recorded at 340 nm. Etiochloroplasts were lysed by addition of 0.1% Triton X-100 or by osmotic shock (see below). Intactness of the plastids was evaluated from the relative rates of $NADP^+$ reduction by unlysed and lysed etiochloroplasts.

Preparation of Stroma and Membranes

For separation of stroma from membranes, purified etiochloroplasts were lysed by osmotic shock (24, 27). Purified etiochloroplasts were suspended in a hypotonic lysing medium composed of 25 mM Tris, 30 mM $MgCl_2$, 7.5 mM EDTA, 37.5 mM methanol, 4.5 mM glutathione, 40 mM NAD^+, and 8 mM methionine at a room temperature pH of 7.7. The lysed plastid suspension was centrifuged at 235,000*g* for 1 h in a Beckman 80 Ti angle rotor at 1°C. This centrifugation separated the suspension into a colorless soluble-protein supernatant (stroma) and a yellowish pellet (membranes) (27). The stromal fraction was decanted and the pelleted membranes were resuspended either in the incubation or lysing medium.

Isolation of Mitochondria

The same cotyledonary homogenate used for the isolation of etiochloroplasts was also used for the isolation of mitochondria. The latter were isolated by differential centrifugation according to Douce *et al.* (9).

Conversion of ALA to Proto

In a total volume of 1 mL containing 0.33 mL of stroma or membrane suspension, the reaction mixture consisted of 0.033 mL of 10 mM ALA, and 330 mM sucrose, 200 mM Tris, 20 mM $MgCl_2$, 5 mM EDTA, 25 mM methanol, 3 mM glutathione, 27 mM NAD^+, 5 mM methionine, and 0.1% (w/v) BSA, at a room temperature pH of 7.7. Incubation was in a flat-bottom glass tube. ALA was added last to begin the reaction. The tubes were wrapped in aluminum foil and were incubated at 28°C for 2 h in darkness in a water bath operated at 50 oscillations/min.

Subcellular Markers

All enzymes were assayed at 25°C. Activity was linear with respect to time and enzyme concentration. Spectrophotometric determinations were conducted on an Aminco dual wavelength spectrophotometer model DW2 operated in the split beam mode. Succinate:Cyt *c* reductase (EC 1.3.99.1), a mitochondrial marker, was monitored in 5 mM potassium phosphate buffer (pH 7.2), 0.05 mM Cyt *c*, 1 mM NaCN, 0.2 mM ATP, and 10 mM sodium succinate (1). The activity of NADP:triose phosphate dehydrogenase (EC 1.2.1.13), an etiochloroplast marker was determined in 100 mM Tris-HCl (pH 8.0), 4 mM ATP, 0.14 mM NADPH, 4.35 mM glutathione, 10 mM $MgCl_2$, 2 mM 3-phosphoglycerate, and 0.12 unit phosphoglycerate kinase (2). Lactate dehydrogenase (EC 1.1.1.27), a cytosol marker, was assayed in 33 mM Mes buffer (pH 6.0), 0.125 mM NADH, and 0.33 mM sodium pyruvate (8). Hydroxypyruvate reductase (EC 1.1.1.81), a microbodies marker, was assayed in 100 mM potassium phosphate buffer (pH 6.7), 0.14 mM NADH, and 5 mM hydroxypyruvate (12). G6PDH (EC 1.1.1.44), a marker of plastid stroma, was measured in 20 mM Tes-NaOH (pH 7.5), 10 mM $MgCl_2$, 0.17 mM $NADP^+$, 0.2 mM gluconate-6-phosphate, and 0.1% Triton X-100 (25). Molar extinction coefficients of (a) 6.22×10^3 M^{-1} cm^{-1} at 340 nm for NADH or NADPH and (b) 21.1×10^3 M^{-1} cm^{-1} at 550 nm for Cyt *c* were used. Protochlorophyllide content was used as a marker of etiochloroplast membranes.

Protein Determination

Protein was determined according to the method of Smith *et al.* (28).

Pigment Extraction

Before or after incubation, pigments were extracted by addition of 5 mL of cold acetone:0.1 N NH_4OH (9:1, v/v) to 1 mL reaction mixture, followed by centrifugation at 39,000*g* for 10 min at 1°C. The ammoniacal acetone extract was retained, and the pellet was discarded. Chl and other fully esterified tetrapyrroles were transferred from acetone to hexane by extraction with an equal volume of hexane, followed by a second extraction with one-third volume of hexane. The remaining hexane-extracted acetone residue contained the Proto and Pchlide, and was used for quantitative pigment determination by spectrofluorometry (22).

Spectrofluorometry

Fluorescence spectra were recorded at room temperature on a fully corrected photon-counting SLM spectrofluorometer model 8000C, interfaced with an IBM model XT microcomputer. Determinations were performed on an aliquot of the hexane-extracted acetone fraction in a cylindrical microcell 3 mm in diameter. The digital spectral data were automatically converted by the computer into quantitative values. All spectra were recorded at emission and excitation bandwidths of 4 nm.

RESULTS

Evaluation of the Purity of Percoll-Purified Plastids

Although the biosynthetic activity responsible for the conversion of ALA to Proto was not adversely affected by Percoll purification, contamination by other subcellular organelles was considerably reduced (Table I). Similar results were reported by numerous other investigators for various plastid preparations (16, 18, 19). Mitochondrial contamination was reduced about ninefold as evidenced by succinate:Cyt *c* reductase activity, a mitochondrial marker. Contamination by cytosol enzymes was reduced fivefold as evidenced by lactate dehydrogenase activity, a cytosol marker enzyme. Reduction in microbodies contamination, amounted to about sevenfold, as evidenced by hydroxypyruvate reductase activity, a microbody marker enzyme.

Washed crude mitochondria also exhibited a certain ALA to Proto conversion activity which on an organelle protein basis amounted to 2.44% of the activity exhibited by the plastids ([8.23/336.80]100 = 2.44%) (Table I). However, the same mitochondrial preparation, on an organelle protein basis, exhibited 13.68% triose phosphate dehydrogenase activity, a plastid marker, in comparison to purified etiochloroplasts ([6.1/44.6]100 = 13.68%). This, in turn, suggested that the ALA to Proto biosynthetic activity exhibited by the washed crude mitochondria was due to etiochloroplast contamination.

In view of their high rates of ALA to Proto conversion, their low level of nonplastidic subcellular organelle contamination, including mitochondrial contamination, and the very low level of ALA to Proto conversion exhibited by mitochondria (Table I), Percoll-purified etiochloroplasts were deemed acceptable as a starting point for further investigations of the intraplastidic localization of the ALA to Proto biosynthetic activity.

Evaluation of the Efficacy of Lysis by Osmotic Shock

Before proceeding with intraplastidic investigations the efficacy of the osmotic shock methodology used in breaking the plastids apart was evaluated. To this end lysis by osmotic shock was compared to lysis by Triton X-100. The latter is widely used in breaking cells and subcellular organelles. Lysis by osmotic shock was as efficient as lysis by Triton X-100. This was evidenced by similar G6PDH activity, a stromal marker, in osmotically shocked (29.69) and Triton X-100-treated (30.40) purified plastids (Table II). From the data reported in Table II, it was also possible to compare the intactness of crude etiochloroplasts with Percoll-purified ones. Percoll purification increased plastid intactness from about 68 to 86%. Percent intactness was calculated from the G6PDH activity of the plastids before and after lysis using the following formula: 100 − (control G6PDH activity/G6PDH activity of lysed plastids)100.

ALA to Proto Biosynthetic Activity Appears to be Membrane-Bound in Osmotically Shocked Plastids

Osmotic lysis of Percoll-purified plastids followed by high speed centrifugation resulted in two fractions. A somewhat

Table I. *ALA to Proto Biosynthetic Activity and Various Marker Enzyme Activities in Crude and Purified Etiochloroplasts and Crude Mitochondria Isolated from Etiolated Cucumber Cotyledons*

Values are means of four replications. The data were analyzed as a randomized complete block.

Enzyme Activity	Crude Etiochloroplasts	Purified Etiochloroplasts	Crude Mitochondria
Proto[a]	346.35 a[b]	336.80 a	8.23 b
TPD[c] (etiochloroplast marker)	37.36 a	44.60 a	6.10 b
SCR[d] (mitochodrial marker)	58.80 b	6.82 c	173.53 a
LDH[e] (cytosol marker)	30.02 b	5.95 c	85.51 a
HPR[f] (microbodies marker)	182.80 a	26.43 b	150.89 a

[a] ALA to Proto biosynthetic activity (nmol Proto formed/2 h/100 mg protein. [b] Means of four replicates. Partitioning of the means is by LSD at the 5% level of significance. Within rows, values followed by different letters are significantly different. [c] NADP: Triose phosphate dehydrogenase activity (μmol NADPH oxidized/min/mg protein). [d] Succinate: Cyt *c* reductase activity (μmol Cyt *c* reduced/min/mg protein). [e] Lactate dehydrogenase activity (μmol NADH oxidized/min/mg protein). [f] Hydroxypyruvate reductase activity (μmol NADH oxidized/min/mg protein).

Table II. *Intactness of Crude and Purified Etiochloroplasts as Measured by the Activity of the Stromal Marker G6PDH*

Values are means of three replications. The data were analyzed as a randomized complete block.

Treatment	G6PDH Activity	
	Crude etiochloroplasts	Purified etiochloroplasts
	μmol NADP⁺ reduced/min/mg protein	
Control[a]	4.04 c[b]	4.02 c
After lysis by Triton X-100[c]	12.56 b	30.40 a
After lysis by osmotic shock[d]	13.36 b	29.69 a

[a] Pelleted etiochloroplasts were resuspended in the incubation medium containing osmoticum. [b] Means of three replicates. Partitioning of the means is by LSD at the 5% level of significance. Values followed by different letters are significantly different. [c] Etiochloroplasts were lysed by addition of 0.1% Triton X-100. [d] Pelleted etiochloroplasts were resuspended in the lysing medium which had no osmoticum.

colorless supernatant and a yellowish pellet that contained about 7.16 and 92.84% of the plastid protein, respectively. The lower protein content of the stroma in comparison to the membrane fraction, probably reflects the lower carboxydismutase content of the etioplasts used in this work. Indeed, carboxydismutase constitutes 27 to 36% of all the chloroplast stromal proteins, and the etioplast content of this enzyme is only 14 to 33% that of the chloroplast (15). On a unit protein basis, the supernatant exhibited a much higher G6PDH activity (81.32%), a plastid stromal marker, than the membrane fraction (18.68%). On the other hand, the Pchlide content, a plastid membrane marker (27), of the pellet which amounted to 91.33% was much higher than that of the supernatant (8.67%) (Table III). These results indicated, in turn, that the supernatant was mostly stromal in nature while the pellet consisted mainly of plastid membranes.

Comparison of the Pchlide content of the stroma and of the G6PDH specific activity of the plastid membranes, also gave an idea of the extent of cross-contamination among the two fractions. Since on a unit protein basis the Pchlide content of the stromal fraction amounted to 8.67% of the total plastid Pchlide content, this, in turn, may be interpreted as an 8.67% contamination of the stromal fraction by plastid membranes (Table III). Likewise, on a unit protein basis, the membrane fraction exhibited 18.68% of the total plastid G6PDH activity. This may also be interpreted as an 18.68% contamination of the plastid membrane fraction by the stroma.

Intraplastidic localization of the ALA to Proto biosynthetic activity was evaluated by comparison of conversion rates of exogenous ALA to Proto by the stromal and plastid membrane fractions (Table III). The specific activity of the membrane fraction (195.96) was about eightfold higher than that of the stroma (25.32). Furthermore, the proportion of ALA to Proto conversion activity, observed in the stroma, which amounted to 11.44% of the total ALA to Proto conversion activity, was within the range of contamination of the stroma by the membrane fraction (8.67%) (Table III). Altogether these results strongly suggested that the ALA to Proto conversion activity in cucumber etiochloroplasts was associated with the plastid membranes.

Solubilization of the ALA to Proto Biosynthetic Activity by High Speed Homogenization

Several attempts were made to solubilize the ALA to Proto biosynthetic activity from the plastid membranes while retaining a reasonable level of biosynthetic activity. The most successful attempt was achieved by high speed shearing and cavitation induced by high speed homogenization.

Percoll-purified etiochloroplasts were suspended in incu-

Table III. *Distribution of ALA to Proto Biosynthetic Activity, Pchlide Content, and G6PDH Activity in Subplastidic Fractions Prepared after Osmotic Shock or High Speed Homogenization Followed by Ultracentrifugation*

Values are means of three replications. The data were analzyed as a randomized complete block. Values in parenthesis represent percentage of total enzymatic activity or total Pchlide content.

Treatment/Fraction	ALA to Proto[a] Biosynthetic Activity	Pchlide Content[b] (Membrane marker)	G6PDH Activity[c] (Stroma marker)
Osmotic shock			
Supernatant	25.32 bc[d]	3.83 b	242.91 a
	(11.44)	(8.67)	(81.32)
Pellet	195.96 a	40.34 a	55.81 b
	(88.56)	(91.33)	(18.68)
High speed homogenization			
Supernatant	94.30 b	12.16 ab	53.87 b
	(99.73)	(27.83)	(100.00)
Pellet	0.26 c	31.54 a	0.00 c
	(0.27)	(72.17)	(0.00)

[a] ALA to Proto biosynthetic activity (nmol Proto formed/2 h/100 mg protein). [b] Protochlorophyllide content (nmol Pchlide/100 mg protein). [c] Gluconate-6-phosphate dehydrogenase activity (μmol NADP⁺ reduced/min/mg protein. [d] Means of three replicates. Partitioning of the means is by LSD at the 5% level of significance. Within columns, values followed by different letters are significantly different.

bation medium composed of 500 mM sucrose, 200 mM Tris, 20 mM $MgCl_2$, 2.5 mM EDTA, 8 mM methionine, 40 mM NAD^+, 1.25 mM methanol, and 0.1% (w/v) BSA, at a room-temperature pH of 7.7. The plastid suspension was subjected to three high speed homogenizations, of 10 s each, using a Brinkman Polytron operated at 7/10 maximal intensity. The homogenized suspension was centrifuged at 235,000*g* for 1 h in a Beckman 80 Ti angle rotor at 1°C. The high speed centrifugation yielded two fractions, both highly colored. On a unit protein basis, the Pchlide content of the highly colored supernatant was 38.56% that of the pellet. This, in turn, indicated that high speed shearing and cavitation of the plastids resulted in some release of membrane components such as Pchlide (Table III). It is not clear at this stage whether Pchlide release was due to Pchlide solubilization, or to formation of minor amounts of small membrane fragments that could no longer sediment at the used centrifugal forces. Pchlide release appeared to be accompanied by solubilization of the ALA to Proto activity all of which (99.73%) was now observed in the colored supernatant (Table III). It also removed all remnants of G6PDH activity from the membrane fraction. The treatment also lowered the overall ALA to Proto biosynthetic activity (Table III).

DISCUSSION

The results described in this work are compatible with a loose, surface association of the enzymes that catalyze the conversion of ALA to Proto, with the plastid membranes. The high Mg^{2+} and NAD^+ concentrations used in the lysing medium were the same as those needed for high conversion rates of ALA to Mg-tetrapyrroles *in vitro* (7). It is unlikely that these concentrations resulted in a nonspecific association of otherwise soluble stromal enzymes with membranes for the following reasons: (a) under the same conditions, 81.3% of the G6PDH, a stromal enzyme, was detected in the soluble fraction; and (b) vigorous homogenization solubilized all of the ALA to Proto biosynthetic activity, despite the high Mg^{2+} and NAD^+ concentrations, while most of the Pchlide, a membrane marker, remained in the membrane fraction. Loose association with the plastid membranes is evidenced by ready solubilization of the ALA to Proto activity by high speed homogenization in the absence of added detergents. Complete solubilization of the ALA to Proto biosynthetic activity (99.73%) as compared to the very partial release of Pchlide (28%), a plastid membrane constituent (6), leads to the conclusion that Pchlide is much more deeply embedded in the plastid membranes than the ALA to Proto enzymes which appear to be loosely associated with the surface of the membranes (Table III). The previous results of other investigators who reported a stromal localization for the ALA to Proto biosynthetic activity (27), and for ALA dehydratase and PBG deaminase (4, 17, 26), reflect the fragility and ease of dissociation of the ALA to Proto conversion activity during experimental manipulations. On the other hand, reports of a membrane-bound location of these enzymes (3, 18) overlooked the loose surface association of these enzymes with the plastid membranes.

During the course of this work no attempts were made to determine whether the ALA to Proto biosynthetic enzymes were located in the inner plastid membranes or in the plastid envelope. On the basis of inhibition of Mg-Proto chelatase, the enzyme that inserts Mg into Proto by *p*-chloromercuribenzene sulfonate, Fuesler *et al.* (10) suggested that in whole developing chloroplasts, Mg-Proto chelatase may be located in the plastid envelope instead of the inner plastid membranes. If this hypothesis proves to be correct, it is then advantageous for the membrane-bound ALA to Proto conversion enzymes also to be located in the plastid membrane, next to the Mg-Proto chelatase which uses Proto as a substrate.

One of the important issues in cell metabolism is whether or not sequential metabolic complexes, also referred to as multienzyme complexes, exist within membranes (29). These complexes are capable of catalyzing sequential reactions on a substrate, without allowing the metabolic intermediates to be in diffusion equilibrium with identical molecules in the bulk phase of the same cell compartment (29). This process offers definite advantages in the cell environment where diffusion coefficients for substrate molecules the size of porphyrins (about 700 in mol wt) may be one-fifth those in H_2O and would result in a considerable slowing down of enzymatic reaction rates (29). The rates of ALA conversion to Proto by the membrane bound enzymes observed after plastid lysis and isolation of the plastid membranes (Table III) were high enough to support the highest rates of ALA conversion to Pchlide (150–290 nmol/100 mg protein) which were reported by Daniell and Rebeiz (7) in isolated etiochloroplasts. Although the efficiency of ALA to Proto conversion reported in this work for isolated plastid membranes is compatible with the existence of the ALA to Proto enzymes as a multienzyme complex, more kinetic information is needed before a conclusive statement addressing this question is made.

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