Succinyl-Coenzyme A Synthetase and its Role in δ-Aminolevulinic Acid Biosynthesis in Euglena gracilis

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ABSTRACT

Euglena gracilis cells synthesize the key tetrapyrrole precursor, δ-aminolevulinic acid (ALA), by two routes: plastid ALA is formed from glutamate via the transfer RNA-dependent five-carbon route, and ALA that serves as the precursor to mitochondrial hemes is formed by ALA synthase-catalyzed condensation of succinyl-coenzyme A and glycine. The biosynthetic source of succinyl-coenzyme A in Euglena is of interest because this species has been reported not to contain α-ketoglutarate dehydrogenase and not to use succinyl-coenzyme A as a tricarboxylic acid cycle intermediate. Instead, α-ketoglutarate is decarboxylated to form succinic semialdehyde, which is subsequently oxidized to form succinate. Desalted extract of Euglena cells catalyzed ALA formation in a reaction that required coenzyme A and GTP but did not require exogenous succinyl-coenzyme A synthetase. GTP could be replaced with ATP. Cell extract also catalyzed glycine- and α-keto-glutarate-dependent ALA formation in a reaction that required coenzyme A and GTP, was stimulated by NADP+, and was inhibited by NAD*. Succinyl-coenzyme A synthetase activity was detected in extracts of dark- and light-grown wild-type and nongreening mutant cells. In vitro succinyl-coenzyme A synthetase activity was at least 10-fold greater than ALA synthase activity. These results indicate that succinyl-coenzyme A synthetase is present in Euglena cells. Even though the enzyme may play no role in the transformation of α-ketoglutarate to succinate in the atypical tricarboxylic acid cycle, it catalyzes succinyl-coenzyme A formation from succinate for use in the biosynthesis of ALA and possibly other products.

The phytoflagellate Euglena gracilis synthesizes the key heme and Chl precursor, ALA, from glutamate via the transfer RNA-dependent five-carbon pathway in the plastids of green cells. Unlike other oxygenic organisms, Euglena also has the ability to form ALA via the route used by animals, yeasts, and some bacteria, in which the condensation of glycine and succinyl-coenzyme A is catalyzed by ALA synthase (succinyl-CoA:glycine C-succinyltransferase [decarboxylating], EC 2.3.1.37) (1, 10). ALA that serves as precursor to Chl is synthesized solely by the five-carbon route in Euglena (15, 22), whereas ALA that is used for mitochondrial tetrapyrrole synthesis is formed exclusively by the ALA synthase route (22). Certain nongreening Euglena mutant strains that do not undergo light-induced plastid differentiation appear to lack the ability to form ALA from glutamate in vivo, and these strains rely on the ALA synthase route for all tetrapyrrole precursor biosynthesis (13, 22).

In the mitochondria of animals and yeasts, the ALA precursor, succinyl-CoA, is formed as the product of the α-ketoglutarate dehydrogenase reaction, a step of the tricarboxylic acid cycle. Euglena has been reported to have an atypical tricarboxylic acid cycle. The α-ketoglutarate dehydrogenase complex (EC 1.2.4.2, EC 2.3.1.61, EC 1.6.4.3) is apparently absent, and succinyl-CoA is not an intermediate of succinate formation from α-ketoglutarate in the tricarboxylic acid cycle of Euglena (8, 18). Instead, Euglena mitochondria contain α-ketoglutarate decarboxylase (EC 4.1.1.5), which decarboxylates α-ketoglutarate to form succinic semialdehyde (17). Another enzyme, succinic semialdehyde dehydrogenase (succinate-semialdehyde:NAD oxidoreductase, EC 1.2.1.16), then oxidizes the succinic semialdehyde to form succinate (20).

The absence of α-ketoglutarate dehydrogenase in Euglena raises the question of the source of succinyl-CoA substrate for ALA synthesis via the ALA synthase reaction. Therefore, we examined desalted Euglena extracts for the presence of succinyl-CoA synthetase (succinylthiokinase and succinate-CoA ligase, EC 6.2.1.4 and EC 6.2.1.5, respectively). Activity was found in light- and dark-grown wild-type and nongreening mutant cell extracts. Euglena extract formed ALA from α-ketoglutarate plus glycine via a coupled reaction in the presence of the cofactors required for α-ketoglutarate decarboxylase, succinic semialdehyde dehydrogenase, succinyl-CoA synthetase, and ALA synthase.

MATERIALS AND METHODS

Growth of Cells

Axenic cultures of Euglena gracilis Klebs var Pringsheim wild-type strain Z and nongreening mutant strain W4ZNalL were grown in glucose-based heterotrophic medium in the light or complete darkness as previously described (1). Cells referred to as dark grown had been subcultured in continuous darkness for at least 1 year before these experiments.

Cell Extraction for Enzyme Preparation

Cell cultures in the exponential growth phase were thoroughly chilled on ice under the light conditions in which they were grown. All subsequent operations were performed at 0 to 4°C. Cells were harvested by centrifugation, washed, resuspended, and disrupted by sonication as previously described (12). Cell debris and unbroken cells were removed by centrif-

1 Supported by National Science Foundation grant DCB91-03253.
2 Abbreviations: ALA, δ-aminolevulinic acid; PALP, pyridoxal-5-phosphate.
uation for 10 min at 10,000g. The clarified supernatant fluid was fractionated by differential (NH₄)₂SO₄ precipitation between 35 and 60% of saturating concentration of (NH₄)₂SO₄, in the presence of 5 mM EDTA and 0.004% PMSF, and desalted by passage through Sephadex G-25 that was prequilibrium and eluted with column buffer (1.0 M glycerol, 50 mM Tricine, 15 mM MgCl₂, 1.0 mM DTT, 0.02 mM PALP [pH 7.9]). The protein-containing effluent was supplemented with 0.004% PMSF and stored at -75°C.

**ALA Synthase Assay**

ALA synthase was assayed by modifications of a previously described method (5). Incubation was for 30 min at 40°C in 1 mL of medium (75 mM HEPES, 50 mM glycine, 5 mM EDTA, 0.1 mM PALP [pH 7.8]) supplemented with either 0.1 mM succinyl-CoA, prepared by the method of Simon and Shemin (19), or a succinyl-CoA-generating system consisting of 25 mM succinate, 1 mM GTP, 0.2 mM CoA, and sufficient succinyl-CoA synthetase to catalyze the formation of 0.2 μmol succinyl-CoA per min. In some incubations where indicated, 5 mM levulinate was included to inhibit the conversion of ALA to porphobilinogen. Reactions were terminated by addition of one-tenth volume of 1.0 M citric acid and one volume of 10% (w/v) SDS followed by heating for 3 min at 95°C. ALA was isolated on Dowex 50W-X8 (Na) as previously described (23), ethylacetocetate was added, and the solutions were heated to 95°C for 15 min to form 1-methyl-2-carboxyethyl-3-propionic acid pyrrole (11). The product was quantitated spectrophotometrically after reaction with an equal volume of Ehrlich-Hg reagent (21), using a Cary model 219 spectrophotometer (Varian Instruments, Palo Alto, CA). The A₅₅₃ of control samples containing heat-denatured desalted extract was subtracted from those of incubated samples to determine net A₅₅₃ values, and ALA was calculated from a standard curve.

In some cases where indicated, the Dowex 50W-X8 ALA isolation step was omitted. These reactions were terminated by addition of 50 μL of 100% (v/v) aqueous TCA, precipitated proteins were removed by centrifugation, and the supernatant fluid was reacted with ethylacetocetate and Ehrlich-Hg reagent as described above.

**Assay for ALA Formation from Glycine and Succinate**

These assays were done essentially as described above for ALA synthase, except that neither succinyl-CoA nor a succinyl-CoA synthetase system was provided. Instead, the incubation mixture was supplemented with 25 mM succinate, 0.2 mM CoA, and 1 mM GTP or ATP.

**Assay for ALA Formation from Glycine and α-Ketoglutarate**

Incubation was for 30 min at 43°C in 1 mL of medium (1 M glycerol, 75 mM glycine, 50 mM Tricine, 10 mM α-ketoglutarate, 5 mM MgCl₂, 5 mM KCl, 5 mM EDTA, 5 mM leuvulinate, 1 mM GTP, 0.5 mM NAD⁺, 0.5 mM NADP⁺, 1 mM DTT, 0.2 mM thiamine pyrophosphate, 0.1 mM PALP, 0.1 mM CoA [pH 8.2]). Reactions were terminated, and ALA was isolated and quantitated as described above.

**Succinyl-CoA Synthetase Assay**

Incubation was for 30 min at 43°C in 1 mL of medium (1 M glycerol, 50 mM Tricine, 25 mM succinate, 10 mM MgCl₂, 5 mM GTP, 1 mM DTT, 0.2 mM CoA, 0.02 mM PALP [pH 8.0]). Reactions were terminated by cooling to 0°C. Succinyl-CoA was quantitated by modifications of the method of Lipmann and Tuttle (9), in which succinic acid is chemically generated from succinyl-CoA and determined by the formation of a colored complex with Fe⁺³. To the cooled incubation mixture was added 0.25 mL of a 1:1 (v/v) mixture of 56% (w/v) aqueous NH₄OH and 28% (w/v) aqueous NaOH. Next, 0.25 mL of 0.2 M Na-acetate (pH 5.4) buffer was added, and the solution was mixed and allowed to stand at room temperature for 10 min. Then, 0.25 mL of 24% (v/v) aqueous TCA was added, followed by 0.25 mL of 6 N HCl. Finally, 0.25 mL of 10% (w/v) FeCl₃·6H₂O in 0.1 N HCl was added. After standing at room temperature for 5 min, the solution was clarified by centrifugation and the A₅₄₀ was determined. Net absorbance was determined by subtraction of the A₅₄₀ of samples derived from incubations containing heat-denatured Euglena protein. Concentration was calculated from a standard curve based on samples containing known amounts of succinic acid, prepared from succinic anhydride as described previously (9).

**Other Procedures**

Cell population densities were determined with a Coulter Counter (model ZBI, Coulter Electronics). Protein concentrations were determined by the dye-binding method of Bradford (2) using BSA as the standard.

**Chemicals**

Succinyl-CoA synthetase (porcine heart succinic thiokinase) was purchased from Sigma. All chemicals were from Sigma, Fisher, and Research Organics.

**RESULTS**

**ALA Formation in the Presence and Absence of Added Succinyl-CoA Synthetase**

In previous studies of ALA synthase activity in Euglena extracts, the incubation mixtures were either supplied with chemically synthesized succinyl-CoA or supplemented with commercial succinyl-CoA synthetase and GTP to ensure the availability of the ALA synthase substrate, succinyl-CoA (1, 4-6, 16). Euglena extract was tested for the ability to catalyze ALA synthesis in the absence of added succinyl-CoA synthetase. Unsupplemented extract formed nearly as much ALA as did extract supplemented with succinyl-CoA synthetase (Table I). The reaction required nonadenatured protein and nucleoside triphosphate.

**Nucleoside Triphosphate Requirement for ALA Formation**

Succinyl-CoA synthetases from different sources have different nucleoside triphosphate requirements, some requiring GTP and others ATP (14). Euglena extract supplied with
Table I. Effect of Added Succinyl-CoA Synthetase on ALA
Synthesis

Desalted protein extract from dark-grown wild-type Euglena cells was assayed for ALA synthase-catalyzed ALA-forming ability at 40°C in the presence and absence of added succinyl-CoA synthetase. Except where indicated, incubations (1 mL) contained 75 mM Hepes (pH 7.8), 50 mM glycine, 25 mM succinate, 5 mM EDTA, 1 mM GTP, 0.2 mM CoA, 0.1 mM PALP, sufficient succinyl-CoA synthetase to catalyze the formation of 0.2 μmol succinyl-CoA per min, and 1 mg of Euglena protein. In this experiment, ALA was not purified by Dowex 50W-X8 chromatography before determination.

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Table II. Nucleoside Triphosphate Requirement for ALA Synthesis

Desalted protein extract from dark-grown wild-type Euglena cells was assayed for ALA synthase-catalyzed ALA-forming ability at 40°C in the presence and absence of added GTP and ATP. Incubations (1 mL) contained 75 mM Hepes (pH 7.8), 50 mM glycine, 25 mM succinate, 5 mM EDTA, 1 mM GTP or ATP, 0.2 mM CoA, 0.1 mM PALP, and 1 mg of Euglena protein. In this experiment, ALA was not purified by Dowex 50W-X8 chromatography before determination.

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either of these nucleoside triphosphates formed approximately equal amounts of ALA (Table II).

Glycine- and α-Ketoglutarate-Dependent ALA Formation

Euglena extract was tested for the ability to form ALA from α-ketoglutarate via the coupling of four enzymic steps: α-ketoglutarate decarboxylase, succinic semialdehyde dehydrogenase, succinyl-CoA synthetase, and ALA synthase. Significant ALA formation occurred only in incubations containing α-ketoglutarate, glycine, GTP, and CoA (Table III). Although the reaction was stimulated by NADP⁺, an absolute requirement could not be established. The residual activity in the absence of added NADP⁺ was probably caused by carryover of endogenous pyridine nucleotide through the purification steps of differential (NH₄)₂SO₄ precipitation and gel filtration, as has been previously observed for other pyridine nucleotide-requiring Euglena enzymes (13). In contrast to the stimulation by NADP⁺, a slight inhibition by NAD⁺ was observed.

Under incubation conditions similar to those used for the above assay, ALA synthesis from glycine and chemically synthesized succinyl-CoA did not require nucleoside triphosphate, and neither GTP nor ATP markedly increased the amount of ALA formed in this reaction (Table IV).

Succinyl-CoA Synthetase Activity

Succinyl-CoA synthetase activity was present in Euglena extracts (Table V). Full activity required nonnondenatured protein, succinate, CoA, and GTP. Apparent activity was also present when ATP was used instead of GTP as the nucleoside triphosphate, but with ATP, strong dependence on added succinate and incubation at temperatures above 0°C could not be demonstrated (Table VI). Some differences in activity were measured in extracts from light- and dark-grown wild-type and nongreening mutant cells (Table VII). The activity levels were lower, but the differences between cell types were greater, in the GTP-linked enzyme assays than in the ATP-linked assays. In all cases, the measured in vitro succinyl-CoA synthetase activity was at least 10 times greater than ALA synthase activity.

Table III. Glycine- and α-Ketoglutarate-Dependent ALA Synthesis

Desalted protein extract from dark-grown wild-type Euglena cells was assayed for ALA formation from α-ketoglutarate and glycine at 43°C. Except where indicated, the incubation mixture (1 mL) contained 1 mM glycerol, 50 mM Tricine (pH 8.2), 75 mM glycine, 10 mM α-ketoglutarate, 5 mM MgCl₂, 5 mM KCl, 5 mM EDTA, 5 mM levulinate, 1 mM GTP, 0.5 mM NAD⁺, 0.5 mM NADP⁺, 1 mM DTT, 0.2 mM thiamine pyrophosphate, 0.1 mM PALP, 0.1 mM CoA, and 5.4 mg of Euglena protein.

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Table IV. Influence of ATP and GTP on ALA Synthesis from Succinyl-CoA

Desalted protein extract from dark-grown wild-type Euglena cells was assayed for ALA formation from chemically synthesized succinyl-CoA at 43°C. Except where indicated, the incubation mixture (1 mL) contained 1 mM glycerol, 75 mM glycine, 50 mM Tricine (pH 8.2), 5 mM MgCl₂, 5 mM EDTA, 5 mM levulinate, 1 mM DTT, 0.1 mM PALP, 0.1 mM succinyl-CoA, and 2.8 mg of Euglena protein.

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DISCUSSION

It was previously demonstrated that Euglena has the ability to form ALA by both the transfer RNA-dependent five-carbon pathway and by ALA synthase-catalyzed condensation of glycine with succinyl-CoA (1, 13, 22). In vivo, the five-carbon pathway is the sole source of Chl precursors in Euglena (15, 22), and this pathway is inactive in dark-grown cells (12, 22). The ALA synthesized from glycine, on the other hand, is the sole source of mitochondrial tetrapyrrole precursors in Euglena and may also supply other cellular tetrapyrrole end products in the dark (22).

Even though ALA synthesis from glycine has been shown to occur in vivo and ALA synthase activity has been detected in vitro, there still remained some uncertainty whether ALA synthase can function in vivo. The uncertainty arose from the existence of an atypical tricarboxylic acid cycle in Euglena. In the atypical cycle, the α-ketoglutarate dehydrogenase and succinyl-CoA synthetase steps are bypassed, and α-ketoglutarate is converted to succinate via succinate semialdehyde (Fig. 1). Because succinyl-CoA is not an intermediate in the atypical tricarboxylic acid cycle, it was possible that this compound is absent from Euglena cells and that ALA is formed from glycine in vivo via some other route.

The ability of Euglena to synthesize succinyl-CoA and to use this compound for ALA biosynthesis was demonstrated in three ways. First, desalted cell extracts catalyzed ALA formation in the absence of added succinyl-CoA synthetase.

The reaction required CoA and a nucleoside triphosphate. Second, succinyl-CoA synthetase activity was detected in cell extracts. The activity level was more than adequate to supply substrate for ALA synthase. Third, cell extracts catalyzed glycine- and α-ketoglutarate-dependent ALA synthesis. This activity required CoA and nucleoside triphosphate. Nucleoside triphosphate was not required for ALA synthesis from glycine and chemically synthesized succinyl-CoA. The nucleoside triphosphate requirement for ALA synthesis from α-ketoglutarate indicates that the succinyl-CoA is not formed from α-ketoglutarate via the α-ketoglutarate dehydrogenase reaction because neither that reaction nor ALA synthase has a requirement for nucleoside triphosphate. The CoA requirement indicates that succinyl-CoA is a required precursor to ALA and that succinic semialdehyde cannot be converted to ALA directly. The stimulation of the reaction by NADP + also supports the proposal that succinic semialdehyde must first be oxidized to succinate before succinate is converted to ALA. These results thus support the previous reports indicating that Euglena contains an atypical tricarboxylic acid cycle that lacks α-ketoglutarate dehydrogenase and that α-ketoglutarate is converted to succinate via an alternative route that does not involve succinyl-CoA (17, 18).

Succinyl-CoA synthetases have been classified into two groups based on the nucleoside triphosphate specificity (7, 14, 24, 25). The enzymes obtained from most animal cells, Gram-positive bacteria, and cyanobacteria (EC 6.2.1.4) are more active with GTP, whereas the enzymes from yeasts, plants, and most Gram-negative bacteria (EC 6.2.1.5) are more active with ATP. Some bacterial enzymes have been reported to have no marked activity difference with ATP and GTP (3). Enzymes of both catalytic classes can exist in "large" or "small" sizes, depending on the number of protein subunits they contain (14). ALA synthesis in Euglena extracts was supported approximately equally by GTP and ATP. This result indicates that both nucleoside triphosphates support sufficient succinyl-CoA synthetase to provide this ALA synthase substrate at nonlimiting concentration. Both nucleoside triphosphates also supported succinyl-CoA synthetase activity.
ity. The measured activity was somewhat greater in the ATP-linked enzyme assay than in the GTP-linked assay. However, because a strong dependence on added succinate or elevated temperature could not be demonstrated for the ATP-linked enzyme assay, it could not be ascertained whether some portion of the measured ATP-linked activity is nonspecific or artifactual. Therefore, we hesitate to classify the *Euglena* succinyl-CoA synthetase on the basis of its nucleoside triphosphate preference at this time.

ALA synthase activity is much higher in extracts of dark-grown wild-type cells than in light-grown cell extracts, and the extractable activity declines precipitously within the first few hours after transfer of dark-grown cells to the light (4, 6). Certain nongreening mutant *Euglena* strains appear to lack the capacity to form ALA from glutamate in vivo (22). In these strains, ALA synthase activity remains high in the light or dark (1). It has been proposed that ALA formation via the ALA synthase pathway in *Euglena* is regulated by the availability of ALA that is synthesized in the plastid via the five-carbon pathway (4). When the plastids are biosynthetically active, they may export heme or a heme precursor for incorporation into other cellular hemoproteins, thus lowering the requirement for mitochondrial ALA synthesis. Conversely, when the plastids are quiescent or inactive, ALA synthase may satisfy most or all cellular requirements for heme precurors. Relatively small differences were found in succinyl-CoA synthetase activity in extracts of dark- and light-grown wild-type and nongreening mutant *Euglena* cells. This result indicates that the reciprocal relationship that exists between the state of plastid development and ALA synthase activity is specific to that enzyme and is not a general pattern exhibited by mitochondrial biosynthetic enzymes.

In conclusion, the results reported here indicate that succinyl-CoA synthetase is present in *Euglena* cells. Even though the enzyme may play no role in the transformation of *α*-ketoglutarate to succinate in the atypical tricarboxylic acid cycle of *Euglena*, it can catalyze succinyl-CoA formation from succinate for use in the biosynthesis of ALA (Fig. 1) and possibly other products.

**ACKNOWLEDGMENTS**

We thank S. Rieble for helpful discussions and A.G. Smith for bringing to our attention the reports of the atypical tricarboxylic acid cycle in *Euglena*.

**LITERATURE CITED**


2. Bradford MM (1976) A rapid and sensitive method for the


