Stimulation of Cytochrome *c* Synthesis in the Developing Polyphemus Moth by δ-Aminolevulinic Acid*

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ABSTRACT: Cytochrome *c* is composed of two vastly different moieties, an apoprotein and a heme molecule. The biosynthesis of cytochrome *c* in the Polyphemus silk moth was studied in an attempt to determine if an interrelationship exists between the synthesis of the heme and apoprotein moieties. Developing adult moths were injected at the same stage of development with δ-aminolevulinic acid, a heme precursor, δ-aminolevulinic acid plus puromycin (an inhibitor of ribosomal protein synthesis), δ-aminolevulinic acid plus chloramphenicol (an inhibitor of mitochondrial protein synthesis), or δ-aminolevulinic acid plus actinomycin D (an inhibitor of mRNA synthesis). The cytochrome *c* content of the moth thoraces was determined spectrophotometrically 24 hr after the pupae were injected with δ-aminolevulinic acid or δ-aminolevulinic acid plus an inhibitor. The results, when compared to results obtained with control animals, showed that δ-aminolevulinic acid stimulated cytochrome *c* synthesis by approximately 68%. Puromycin was the only antibiotic capable of blocking stimulation by δ-aminolevulinic acid. The results indicate that heme or a heme precursor controls the level of cytochrome *c* by some undefined mechanism. The results obtained with puromycin and chloramphenicol agree with previous studies which demonstrated that cytochrome *c* was synthesized on the cytoplasmic ribosomes. Since the stimulation of cytochrome *c* synthesis by δ-aminolevulinic acid was not inhibited by actinomycin D, it appears that the mRNA for apocytochrome *c* is stable over the 24-hr period studied.

Studies demonstrating a regulatory role of the heme molecule in the biosynthesis of many heme proteins have appeared in the literature since the 1940's. *Leuconostoc mesenteroides*, a facultatively anaerobic bacterium, contains no catalase when grown in the absence of heme. When heme is added to the medium, catalase appears after a lag period, suggesting *de novo* synthesis of the apoprotein. A similar situation was found in the anaerobic bacterium, *Aerobacter indolgenes*. The authors concluded that the synthesis of heme permitted the synthesis of these heme proteins (Granick and Gilder, 1947). Heme also appears to induce the *de novo* synthesis of the protein moiety of hemoglobin (Levere and Granick, 1967; Levere *et al.*, 1967; Zucker and Schulman, 1968).

Jacob's group (1967) found that heme added to suspensions of anaerobically grown *Staphylococcus epidermidis* resulted in a marked rise in catalase accompanied by an increased respiratory activity. In this instance chloramphenicol, a known inhibitor of protein synthesis, did not affect the changes, suggesting that the apoenzymes of the heme proteins were present but inactive until the heme was made available. These results differ strikingly from the others discussed above.

Tuppy and Birkmayer (1969) demonstrated that in a "petite" mutant of yeast a situation similar to that described by Jacob's group exists in the case of cytochrome oxidase. Miyaka and Sugimura (1970) working with a respiratory-deficient mutant of yeast, lacking all heme proteins, have reported that the addition of protoporphyrin IX (a heme precursor) to the medium restored respiratory activity.

Cytochrome *c* is synthesized *de novo* in the Polyphemus silk moth as demonstrated by the incorporation of [^{14}C]lysine into cytochrome *c* during adult development (unpublished results). *De novo* synthesis of cytochrome *c* was also demonstrated in the closely related *Samia cynthia* (Chan and Margoliash, 1966). The rate of synthesis is very rapid within the last 7 days of adult development. Thus, the developing Polyphemus moth represents an excellent system to study the role heme might play in the biosynthesis of cytochrome *c*. This paper reports on the stimulation of cytochrome *c* synthesis by heme or a heme precursor.

Methods

Determination of Stage and Development of Polyphemus. The time table for the adult development of male Cecropia at 25° (Schneiderman and Williams, 1954) was used. Stages estimated to within 12 hr were assigned according to days of development of Cecropia, although the terminology is only a rough approximation for days of development in Polyphemus.

Injection Technique. Materials for injection were dissolved into insect Ringer (Ephrussi and Beadle, 1936) and injected into pupae anesthetized by CO_2 gas. The wounds were sealed with paraffin wax (Williams, 1959).

Isolation of Mitochondria. Animals were chilled on ice for at least 15 min prior to dissection. The muscles were isolated and the tissue blotted and weighed. All subsequent steps were carried out at 4°. The tissue was minced with a pair of scissors and suspended in ten volumes of a solution consisting of 0.32 M sucrose–0.02 M Tris buffer (pH 7.2) and 5×10^{-5} M EDTA. The suspension was homogenized with 25 passes of a loose pestle in a Dounce homogenizer. The homogenate was spun down at 800*g* in an I.E.C. refrigerated centrifuge for 15 min, yielding a bottom layer of unbroken cells and cell debris, a cloudy and viscous middle layer and a relatively clear top layer. The top layer of the supernatant was removed and saved; the remainder of the homogenate was resuspended with

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two volumes of the sucrose solution and recentrifuged at 800*g* for 10 min. The supernatants (the relatively clear top layer) were pooled and centrifuged in a Beckman Model L ultracentrifuge at 10,000*g* for 10 min. The supernatant from the 10,000*g* run and cell debris fractions were combined and held for later use. The 10,000*g* (mitochondria) pellet was washed twice with sucrose solution, agitating the supernatant slightly each time in order to wash off a lipid-containing layer that sediments just above the mitochondria. These supernatants were also added to the cell debris fraction, and the washed mitochondrial pellet was suspended in 1 ml of the sucrose solution.

Two-tenths ml of the mitochondrial suspension was removed for an oxidase assay and centrifuged at 37,700*g* for 30 min. The mitochondrial yield was between 50 and 80% as determined by the oxidase assay. The cell debris material was centrifuged in the same run to yield a cell debris pellet. The mitochondrial pellet and the cell debris pellet were then used for the oxidase assay described below. The remaining 0.8 ml of the mitochondrial suspension was used for the spectral determination of the cytochromes.

Oxidase Assay. An assay for the mitochondrial marker, cytochrome oxidase, was performed on the cell fractions in order to determine the fraction of mitochondria isolated. The mitochondrial pellets and cell debris pellets were stored at −20° for no longer than 7 days. When desired, the samples were suspended in 0.2 M PO_4 buffer (pH 7.2–7.4), containing 2% sodium cholate and stored overnight at 4°. The suspensions were then centrifuged at 37,700*g* for 15 min and the supernatants removed. The cell debris pellets were washed twice with the 0.2 M PO_4–cholate buffer. The mitochondrial pellet was washed once. A solution of 0.01 M PO_4 buffer (pH 7.2–7.4) containing 1.0% Tween 80 was added to the pooled supernatants of each cell fraction to give a final solution of 0.1% with respect to Tween 80. The supernatants were then dialyzed for 48 hr, with six changes, against 0.01 M PO_4 buffer (pH 7.2–7.4), containing 0.1% Tween 80 to remove the cholate which inhibits oxidase activity. The dialyzed material, containing partially purified cytochrome oxidase, was then placed in a test tube and the dialysis bag rinsed once with the 0.01 M PO_4 buffer containing 0.1% Tween 80 and this wash solution combined with the dialyzed material. The total volume for each sample of the dialyzed material was carefully determined. These samples were used immediately for the oxidase assays according to the procedure of Smith (1955).

The reduced cytochrome *c* used in the oxidase assay was prepared the same day as the assay. Three-times-recrystallized equine cytochrome *c* (Calbiochem) was reduced with sodium dithionite and passed through a Sephadex G-25 (fine) column that had been previously equilibrated with the 0.01 M PO_4 (pH 7.4) buffer. Only the central portion of the cytochrome *c* band was collected and used. Absence of dithionite was confirmed by analysis of the spectra below 400 mμ.

All assays were run with 0.3 ml of the sample, 0.7 ml of 0.1 M PO_4 buffer (pH 7.4), and sufficient reduced cytochrome *c* (between 0.1 and 0.2 ml of the collected sample) to render the solution approximately 35 μM with respect to cytochrome *c*. The assays were run on a Unicam 800 and recorded on a Westronics external recorder. The assay was checked (results not shown here) by dividing a mitochondrial suspension into known fractions and following the above procedure. The experimentally determined fractions agreed with the theoretical calculations within 5%.

Simultaneous Determination of the Mitochondrial Cytochromes. The remaining 0.8 ml of the mitochondrial suspension (as described in the previous section) was kept frozen at −20° for up to 1 week. The procedure followed was that of Williams (1964). The spectra were determined on a Cary 14 spectrophotometer, on an expanded optical density scale from 0.00 to 0.10. Base-line corrections were made using the isosbestic points at 530, 580, and 615 mμ. The points at 530 and 615 mμ were taken from work by Chance and Schoener (1966). The 580-mμ point is an approximation based on the fact that 577 mμ is a valley point for cytochrome *b* (lies below 0 optical density unit in the difference spectrum) and that the absorption at 580 mμ due to cytochromes *a* and *c* are negligible (Yonetani, 1960; Margoliash *et al.*, 1959).

Results

Groups of female Polyphemus at stage 13 were injected with 0.1 ml of δ-aminolevulinic acid (2 mg of δ-aminolevulinic acid/ml of insect Ringer), δ-aminolevulinic acid plus puromycin (2 mg of δ-aminolevulinic acid + 1.6 mg of puromycin per ml of insect Ringer), δ-aminolevulinic acid plus actinomycin D (2 mg of δ-aminolevulinic acid + 0.1 mg of actinomycin D per ml of insect Ringer), and δ-aminolevulinic acid plus chloramphenicol (2 mg of δ-aminolevulinic acid + 50 mg of chloramphenicol per ml of insect Ringer). The δ-aminolevulinic acid was purchased from Nutritional Biochemicals Corp. in the form of δ-aminolevulinic acid hydrochloride. The 2-mg of δ-aminolevulinic acid/ml of solution has an approximate pH of 2.5 and is fully buffered by 0.02 M Tris buffer, pH 7.3 (0.1 ml of acid and 1 ml of buffer). Therefore, the pH of a 3–5 g of moth will not be affected by 0.1 ml of δ-aminolevulinic acid. The dose of puromycin used results in 50% or more inhibition of protein synthesis (Lockshin, 1969). Insect Ringer solution (Ephrussi and Beadle, 1936) was injected into pupae in a control experiment.

Mitochondria were isolated from the thoraces by the method described earlier, 24 hr after injection. As was previously stated 0.8 ml of the mitochondrial suspension was used for spectroscopic analysis and 0.2 ml of the suspension along with the cell debris pellet were used for the analysis of cytochrome oxidase.

The concentrations of the mitochondrial cytochromes obtained by the spectrophotometric analysis method are corrected for the fraction of mitochondria actually isolated (as determined by the oxidase assay) to give a final concentration of cytochromes in the whole tissue. The results of the corrected spectroscopic analysis of the mitochondrial preparations are given in Table I.

The per cent increase of the cytochrome *c* in animals treated with a total of 0.2 mg of δ-aminolevulinic acid as compared to Ringer-treated animals is approximately 68%. The concentration of cytochrome *c* in untreated stage 14 animals was the same as in those injected with Ringer. The results also show that puromycin inhibits the rise in the cytochrome *c* level provoked by δ-aminolevulinic acid while chloramphenicol and actinomycin D have no inhibitory effects.

Attempts were made to demonstrate the possible inductive properties of heme and protoporphyrin IX. These experiments were designed in the same manner as those with δ-aminolevulinic acid. However, since heme and protoporphyrin IX are not water soluble a different solvent had to be employed. First a dimethyl sulfoxide solution (Me_2SO–insect Ringer, 1:1, v/v) and then a 0.04 N NaOH solution were used as solvents for heme and protoporphyrin IX. Some of the pupae 24 hr after injection were in poor condition and inhibition of cytochrome synthesis was observed, possibly due to cell death.

TABLE I: Stimulation of Cytochrome *c* Synthesis by δ-Aminolevulinic Acid.[a]

Sample (Concn/ml of Insect Ringer)	Cytochrome *c* (mμmoles/g of Tissue Wet Wt
Insect Ringer	1.36
	0.96
	1.25
	0.96
	1.14
2 mg of δ-aminolevulinic acid	1.70
	2.21
	1.77
2 mg of δ-aminolevulinic acid + 50 mg of chloroamphenicol	1.71
	1.90
	1.23
2 mg of δ-aminolevulinic acid + 1.6 mg of puromycin	0.94
	1.27
	1.21
	1.12
2 mg of δ-aminolevulinic acid + 0.1 mg of actinomycin D	1.90
	4.55
	3.46
Average values ± extremes	
Insect Ringer	1.13 ± 0.23
2 mg of δ-aminolevulinic acid	1.89 ± 0.32
2 mg of δ-aminolevulinic acid + 50 mg of CAP	1.61 ± 0.38
2 mg of δ-aminolevulinic acid + 1.6 mg of puromycin	1.14 ± 0.20
2 mg of δ-aminolevulinic acid + 0.1 mg of actinomycin D	3.30 ± 1.40

[a] ALA and δ-aminolevulinic acid plus various inhibitors were injected into female, stage 13, pupae (0.1 ml of each solution/pupa) and the cytochrome content determined. Control pupae were injected with insect Ringer. The number of pupae used for each determination varied from 2 to 8.

Discussion

δ-Aminolevulinic acid was used to test for possible inductive properties of heme or a heme precursor, because it is soluble in water. The water-insoluble protoporphyrin IX and heme could not be used owing to the difficulties already discussed under Results. As indicated in Table I, δ-aminolevulinic acid stimulates cytochrome *c* synthesis. The average concentration of cytochrome *c* per gram of thorax tissue (wet weight) determined from several experiments increased 68% over the average control values when stage 13 female animals were injected with 0.2 mg of δ-aminolevulinic acid and killed 24 hr later.

Several important conclusions can be derived from the results of the experiments in which inhibitors were injected simultaneously with δ-aminolevulinic acid. Developing animals which had been injected with 160 μg of puromycin, an inhibitor of ribosomal protein synthesis, along with the δ-aminolevulinic acid were found to contain only control levels of cytochrome *c*. The reason that the concentration of cytochrome *c* was not below control levels (at the stage 13 concentration) is presumably the fact that this concentration of puromycin only inhibits 50% of the ribosomal protein synthesis. On the other hand, chloramphenicol, which is a specific inhibitor of mitochondrial protein synthesis (Clark-Walker and Linnane, 1966) had little effect on the stimulation of cytochrome *c* synthesis when added together with δ-aminolevulinic acid. The low value of one determination with δ-aminolevulinic acid plus chloramphenicol may be due to infection of one or more of the animals after injection. These results concur with previous studies (Gonzalez-Cadavid and Campbell, 1967; Sherman *et al.*, 1966) which showed that cytochrome *c* is synthesized on the cytoplasmic ribosomes and not in the mitochondria. When actinomycin D, an inhibitor of the synthesis of mRNA, was injected in conjunction with δ-aminolevulinic acid, stimulation of cytochrome *c* synthesis was still observed, suggesting that the mRNA for apocytochrome *c* is stable within the 24-hr period studied. Actinomycin D seems to enhance the stimulatory action of δ-aminolevulinic acid. This may be due to a stable mRNA for cytochrome *c*, in the actinomycin D treated cells, while the less stable mRNA species are lost. This condition may permit an increased translation of the mRNA for cytochrome *c*. Kadenbach found an increase in mitochondrial protein synthesis in rat liver slices with low concentrations of actinomycin C (1–10 μg/ml) (Kadenbach, 1968). The concentration of actinomycin D used in our studies falls within this range. The mechanism involved in both cases may be similar. The large variation in the concentration of cytochrome *c* in the latter experiment cannot be accounted for.

δ-Aminolevulinic acid stimulated the synthesis of cytochrome *c* by an as yet undefined mechanism. Stimulation of the biosynthesis of apocytochrome *c* by heme or a heme precursor is strongly implied. It has already been demonstrated that the protein moiety of cytochrome *c* is synthesized *de novo* during adult development of the Polyphemus. The 68% increase of cytochrome *c* synthesized within the 24-hr period studied, in the presence of added δ-aminolevulinic acid supports a stimulatory role for heme or a heme precursor. The inhibition of the stimulation of cytochrome *c* synthesis by puromycin added with δ-aminolevulinic acid further supports the conclusion that the apocytochrome *c* is synthesized *de novo* and that it is not present prior to the addition of δ-aminolevulinic acid. However, the argument that the apocytochrome *c* may be first synthesized and then attached to a heme group cannot be fully ruled out by the present study.

Recent studies have shed new light on the rate-limiting enzyme δ-aminolevulinic acid synthetase, of heme synthesis (Hayashi *et al.*, 1969, 1970; Kurashima *et al.*, 1970). The authors found that δ-aminolevulinic acid synthetase is synthesized on the cytoplasmic ribosomes and then transported into the mitochondria of rat liver. If this process is common to all eucaryotic cells then a nuclear control mechansim for the biosynthesis of functioning mitochondria may operate. If the controlling factor of heme protein biosynthesis is the level of heme or a heme precursor present in the cell then the nuclear control of the rate-limiting enzyme for heme synthesis, δ-aminolevulinic acid synthetase, could direct the formation of functional mitochondria.

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Metabolic Pathways in *Tetrahymena:* Distribution of Carbon Label by Reactions of the Tricarboxylic Acid and Glyoxalate Cycles in Normal and Desmethylimipramine-Treated Cells*

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ABSTRACT: A metabolic scheme is presented showing the interaction of the tricarboxylic acid and glyoxalate cycles with gluconeogenesis, glycolysis, and lipogenesis in *Tetrahymena*. A steady-state kinetic analysis of the redistribution of label from acetate according to this scheme is developed. The following data were obtained from measurements under steady-state conditions with [1-^{14}C]acetate and [2-^{14}C]acetate as substrates: O_2 consumption; the incorporation of label into CO_2, lipid, and glycogen; cell glycogen content before and after incubation. The data were used in conjunction with the theoretical analysis to obtain estimates of the rates of component pathways within the metabolic scheme. For normal cells our results indicate that: total rate of acetyl-CoA production is approximately constant; the rate of the glyoxalate cycle varies from 20 to 75% of the rate of the tricarboxylic acid cycle in a manner dependent on culture age; the rate of lipogenesis is comparable to the rate of the tricarboxylic acid cycle except in stationary cultures, where the rate of lipogenesis falls. Data were also obtained for cells treated with desmethylimipramine. It was found that treatment with this drug depressed the rates of: O_2 consumption, the glyoxalate cycle, the tricarboxylic acid cycle, and glycogen synthesis. Desmethylimipramine generally increased the rate of oxidation of glyoxalate *via* glyoxalate oxidase. Possible modes of action of desmethylimipramine on *Tetrahymena* are discussed.

The pathways of intermediary metabolism in *Tetrahymena* resemble those in mammalian cells in many ways (Kidder, 1967; Hogg and Elliott, 1951; Ryley, 1952; Barber *et al.*, 1965), but one major difference lies in the ability of *Tetrahymena* to carry out the net synthesis of glycogen from 2-carbon precursors (Levy and Scherbaum, 1965). The ability to perform this net synthesis of glucose units from acetyl-CoA is due to the presence of the enzymes of the glyoxalate cycle in *Tetrahymena* (Hogg and Kornberg, 1963). The anaplerotic function of the glyoxalate cycle serves to replenish the 4-carbon compounds which are drained from the tricarboxylic acid cycle and thus permits gluconeogenesis to proceed without disruption of the energy-yielding steps of the tricarboxylic acid cycle (Kornberg, 1967).

The original work of Hogg and Kornberg (1963) demon-

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