Synthesis, Assembly, and Localization of Periplasmic Cytochrome c*

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SUMMARY

The kinetics of synthesis, assembly, and localization of periplasmic cytochrome c-550 was examined in the gram-negative bacterium, Spirillum itersonii. For this purpose, a disc electrophoresis technique was developed to isolate and quantitate radioactive pigment produced from isotopic tracers of amino acids and iron. The synthesis of heme which was not covalently bonded to protein (NCBP-Heme) was also measured for comparison.

Pulse amino acid-labeled cytochrome c-550 entered the periplasmic space with no significant lag. Periplasmic radioactivity did not increase or decrease upon chase, or in the presence of chloramphenicol. A cytoplasmic precursor pool could not be demonstrated. It is concluded that the periplasmic hemoprotein is not a precursor to, or a product of, a membrane-bound or soluble cytoplasmic pigment. Indeed, stable localization seems to occur upon completion of the pigment's polypeptide chain, or within a few seconds thereafter.

The initial rate of iron incorporation into periplasmic cytochrome c-550 followed an upward-shaped profile, while uptake of tracer into NCBP-Heme was linear. Addition of chloramphenicol completely blocked further iron incorporation into the protein, but stimulated NCBP-Heme accumulation 16-fold. These results suggest that assembly of the pigment is tightly coupled with protein synthesis, whereas prosthetic group synthesis is not.

The possibility that cytochrome c-550 might be assembled from a pool of iron-tetrapyrole precursor was tested using the inhibitor of heme biosynthesis, levulinate. When levulinate and radioactive iron were added simultaneously to cells, uptake of isotope into both cytochrome c-550 and NCBP-Heme was inhibited by over 85%. In contrast, when levulinate was added to cells previously labeled with radioactive iron, isotope uptake into cytochrome c-550 continued unaffected for 15 min, and at a decreasing rate for an additional 45 min. This increase was accompanied by a 20% decrease in previously formed NCBP-Heme which was dependent on protein synthesis. These and other results suggest that c-type cytochromes in this microorganism are assembled from an iron-tetrapyrole precursor pool. The intracellular concentration of the putative intermediate was estimated to be 10 μM.

Upon depletion of the iron-tetrapyrole precursor pool by exposure to levulinate, no evidence was gained for the continued synthesis of cytochrome c-550 protein. These results suggest that the availability of prosthetic group precursor may influence the production of the cytochrome c-550 protein counterpart.

Cytochrome c is a hemoprotein which contains a covalently linked prosthetic group. The porphyrin nucleus is linked to the protein component by two thioether bridges, from reduced vinyl side chains to cysteinyl residues (1, 2). Little is known about the sequence of intermediary steps leading to the assembly of holocytochrome c from its precursors. The nature of the control mechanisms which coordinate the synthesis of the protein moiety with the formation of its prosthetic group are also not clear.

In an effort to define more clearly the biosynthesis of cytochrome c, a study of the formation of this pigment in the gram-negative aerobic bacterium, Spirillum itersonii, was performed. In this organism, soluble c-type hemoproteins are localized in the surface layers of the cell, and can be selectively released along with other periplasmic proteins by Tris-EDTA treatment (3). The major pigment, periplasmic cytochrome c-550 (4),1 with a molecular weight of 10,800 and an isoelectric point at pH 9.8 (5), was specifically chosen for radioisotope studies. Data are presented which indicate that the assembly and localization of periplasmic cytochrome c-550 are tightly coupled with protein synthesis; assembly occurs by the utilization of iron-tetrapyrole molecules from a large precursor pool. It is shown further that prosthetic group production is not dependent upon the formation of the cytochrome protein, but that the availability of prosthetic group may influence the production of the protein.

EXPERIMENTAL PROCEDURE

The following isotopes were purchased from New England Nuclear Corp.: [55Fe]Ferric chloride (Fe-55R, 1.72 Ci per mmole

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1 Microfilm copies of Dr. Garrard's thesis may be obtained from the University Microfilm, Inc., 300 North Vebo Road, Ann Arbor, Michigan 48106.
of iron), [59Fe]ferric chloride (Fe-59, 0.51 to 1.42 Ci per mmole of iron), L-[U-14C]leucine (200 mCi per mmole), and [G-H]urate (5.61 Ci per mmole). L-[G-14H]phenylalanine (788 mCi per mmole) and L-[U-14C]phenylalanine (155 mCi per mmole) were obtained from International Chemical and Nuclear Corp. Materials from the following commercial sources were also used: levulunie acid and 2,6-lutidine (Eastman Organic Chemicals), rifanyein SV (Calbiochem), chloramphenil (Sigma Chemical Co.), cyclohexanone (J. T. Baker Chemical Co.), and “Aqualos” (New England Nuclear Corp.).

Culture System—Soluble cytochrome c of S. iberidii (ATCC 11331) is maximally produced during the second phase of diauxic growth (6) in nitrate-supplemented medium (7). This condition was reproducibly achieved by first growing cells to stationary phase in iron-limited (0.5 to 2.0 μM iron) glycine-glutamate-succinate medium (7) containing 0.07% (w/v) ammonium persulfate. The upper and lower tray of the resulting periplasmic extract were analyzed for estimating the recovery of 59Fe- or 3H-labeled cytochrome c-550. This standard was prepared and tested by procedures similar to those outlined above.

Culture Labeling and Sample Preparation— Radioactive iron dissolved in 0.1 N HCl was normally added to cultures 3 to 6 hours after the initiation of cytochrome synthesis by unlabeled iron citrate. The labeled iron was incorporated at increasingly higher rates the later it was added. This could be due to the decreasing solubility with time of the unlabeled iron. The high rate of iron incorporation which occurs when isotope is added at later times was used as an advantage for rapid kinetic studies. When it was necessary to know the specific activity of iron, unlabeled iron and isotope were mixed before addition to cells.

Incorporation of 59Fe or L-[14C]leucine was terminated by pipetting 1.0-ml culture samples into tubes containing 3.0 ml of cold 0.5 M Tris (pH 8.5)-0.05 M EDTA-1.0 mM unlabeled L-leucine. After 30-min incubation at 4°C, cells were removed by centrifugation at 20,000 × g for 15 min at 4°C. Supernatant fractions were dispensed into 15.0-ml conical glass centrifuge tubes, and to each tube 50 μg of purified cytochrome c-550 and 1 × 106 cpm of cytochrome c-550 ([59Fe]-labeled standard) were added. After oxidation of cytochrome by addition of 1 drop of 1.0 mM potassium ferricyanide and vigorous agitation, an equal volume of cold 25% (w/v) trichloracetic acid was added. Protein was pelleted after 1-hour incubation at 4°C by centrifugation in a swinging bucket rotor for 30 min at 4,000 × g. Resulting supernatants were discarded and pellets were blotted dry of excess acid. To each tube 20 μl of 2.5 N NaOH were added, and after pellets were dissolved (15 to 30 min at 24°C) the pH was adjusted to neutrality by the addition of about 20 μl of 1.0 N HCl. After addition of 20 μl of 80% sucrose, sample volumes were adjusted to 100 μl, and portions were subjected to pH 6.6 disc electrophoresis (see below).

When the incorporation of 59Fe or L-[14C]phenylalanine was used to measure cytochrome c-550 synthesis, all of the above operations were the same with the two following exceptions: samples were treated with Tris-EDTA containing 1.0 mM unlabeled L-phenylalanine, and 59Fe-labeled standard was used to determine recovery.

Cytochrome c-550 remaining in cells after Tris-EDTA treatment was also determined. This measurement was an important control, especially in cases where inhibitors were used and the possibility existed that the pigment might accumulate in the cytoplasm. Tris-EDTA-treated cells were resuspended, disrupted by sonic oscillation (3), and debris was removed by centrifugation at 105,000 × g for 4 hours at 4°C. The resulting supernatants were prepared for disc electrophoresis as described above.

Isolation of Cytochrome c-550 by pH 6.6 Disc Electrophoresis—Polyacrylamide lower gels, 8 cm in length and 6 mm in diameter, were composed of the following ingredients: 7.5% (w/v) acrylamide, 0.2% (w/v) N,N',N'-tetramethylethylenediamine, 0.06% (v/v) N,N',N'-tetramethylethylenediamine, 6% (v/v) 1 KOH, 14.3% (w/v) glycine, 0.003% (w/v) potassium ferricyanide, and 0.07% (w/v) ammonium persulfate. The upper gel, 0.5 cm in length, contained: 2.5% (w/v) acrylamide, 0.63% (w/v) N,N'-methylene bisacrylamide, 0.06% (v/v) N,N',N'-tetramethylethylenediamine, 6% (v/v) 1 KOH, 14.3% (w/v) glycine, 0.003% (w/v) potassium ferricyanide, and 0.07% (w/v) ammonium persulfate. The upper and lower tray buffer was 0.382% (v/v) 2,6-lutidine-0.137% (w/v) glycine. Electrophoresis was performed at constant current (2.5 mA per gel) for 3 hours at 4°C in a standard vertical gel system with the direction of migration toward the cathode. The resulting faint
Fig. 1. Separation of soluble cytochrome c-550 from periplasmic and cytoplasmic proteins by pH 6.6 disc electrophoresis. A culture (20 ml) at 6-hours incubation time was labeled for 10 min with L-[14C]leucine (1.5 μCi per ml of culture, 6 μM). A 1.0-ml sample was treated with Tris-EDTA to obtain periplasmic extract. The remaining cytoplasmic proteins were isolated after sonication and high speed centrifugation. 55Fe-labeled periplasm (3,500 cpm of cytochrome c-550) and carrier cytochrome c-530 (50 μg) were each added to both of the 14C-labeled cytoplasmic and periplasmic fractions. Samples were then prepared for and subjected to pH 6.6 disc electrophoresis. —O--O, 55Fe-cpm; •--•, 14C-cpm. c-550 indicates locations of red bands.

Red bands were sliced out of the gels and uniformly macerated onto Whatman No. 3MM filter discs. Discs were placed in vials and dried at 110° for 1 hour. Radioactivity was determined after addition of toluene based scintillation fluid. The original counts contained in experimental samples were calculated by the method of isotopic dilution. Recovery of 55Fe-labeled marker cytochrome c-550 ranged from 15 to 25%.

Fig. 1 shows typical results obtained by the disc electrophoresis technique. Comparison of the amount of cytochrome c-550 present in cytoplasmic and periplasmic fractions reveals that the pigment is entirely periplasmic. The separation of this pigment from other contaminating proteins present in the periplasmic extract is essentially complete, since throughout the symmetrical peak the ratio of 55Fe to 14C is nearly constant. Symmetrical peaks of each isotope throughout the cytochrome c-550 band, having similar isotope ratios, were observed with 45, 7.5%, and 12% (w/v) acrylamide gel columns, and with samples precipitated with trichloroacetic acid.

NCBP-Heme synthesis—NCBP-Heme synthesis was measured by 55Fe incorporation into material which was extractable by acid-cyclohexanone (8, 9). Culture samples (1.0 ml) were pipetted into tubes containing 2.0 ml of cold 0.1 M HCl. After incubation for 30 min at 4°, 3.0 ml of cold cyclohexanone were added, and the resulting two phases were enulsified by vigorous agitation. After an additional 2 hours at 4° the phases were separated by centrifugation. Portions of the organic phase were counted using "Aquasol" scintillation fluid to dissolve dry residues.

Assay of RNA and Protein Synthesis—RNA and protein synthesis were measured by the incorporation of 3H]uracil and 14C-labeled amino acid into acid-insoluble material. Culture samples were precipitated into cold trichloroacetic acid to give a final concentration of 10% (w/v) acid. When the final amount of protein per sample was less than 0.1 mg, carrier bovine serum albumin (0.1 mg) was added. After 1 hour at 4°, precipitates were collected by filtration onto Whatman GF/C filter discs and washed with 20 ml of cold 5% (w/v) trichloroacetic acid containing the appropriate unlabeled compound (1.0 mM).
In an attempt to answer these questions, a pulse-chase experiment was performed with cells actively synthesizing a membrane-associated hemoprotein. The precise conditions of labeling for Experiments I, II, and IV are described in Fig. 2. Experiment III cells (40 ml) were labeled from 5- to 10-hour incubation time with 5 μCi per ml of culture L-[3H]phenylalanine (0.5 mM). Experiment V cells (20 ml) were labeled as in Fig. 2, except after 20 min 1.2 μCi unlabeled L-leucine was added.

Harvested cells were treated with Tris-EDTA to obtain periplasmic extracts. The remaining cytoplasmic proteins were isolated after sonication and high speed centrifugation. The amount of radioactive cytochrome c-550 was then determined in each fraction by pH 6.6 disc electrophoresis. The percentage of the total labeled hemoprotein extracted by Tris-EDTA is defined as periplasmic.

The possibility that a cytoplasmic pool of soluble cytochrome c-550 might exist during some stage in the synthesis of the hemoprotein was explored. The cellular distribution of radioactive soluble cytochrome c-550 was determined by pH 6.6 disc electrophoresis. The possibility that a cytoplasmic pool of soluble cytochrome c-550 might exist during some stage in the synthesis of the hemoprotein is defined as periplasmic.

### Table I

| Experiment | Labeling timea | Radioactive soluble cytochrome c-550 | % periplasmicb |
|------------|----------------|-------------------------------------|----------------|
| I          | 2.5 min        | 0                                   | 98.4           |
| II         | 10.0 min       | 0                                   | 98.0           |
| III        | 330.0 min      | 0                                   | 98.4           |
| IV         | 20.0 min       | 40.0                               | 98.6           |
| V          | 20.0 min       | 330.0                              | 97.7           |

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b Harvested cells were treated with Tris-EDTA to obtain periplasmic extracts. The remaining cytoplasmic proteins were isolated after sonication and high speed centrifugation. The amount of radioactive cytochrome c-550 was then determined in each fraction by pH 6.6 disc electrophoresis. The percentage of the total labeled hemoprotein extracted by Tris-EDTA is defined as periplasmic.

### RESULTS

#### Kinetics of L-[14C]Leucine Incorporation into Periplasmic Cytochrome c-550

- The observation that soluble cytochrome c-550 is localized in the surface layers of the cell (3) raises several questions concerning its synthesis. How long does it take to reach the periplasmic space from the time it is synthesized on polyribosomes? Is periplasmic cytochrome c-550 a precursor to, or a product of, a membrane-associated hemoprotein?

In an attempt to answer these questions, a pulse-chase experiment with L-[14C]leucine was performed with cells actively synthesizing both soluble and membrane-bound hemoproteins. At various times periplasmic and remaining cytoplasmic proteins were separated. The radioactivity present in periplasmic cytochrome c-550 was determined after isolating the protein by disc electrophoresis. For comparison, the kinetic profiles of L-[14C]leucine incorporation into total periplasmic and remaining cytoplasmic protein were also measured (Fig. 2).

In the pulse stage of incorporation, the rate of appearance of radioactivity in periplasmic cytochrome c-550 followed linear kinetics, without a significant lag (Fig. 2A). The kinetics of L-[14C]leucine incorporation into periplasmic and remaining cytoplasmic protein also exhibited linear profiles. The three incorporation curves each extrapolate to about a 5-s lag (Fig. 2B). During the chase stage of the experiment, no significant increase or decrease in the radioactivity of periplasmic cytochrome c-550 was observed (Fig. 2B). The ratio of total periplasmic to remaining cytoplasmic protein also remained constant during the chase; no obvious precursor-product relationships were noted.

The possibility that a cytoplasmic pool of soluble cytochrome c-550 might exist during some stage in the synthesis of the hemoprotein was explored. The cellular distribution of radioactive soluble cytochrome c-550 was examined in cultures labeled with radioactive amino acid and chased for various periods of time. Regardless of the labeling and chase conditions tested, over 98% of the total cellular soluble cytochrome c-550 was recovered in the periplasmic fraction (Table I). The trace of pigment detected in the cytoplasmic fraction is probably due to periplasmic contamination.

#### Kinetics of 55Fe Incorporation into Periplasmic Cytochrome c-550

- The question is now asked, how long from the time of synthesis of the polypeptide chain of periplasmic cytochrome...
c-550 does it take for the prosthetic group to become covalently attached? The kinetics of \[^{14}C\]leucine incorporation revealed that in less than 15 s material became labeled which migrated with native cytochrome c-550 during electrophoresis (Fig. 2A). Although this observation suggests that the pigment is rapidly assembled, the possibility exists that a precursor might co-migrate with holoprotein. A study of cytochrome c-550 synthesis by iron incorporation was therefore performed.

The kinetic profile of \[^{55}Fe\] incorporation into periplasmic cytochrome c-550 is shown in Fig. 3A. An exponential type curve was obtained. In contrast, the same population of cells incorporated \[^{14}C\]leucine into cytochrome c-550 protein at a linear rate (Fig. 3B). The upward shape of the iron uptake profile was not a result of a lag in the conversion of a precursor to native cytochrome c-550, since incorporation was prevented completely upon addition of 100 μg per ml of chloramphenicol (Fig. 3A).

This antibiotic also completely blocked \[^{14}C\]leucine incorporation into the hemoprotein (Fig. 3B). These results suggest that cytochrome c-550 is rapidly assembled, but radioactive iron equilibrates relatively slowly with the form of iron used in holoprotein synthesis.

NCBP-Heme synthesis—NCBP-Heme synthesis was studied to explore further the process of assembly of cytochrome c-550. NCBP-Heme is defined as both free heme and heme associated noncovalently to heme proteins (for example, cytochrome b heme). The prosthetic groups of c-type cytochromes are covalently linked to their protein components, and thus are not included in this classification. Cells actively producing hemoproteins incorporated \[^{55}Fe\] into NCBP-Heme at a linear rate with no significant lag (Fig. 4). The slight upward shape of the curve was due to a corresponding increase in cell mass. Addition of 100 μg per ml of chloramphenicol at 11 hours or at later times after the addition of isotope resulted in an apparent increase of about 1.6-fold in the rate of NCBP-Heme accumulation (Fig. 4). Addition of rifamycin (100 μg per ml) gave similar results. In contrast, only slight stimulation was observed when chloramphenicol was added 15 min after the addition of tracer (Fig. 4).

The second step in heme biosynthesis is catalyzed by the enzyme \(\delta\)-aminolevulinate dehydratase. This enzyme has been purified from \(S.\) \(i\)ersonii and is competitively inhibited in vitro by the substrate analog levulinate (13). When 20 mM levulinate was added to cultures actively producing hemoproteins, at the same time as \[^{55}Fe\], greater than 85% inhibition of NCBP-Heme synthesis was observed (Fig. 5A). Addition of levulinate at later times resulted in the rapid disappearance of about 20% of the total radioactivity (Fig. 5A). This decline did not occur when chloramphenicol was also present (Fig. 5B).
Fig. 6. Effect of levulinate and chloramphenicol on general protein and stable RNA synthesis. The precursors L-[14C]phenylalanine and [3H]uracil (0.05 μCi per ml of culture, 0.5 μm, and 0.5 μCi per ml of culture, 0.1 μm, respectively) were added to cultures (20 ml) previously incubated for 4 hours. Inhibitors (100 μg of chloramphenicol (CAM) per ml of culture, 20 mM sodium levulinate, pH 7.0 (LEV)) were added after 45 min. Samples (1.0 ml) were withdrawn at 15-min intervals and precipitated with cold 10% (w/v) trichloroacetic acid. O--O, control cells; •-•, cells incubated with inhibitor(s).

Effects of Levulinate and Chloramphenicol on General Protein and Stable RNA Synthesis—Fig. 6 shows the effects of levulinate and chloramphenicol on the incorporation of L-[14C]phenylalanine and [3H]uracil into acid-insoluble material. Addition of chloramphenicol (100 μg per ml), in the presence or absence of levulinate (20 mM), lead to an abrupt 98% inhibition of protein synthesis. In contrast, this antibiotic affected the rate of RNA accumulation only after a 30-min lag. Initial exposure to levulinate (20 mM) caused a 30% inhibition of protein synthesis. After 15 min, however, cells partially recovered to a rate of protein synthesis 70% of the control. Levulinate (20 mM) did not significantly inhibit stable RNA synthesis.

Effect of Inhibition of Heme Synthesis on the Incorporation of 59Fe into Periplasmic Cytochrome c-550—The observation that about 20% of the 59Fe-labeled NCBP-Heme disappeared upon addition of levulinate (Fig. 5A) suggests that a portion of the total NCBP-Heme might contain precursors used for the covalent assembly of c-type cytochromes. This possibility is consistent with the apparent stimulation in the rate of NCBP-Heme accumulation observed upon inhibition of protein synthesis (Fig. 4). Under such conditions utilization of iron-tetrapyrrrole precursors for the synthesis of c-type hemoproteins might be curtailed.

More direct evidence for the existence of a pool of heme which was used for the covalent assembly of c-type pigments was obtained by studying the kinetics of 59Fe incorporation into periplasmic cytochrome c-550, in the presence and absence of inhibitors of heme and protein synthesis. When levulinate (20 mM) was added to cells previously labeled with 59Fe, incorporation of radioactivity into cytochrome c-550 continued unaffected for 15 min, and at a decreasing rate for an additional 45 min.
per ml), in the presence or absence of levulinate (20 mM), abruptly halted further incorporation of $^{59}$Fe into cytochrome c-550 (Fig. 7A). This observation confirms the previous experiment (Fig. 3A) in which complete inhibition of iron uptake was noted during brief periods of exposure to this antibiotic.

Size of Precursor Heme Pool—The pool size of iron-tetrapyrrrole precursor can be estimated from the data of the previous experiment (Fig. 7A). Such cells were making total c-type cytochromes at a rate of 0.18 nmole per hour per ml of culture, as determined by spectroscopic measurements. When levulinate was added to the previously labeled culture, the resulting increase in cytochrome c-550 radioactivity equaled about 28 min of normal synthesis. Assuming other c-type cytochromes are not assembled from the same pool of precursor, the total pool size is estimated to be 0.084 nmole per ml of culture, or about 25,000 molecules per cell. Given a volume of $4 \times 10^{-12}$ ml for the S. bensantii cell, the intracellular concentration of heme precursor would be about 10 $\mu$M.

The above method of approximation could result in an underestimate if the precursor prosthetic group pool was not completely utilized during inhibition of heme synthesis; levulinate might cause secondary effects on the synthesis of c-type cytochrome polypeptide chains and on their conversion to holocytochromes. On the other hand, the above method could lead to an overestimate since inhibition of heme synthesis by levulinate is never complete; levulinate is a competitive inhibitor (13).

Considering the above limitations, the precursor pool size was estimated by another technique. Equal portions of cells, previously grown under conditions of iron limitation, were incubated in media containing varying amounts of iron with known specific activities of $^{59}$Fe. After 54 hours, levulinate was added, and the decrease in labeled NCBP-Heme was followed with time to the plateau values. The amount of the decline occurring in response to levulinate can be considered to represent a minimum estimate for the pool size of the putative intermediate. The average size of the precursor pool determined by this technique was 0.084 nmole of iron per ml of culture (Table II), a value in excellent agreement with the above approximation. The size of the precursor pool was not significantly influenced by the concentration of iron in the incubation media, although the rate of total NCBP-Heme accumulation varied 1.6-fold (Table II).

**Effect of Inhibition of Heme Synthesis on Incorporation of L-$^{14}$C-Leucine into Periplasmic Cytochrome c-550**—To find out what happens to the synthesis of the polypeptide chain of periplasmic cytochrome c-550 when heme synthesis is curtailed, the effect of levulinate (20 mM) on the incorporation of L-$^{14}$C-leucine into material which migrated with native cytochrome c-550 during electrophoresis was examined. As a control, the effect of this inhibitor on holocytochrome c-550 synthesis was measured in the same population of cells by $^{59}$Fe incorporation (Fig. 8). In the presence of levulinate, the kinetics and extent of inhibition of L-$^{14}$C-leucine incorporation were indistinguishable from those measured by $^{59}$Fe uptake (Fig. 8). No evidence for differential inhibition was found.

If an apoprotein precursor component of cytochrome c-550 exists, then the interpretation of the above results depends on its electrophoretic properties. Assuming apocytochrome and native hemoprotein undergo co-electrophoresis, then the above results indicate that only enough polypeptide is made to which prosthetic group can be complexed. The synthesis of the protein counterpart would be coordinated with the synthesis of heme.

On the other hand, precursor cytochrome c-550 polypeptide might not undergo co-electrophoresis with the native pigment. During inhibition of prosthetic group synthesis, such an intermediate might accumulate but not be detected. This latter possibility was tested by several experiments.

The electrophoretic profile of radioactive basic proteins, produced by cells which had their prosthetic group pool depleted, was examined. Cells were treated for 1 hour with levulinate (20 mM), and then pulse-labeled with L-$^{14}$C-leucine for 10 min. The $^{14}$C-labeled periplasmic protein was subjected to disc electrophoresis in the presence of marker $^{59}$Fe-labeled cytochrome c-550, and the gel was fractionated for the determination of radioactivity. No new $^{14}$C-labeled peaks were observed compared to control cells not treated with levulinate. In addition, throughout the native cytochrome c-550 band, the ratio of $^{14}$C to $^{59}$Fe was constant. No evidence was gained for the accumulation of a precursor during prosthetic group starvation.

A further experiment was performed in an effort to rescue a hypothetical precursor of cytochrome c-550 which might be produced in the absence of prosthetic group synthesis. Cells were exposed to levulinate for 1.5 hours, levulinate was removed, and cytochrome c-550 synthesis was assayed by the incorporation of either $^{59}$Fe or L-$^{14}$C-leucine, in the presence or absence of 100 $\mu$g per ml of chloramphenicol. After 2 hours of incubation, the inhibition of cytochrome c-550 synthesis by chloramphenicol was 91.1% as judged by L-$^{14}$C-leucine incorporation, and also 99.1% as determined by $^{59}$Fe uptake. No evidence for the rescue of a precursor was found.

**DISCUSSION**

The possibility that periplasmic cytochrome c-550 might be a precursor to, or a product of, a protein firmly bound to the cytoplasmic membrane was eliminated by an amino acid pulse-chase experiment. A particular form of cytochrome c-550 might be produced, but its implantation into the cytoplasmic membrane would have to occur rapidly, and without exchange with its periplasmic counterpart.

Examination of the kinetics of entry of amino acid-labeled cytochrome c-550 into the periplasmic space revealed no significant lag. Furthermore, upon addition of chloramphenicol no

![Table II](http://www.jbc.org/Downloadedfrom)
detectable increase in periplasmic hemoprotein occurred. These observations suggest that if a cytoplasmic precursor pool of the protein exists, its half-life can only be a few seconds. Direct analysis of cytoplasmic fractions for cytochrome c-550 also support this conclusion. The kinetics of localization of total nascent periplasmic protein was also rapid. This observation suggests that the majority of the proteins residing in the surface layers of the cell becomes localized in a manner not depending on large cytoplasmic precursor pools. Possibly, membrane-bound ribosomes are involved in the synthesis of such proteins. Externalization could be facilitated by the process of peptide chain elongation. Work by other investigators has implicated a membrane localization for ribosomes involved in the synthesis of extracellular proteins (14, 15).

Several lines of evidence suggest that a large pool of iron-tetrapyrole precursor is an intermediate in the assembly of cytochrome c-550, as well as in the formation of other c-type cytochromes in S. itersonii. (a) The profile of iron incorporation into cytochrome c-550 followed an upward-shaped curve, although no significant lag was observed for the labeling of NCDP-Heme. (b) Inhibition of heme synthesis by levulinate allowed synthesis of cytochrome c-550 to proceed for about 30 min from previously formed precursor, but not from newly labeled intermediate. (c) The apparent rate of NCDP-Heme synthesis was stimulated upon addition of inhibitors of RNA and protein synthesis. (d) Addition of levulinate to previously labeled cells resulted in a decrease in NCDP-Heme which was dependent on protein synthesis. (e) The extent of the decrease agreed well with the amount of increase in cytochrome c-heme, assuming all c-type cytochromes in S. itersonii were assembled in a manner similar to cytochrome c-550.

The chemical nature of the postulated iron-tetrapyrole precursor has not been elucidated. Since relatively large amounts of this material are present in NCDP-Heme extracts, elaboration of the structure of the intermediate should be an approachable problem. Earlier it was suggested that cytochrome c is assembled by first forming a covalent intermediate with protoporphyrinogen, followed by the insertion of iron (16, 17). This proposal is not consistent with the observations of the present investigation, nor with those previously reported (18).

Formation of the covalent linkages between the protein component of cytochrome c-550 and its prosthetic group occurs very rapidly. Since L-[14C]leucine was incorporated within 15 s into material that underwent co-electrophoresis with native cytochrome c-550, the possibility was considered that a precursor component might have migrated with the completed molecule. However, when protein synthesis was curtailed, radioactive iron incorporation into the protein was also abruptly halted. It can thus be concluded that assembly of holoprotein is tightly coupled with protein synthesis.

A consideration of the stage of assembly of cytochrome c-550 must include the time it takes to polymerize the protein component's polypeptide chain. Previous studies indicate that the molecule contains about 94 amino acid residues (4). Assuming the rate of peptide chain elongation in S. itersonii at 30° is similar to those reported in other bacteria at 37° (19, 20), the polypeptide chain would take about 6 s to be polymerized. An indirect estimate of the polymerization time of cytochrome c-550 by another method agrees well with this approximation (4). The sensitivity of the experiment demonstrating curtailment of 55Fe incorporation into cytochrome c-550 upon addition of chloramphenicol was limited to the detection of an apoprotein pool with about a 1 min turnover time. Thus no conclusions can be drawn as to when the prosthetic group becomes attached to the polypeptide. Assembly could occur during peptide chain elongation, during chain termination, or after release of completed apoprotein molecules from polyribosomes. Nevertheless, the results of the present study are not inconsistent with the possibility that assembly occurs at the translational level.

The synthesis and assembly of cytochromes requires the delicate cooperation of two complex biosynthetic processes. A balance in the rates of production of precursor prosthetic groups and polypeptide chains must be maintained by yet unknown regulatory devices. In an attempt to explore such relationships, the effect of inhibition of synthesis of one component upon the production of its counterpart was examined. Heme synthesis in S. itersonii does not appear to be tightly coupled with the production of cytochrome protein counterparts. Inhibition of either RNA or protein synthesis did not curtail prosthetic group formation.
On the other hand, the availability of prosthetic groups may influence production of their protein counterparts. When the rate of heme synthesis was retarded by levulinate, no evidence was found to suggest that a precursor polypeptide of cytochrome c-550 accumulated. More likely, only enough protein counterpart was produced to which prosthetic group could be complexed. Perhaps maintenance of constant pool sizes of prosthetic group precursors may regulate the rate of production of their cytochrome protein counterparts. When the rate of NCBP-Heme accumulation was varied by the amount of iron supplied, the size of the putative prosthetic group pool of c-type pigments remained relatively constant. This suggests that the rate of removal of prosthetic group precursors by the covalent assembly of c-type cytochromes had carefully adjusted itself to keep in step with the rate of their supply.

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