

Cytochrome Synthesis and Its Regulation in *Spirillum itersonii*

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Spirillum itersonii contains *b*- and *c*-type cytochromes as well as a carbon monoxide-binding pigment of the cytochrome *o* type. Synthesis of cytochromes *b* and *c* is increased by about two- and fourfold, respectively, when cells are transferred from high to low aeration. The increased concentration of cytochrome is not accompanied by an increase in the respiration rate of the cells. Both cytochrome *b* and cytochrome *c* are located in the particulate fraction of cells grown under high or low aeration, and both pigments are fully reducible by succinate. No evidence was found for the accumulation of the protein component of either cytochrome when synthesis of the prosthetic group was limited by iron deficiency, nor did heme or precursors accumulate when protein synthesis was prevented. It was therefore concluded that the formation of the heme prosthetic group is closely integrated with the synthesis of the protein moiety. δ -Aminolevulinic synthase was detected in extracts of the organism. Its activity was correlated with cytochrome synthesis; it was reduced by high aeration and increased under low aeration. The synthase was inhibited by hemin at concentrations of 10 μ M or higher. The observations are consistent with a central role for the heme prosthetic group in the regulation of cytochrome synthesis.

The synthesis of cytochrome by bacteria is strongly influenced by the environment, in particular by the degree of aeration, and, in many instances, the cytochrome content is increased as the oxygen pressure diminishes (14, 17). High concentrations of cytochromes are also found in organisms which are grown anaerobically with nitrate as terminal acceptor (24, 26). The effect of environment on cytochrome synthesis has been extensively examined in *Haemophilus influenzae* (29, 31, 32). This organism makes a variety of such pigments, whose synthesis is influenced to various extents by the conditions of aeration and by anaerobic growth with nitrate.

Cytochrome synthesis involves the formation of the iron tetrapyrrole prosthetic group (referred to as heme) and the specific protein associated with it. The regulation of tetrapyrrole synthesis has been studied previously in the photosynthetic bacterium *Rhodospseudomonas spheroides*, which makes both hemes and chlorophyll (the latter predominating). The first enzyme of the tetrapyrrole biosynthetic pathway, δ -aminolevulinic (ALA) synthase, is repressed by high aeration and is subject to feedback inhibition by iron protoporphyrin (hemin) (1, 12, 13). There is no information about the regulation of heme synthesis by nonphotosynthetic bacteria. Another

unknown area is the integration of synthesis of the heme prosthetic group with that of the protein moiety of the cytochromes. In animal tissues, there is evidence that the prosthetic group has an important role in regulating the formation of the protein components of the hemoproteins, hemoglobin and tryptophan pyrrolase (5-7, 18, 20).

In the present work cytochrome synthesis and its regulation have been studied in *Spirillum itersonii*. This organism grows aerobically on a chemically defined medium with glutamate as sole carbon source, and it also grows anaerobically with nitrate as terminal electron acceptor (33). It was chosen for this work since the cells were found to be rich in *b*- and *c*-type cytochromes when grown under low aeration. Also, ALA synthase, a likely locus in the regulation of heme synthesis, was readily detectable in extracts of the organism; previous attempts to detect this enzyme in nonphotosynthetic bacteria have been unsuccessful (1).

MATERIALS AND METHODS

Culture of S. itersonii. This organism was obtained from R. Martinez. Stock cultures were maintained on agar slants of GGS medium (see below) with 0.1% Difco yeast extract; these were transferred every 2

weeks and were incubated for 24 hr at 30 C. They were stored at 5 C.

Growth of organism. The standard medium (GGG medium) contained per liter: $(\text{NH}_4)_2\text{SO}_4$, 2 g; $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 7 g; KH_2PO_4 , 3 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 200 mg; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 53 mg; MnSO_4 , 7.5 mg; iron citrate, 0.02 mM; sodium L-glutamate, 3.75 g; sodium succinate $\cdot 6\text{H}_2\text{O}$, 1.35 g; glycine, 0.375 g. In some experiments, noted in the text, iron citrate was omitted. KNO_3 (20 mM) was added as stated in the text. Organisms were grown under high or low aeration. For high aeration, 400-ml volumes of medium in 1-liter Erlenmeyer flasks were shaken on a gyratory shaker at 230 rev/min. For low aeration, flasks were filled to 80% of their normal capacity and were shaken on a gyratory shaker at 200 rev/min. Cultures were inoculated with 0.5% of a culture grown for 18 hr under low aeration in GGG medium + 0.1% yeast extract. Growth was measured by the optical density at 540 $m\mu$; a value of 1.0 (1 cm) corresponded to 0.26 mg of bacterial protein/ml. All cultures were grown at 30 C.

Adaptation system. In some experiments, synthesis of cytochromes and of ALA synthase was studied in the following conditions. Cells were grown in GGG medium under high aeration and were harvested at the end of the logarithmic phase of growth. They were resuspended in fresh GGG medium + 20 mM KNO_3 to a concentration of 0.1 to 0.2 mg of protein/ml and incubated under low aeration. This experimental system permitted rapid and reproducible increases in cytochromes and enzyme (see Fig. 5a).

Preparation of cell-free extracts. Harvested organisms were washed once in one-half of the original culture volume with 40 mM potassium phosphate buffer, pH 7.5, and were resuspended in the same buffer, or in 40 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.5, when assays for ALA synthase were to be done. The cell concentration was from 2 to 5 mg of protein/ml. Disruption was with a French pressure cell (American Instrument Co., Silver Spring, Md.) or by sonic treatment for 60 to 90 sec with a Branson Sonifier (Branson Instruments, Inc., Stamford, Conn.). Extracts were centrifuged for 10 min at $3,000 \times g$ and the supernatant fluid was used routinely for determination of cytochrome and for ALA synthase. In some experiments, the crude extract was fractionated into soluble and particulate components by centrifugation for 90 min at $100,000 \times g$. The particles were resuspended in 40 mM Tris or phosphate buffer, pH 7.5.

Measurement of respiratory activity. This was measured with a Rank oxygen electrode (Rank and Co., Bottisham, Cambs., England), which is similar in principle to the Clark electrode. The reaction vessel contained organisms (about 1 mg of protein) in 2 ml of 50 mM potassium phosphate buffer with 40 μmoles of substrate; measurements were made at 25 C. The cells used for these determinations were harvested and washed as described above.

Estimation of cytochromes. These were determined by measurement of the α band of the reduced minus oxidized spectrum in crude cell-free extracts prepared as above; potassium ferricyanide and sodium dithionite were used as oxidant and reductant, respectively.

The concentration of cytochromes *b* and *c* was calculated by the method of Estabrook and Holowinsky (4), using the extinction values for the analogous pigments in rat liver mitochondria.

The reduced spectrum of the whole cells was determined with a suspension of starch and flour in the reference cuvette (8). Carbon monoxide-binding pigments were demonstrated in cell-free extracts as described previously (24).

The protoheme prosthetic group of cytochrome *b* was extracted and estimated by a procedure similar to that described by Jacobs and Wolin (9). Cell-free extract was precipitated with 5 volumes of acetone at 0 C, and the precipitate was washed with the same volume of acetone. The precipitate (20 to 30 mg of protein) was extracted twice with 10 ml of acetone containing 1% 2 N HCl. The acid extract, containing protoheme, was dried in vacuo at 25 C, and the residue was extracted twice with 10 ml of ether. The ether extract was washed with an equal volume of water and dried. The residue was dissolved in 0.01 N NaOH, and the protoheme was determined as the pyridine hemochromogen (22). Cytochrome *c* heme, which remained in the residue after the extraction with acid acetone, was also determined as the pyridine hemochromogen (23).

All spectroscopic measurements were made with the Cary model 14R spectrophotometer using the 0.1 optical density full scale slide wire.

Tetrapyrroles and precursors. Free and total heme and porphyrins were estimated as described previously (16). δ -Aminolevulinic acid and porphobilinogen were determined by the method of Mauzerall and Granick (21).

ALA synthase activity. This was assayed in cell-free extracts prepared with a French press (1). Optimal conditions (pH, temperature, concentration of substrates and cofactors) for assay of the synthase in *Spirillum* extracts were found to be similar to those established with extracts of *R. spheroides* (1). Under the standard conditions of assay, ALA formation was proportional to the concentration of protein over the range 0.2 to 1 mg/ml of reaction mixture. Assays were done within 2 hr after the preparation of the extract because of the instability of the enzyme; 50% of the activity was lost after 24 hr of storage at 0 C. One unit of enzyme is the amount which catalyzes the synthesis of 1 μmole of ALA in 1 hr at 37 C.

Protein. The Lowry method was used with bovine serum albumin as standard (19). Whole cells were digested for 7 min at 100 C in 0.1 N NaOH for determination of their protein content.

RESULTS

Cytochrome system of *S. itersonii*. The reduced spectrum of cells grown with high aeration showed the α bands of *b*- and *c*-type cytochromes at 558 and 552 $m\mu$, with the corresponding β bands at 528 and 522 $m\mu$ (Fig. 1a). No evidence was found for pigments of the *a* type, which would normally absorb in the region of 580 to 650 $m\mu$. The possibility of a terminal oxidase of the *o* type was investigated with carbon monoxide

(2, 26). A carbon monoxide-binding pigment was demonstrated in the particulate fraction by use of succinate as the reductant; it exhibited a slight trough at 445 $m\mu$ with a peak at 421 $m\mu$ (Fig. 1b). This pigment did not react readily with carbon monoxide under conditions found to be effective with other organisms (24, 27), and no attempt was made to determine it quantitatively. The peak at 558 $m\mu$ in the reduced spectrum is presumably a composite of cytochrome *b* and *o*.

Effect of aeration on the cytochrome system of S. itersonii. Cells grown with high aeration formed about the same quantities of *b*- and *c*-type cytochrome, and their concentration remained constant throughout the growth curve (Fig. 2a and

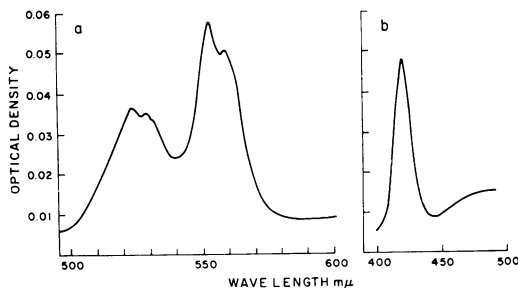


FIG. 1. Absorption spectrum of cytochromes of *Spirillum itersonii*. (a) Dithionite-reduced spectrum of whole cells grown under high aeration on GGS medium; the suspension of cells contained 6.5 mg of protein/ml. (b) Carbon monoxide minus reduced spectrum of particles (4 mg of protein/ml) prepared from cells grown under low aeration on GGS medium; succinate (10 mM) was the reductant.

2b). Under low aeration, the total cytochrome content was considerably higher, and this increase was represented largely by cytochrome *c* (Fig. 2a and 2b; Table 1). The highest concentration of the pigments was found in cells grown under low aeration with nitrate; cytochrome *c* predominated under these conditions (Table 1).

The rise in cytochrome synthesis was evident from the earliest stages after transition of the organisms from conditions of high to low aeration, though the cultures grew initially at the same rate (Fig. 2 and 2b). Enhanced synthesis of cytochrome is not, therefore, a consequence of a slower growth rate.

The increase in cytochrome *c* synthesis relative to cytochrome *b* synthesis under low aeration is shown by comparison of the reduced minus oxidized spectrum of extracts from cells grown under the two conditions (Fig. 3). Under low aeration there is about twice as much cytochrome *c* as *b*.

Measurement of cytochrome *b* by the reduced minus oxidized difference spectra of extracts containing excess cytochrome *c* could give falsely high values, due to the proximity of the α peaks of the two pigments. To check the validity of the values for cytochrome *b* calculated from the difference spectrum, representative extracts were treated with acid-acetone to remove the protoheme prosthetic group. Estimation of protoheme and of cytochrome *c* heme (in the residue) as the pyridine hemochromogens gave values which were in reasonable accord with the concentration of cytochrome calculated from the difference spectrum. For instance, the values for protoheme and for

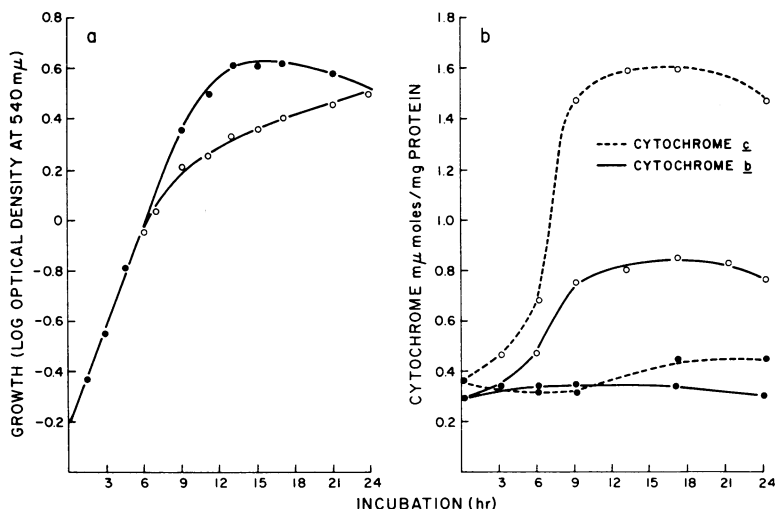


FIG. 2. Growth rate (a) and cytochrome synthesis (b) in cultures of *Spirillum itersonii* growing on GGS medium under high aeration (●) and low aeration (○).

TABLE 1. Effect of growth conditions on cytochrome content and δ -aminolevulinic acid (ALA) synthase activity^a

Medium	Aeration conditions	Cytochromes (μ moles/mg of protein)			ALA synthase (units/mg of protein)
		<i>b</i>	<i>c</i>	Total	
GGS	High	0.32	0.28	0.60	43
GGS	Low	0.63	1.18	1.81	110
GGS + 20 mM KNO ₃	Low	0.85	1.93	2.78	224
GGS without iron	Low	0.17	0.12	0.29	106

^a Determinations were made with organisms harvested at the end of the exponential phase of growth.

cytochrome *c* heme were 76 and 85%, respectively, of those calculated from the difference spectrum of an extract, which gave values of 0.52 and 0.84 μ mole/ml of cytochrome *b* and *c*, respectively. It was concluded that both cytochromes increase under low aeration.

Preferential synthesis of cytochrome *c* did not occur under low aeration when iron was limiting (Table 1). This suggests that the availability of the prosthetic group is a dominant factor in determining the synthesis of excess cytochrome *c* in response to low aeration.

Physiological role of the cytochromes in S. itersonii. The respiratory rate with succinate or glutamate as substrate did not differ significantly with cells grown with high or low aeration. The rate observed with these substrates was about 13 μ moles of O₂ per hr per mg of protein. The rise in cytochrome content in response to low aeration was not therefore reflected by increased respiratory activity as occurs in *Haemophilus parainfluenzae* (30).

Nevertheless, the excess cytochrome formed by *S. itersonii* under low aeration was fully functional in electron transport with succinate as hydrogen donor. Spectrophotometric examination of extracts from such cells showed that succinate reduced cytochrome *b* and *c*, and the addition of dithionite caused no further reduction. The succinate-reduced pigments were reoxidized upon aeration.

The localization of the cytochromes in extracts prepared by lysozyme and by sonic treatment was determined by centrifugation at 100,000 $\times g$ for 90 min. In preparations from cells grown with low aeration, 85% of cytochrome *c* and 90% of cytochrome *b* were recovered in the particulate fraction. The cytochrome remaining in the supernatant fraction after centrifugation for 90 min at 105,000 $\times g$ probably represents material associated with small particles. A similar distribution of both cytochromes was found in extracts from cells grown with high aeration. The enhanced synthesis of cytochrome *c* under low aeration does

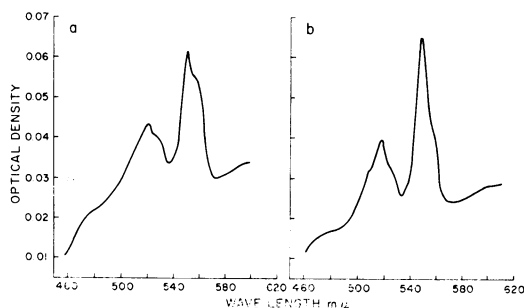


FIG. 3. Difference spectra of extracts from *Spirillum itersonii* grown on GGS medium under high aeration (a) and under low aeration (b). Extracts were prepared by sonic treatment and contained 4.1 and 1.7 mg of protein/ml respectively in (a) and (b). Difference spectra measurements were made as described in Materials and Methods.

not, therefore, represent synthesis of an unbound form of the hemoprotein.

Synthesis of prosthetic group and protein moieties of the cytochromes. Attempts were made to determine whether formation of the protein component of the cytochromes occurred when synthesis of the heme prosthetic group was limited by iron deficiency. Organisms were first incubated with low aeration in GGS medium without added iron. Iron and heme were then added, together with chloramphenicol, and the incubation was continued; samples were withdrawn at intervals for determination of cytochromes. There was no evidence for the accumulation of apo-cytochrome during the initial incubation, since formation of both cytochromes upon addition of iron and heme was completely prevented by chloramphenicol (Fig. 4). Similar results were obtained when DL-4-methyltryptophan (0.2 mM) was used to inhibit protein synthesis.

The possibility that free heme or precursors might accumulate when synthesis of the protein moiety was prevented was also examined in these experiments. However, neither heme, porphobilinogen, nor porphyrins were detected in the culture fluids of either the control experiments or those

with inhibitor present. Traces of ALA were detected (1 to 5 μ moles/ml), but the amount was the same in both control and inhibited cultures. Determination of the total heme content of cells incubated with and without the inhibitors gave values which corresponded to the cytochrome content; i.e., there was no evidence for the accumulation of free heme when formation of the protein component of the cytochromes was blocked.

Effect of growth conditions on the activity of ALA synthase. ALA synthase was readily detectable in extracts of *S. itersonii*, as the enzyme was located exclusively in the soluble fraction of cell extracts. The activity was highest in extracts grown under low aeration, particularly with nitrate present (Table 1).

Derepression of enzyme synthesis occurred upon transfer of cells from high to low aeration, and the rise in enzyme activity paralleled the increase in cytochrome content (Fig. 5a). The increase in enzyme in response to low aeration probably represented de novo synthesis, since it was prevented by chloramphenicol (Fig. 5a) or by deprivation of tryptophan in the case of a mutant strain requiring this metabolite. In the converse type of experiment, repression of enzyme synthesis and of cytochrome formation occurred when cells were trans-

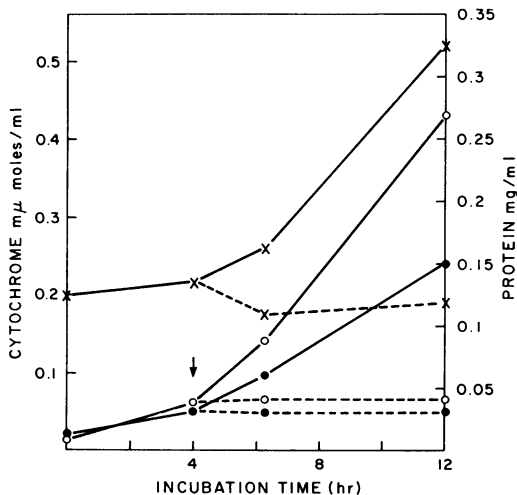


FIG. 4. Effect of chloramphenicol on cytochrome synthesis. Cells were grown in GGS medium without iron under high aeration. After harvesting, they were resuspended in fresh medium without iron and incubated under low aeration for 4 hr. Iron citrate (20 μ M) and hemin (5 μ M) were then added together with chloramphenicol (0.2 mM), and the incubation was continued (dashed lines). Control flasks without chloramphenicol are shown by solid lines. Protein (X), cytochrome b (●), and cytochrome c (O) were determined on samples withdrawn at intervals.

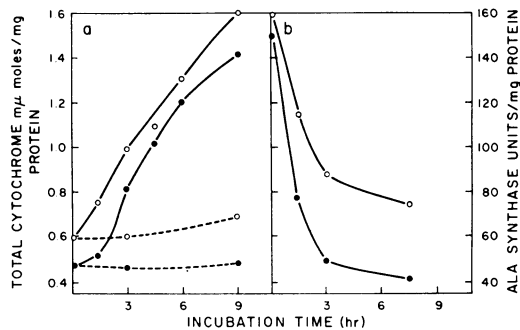


FIG. 5. Derepression and repression of cytochromes and δ -aminolevulinic acid synthase. In (a), organisms were grown with high aeration and then transferred to fresh GGS medium with 20 mM KNO_3 and incubated under low aeration. Chloramphenicol (0.1 mM) was added to some flasks (dashed lines). In (b), cells were previously grown with low aeration in GGS medium with 20 mM KNO_3 and were then transferred to fresh medium and incubated with high aeration. Cytochrome content (O) and ALA synthase activity (●) were measured on samples removed at intervals. In both experiments, the initial cell concentration was 0.1 mg of protein/ml.

ferred from low to high conditions of aeration (Fig. 5b). Experiments with mixed extracts from cells grown under low and high aeration gave no evidence for the formation of an inhibitor of the enzyme under the latter conditions.

There is indirect evidence that iron is concerned in the action of ALA synthase in red blood cells and in plants (15). In *S. itersonii*, however, omission of iron from GGS medium did not affect enzyme activity, although cytochrome synthesis was markedly reduced (Table 1).

Inhibition by hemin of activity of ALA synthase. The effect of hemin on the synthase activity in the soluble fraction of extracts from *S. itersonii* was examined (Table 2). Considerable inhibition occurred at concentrations of 10 μ M or higher. The enzyme, however, was less sensitive to hemin than that from *R. spheroides*; when tested under the same conditions, 1 μ M hemin inhibited the activity from *R. spheroides* by 50%, whereas that from *S. itersonii* was not affected.

Growth in the presence of hemin (10 μ M) did not repress formation of ALA synthase by *S. itersonii*; extracts from organisms grown under high or low aeration had the same enzyme activity as that in controls without hemin.

DISCUSSION

In animal tissues there is evidence that the heme prosthetic group influences the synthesis of the protein component of some hemoproteins. Hemin stimulates globulin synthesis in reticulocytes and in cell-free systems (5, 6, 7). In chick blastoderms,

TABLE 2. Inhibition of δ -aminolevulinic acid (ALA) synthase by hemin^a

Hemin (μ M)	ALA formed (μ moles/ml)	Inhibition (%)
0	70	—
1	72	0
10	40	43
40	26	63
100	18	74

^a The enzyme activity was assayed in the soluble fraction of extracts prepared from cells which had been grown with low aeration in GGS medium with 20 mM KNO₃. The protein concentration was 0.6 mg/ml, and the incubation was for 60 min at 37 C.

globulin synthesis is increased by ALA, which bypasses ALA synthase, the rate-limiting step of heme synthesis in this tissue (18). The formation of tryptophan pyrrolase in liver is apparently determined by the activity of ALA synthase, which limits the rate of synthesis of the heme prosthetic group (20). Several possible mechanisms have been advanced to account for the role of heme in stimulating hemoprotein synthesis, such as promotion of the release of newly formed protein from the ribosomes (18).

The observations with *S. itersonii* are consistent with a central role for the iron porphyrin prosthetic group in the regulation of cytochrome synthesis, though they do not provide direct evidence for this (Fig. 6). Synthesis of the heme group and of the protein moieties are closely linked, since there was no evidence for the independent synthesis of either component. Accumulation of free heme or precursors did not occur when protein synthesis was inhibited, nor was there any indication of a protein precursor when heme synthesis was limited by iron deficiency. The behav-

our of the ALA synthase accords with its possible role in the regulation of heme synthesis. The enzyme was repressed by oxygen, and it was inhibited by hemin, a possible feedback regulator.

Repression by oxygen is a feature common to chlorophyll and to cytochrome synthesis in some bacteria. Each process involves the formation of a tetrapyrrole, and it is possible that oxygen influences cytochrome formation by acting on the synthesis of the heme prosthetic group. Repression of ALA synthase by high aeration supports this possibility. Oxygen might also act directly on heme synthesis by regulating the amount of succinyl-coenzyme A (CoA) available for the synthase (Fig. 6). In the tricarboxylic cycle, oxidation of succinate becomes rate-limiting under low oxygen pressure, and succinate may accumulate (10); the accumulation of succinyl-CoA might then occur by the action of succinyl-CoA synthetase. The participation of this enzyme has been suggested in the control of heme synthesis by liver mitochondria (11).

Synthesis of the *b*- and *c*-type cytochromes by *S. itersonii* does not appear to be under strict coordinate control, since there is a disproportionate increase in cytochrome *c* in response to low aeration. There are other instances of uncoordinated synthesis of *c*-type cytochromes. *Haemophilus parainfluenzae* forms cytochrome *c* preferentially in the presence of nitrate (31); in yeast and *Neurospora*, large amounts of cytochrome *c* are formed when synthesis of the other cytochromes is prevented by mutation or by chloramphenicol (3, 25, 28).

All the cytochrome formed by *S. itersonii* in response to low aeration is apparently integrated with the electron transport chain, since complete reduction was observed with succinate. The high concentration of hemoproteins under these conditions did not, however, give an obvious ad-

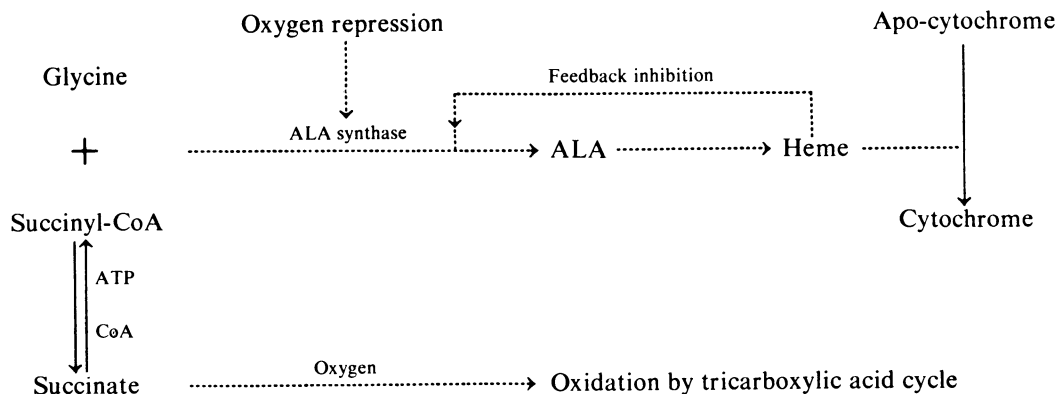


FIG. 6. Possible mechanisms involved in the regulation of cytochrome synthesis.

vantage to the organism, since the respiratory rate of the cells was not enhanced. In *H. parainfluenzae*, which makes a variety of terminal oxidases (cytochromes a_1 , a_2 , and o), respiration increases in response to low aeration (30, 32).

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