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Rapid Reactions of Cytochromes of *Hemophilus parainfluenzae* on Addition of Substrates or Oxygen*

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SUMMARY

Addition of oxidizable substrates to aerobic suspensions of *Hemophilus parainfluenzae* containing no endogenous substrate results in rapid reduction of *b* and *c* type cytochromes to the aerobic steady state. The extent of reduction depends upon the relative activities of the dehydrogenases and oxidases involved. Addition of oxygen to anaerobic cells containing substrate rapidly oxidizes the cytochromes to the aerobic steady state. The rapid kinetics observed on mixing with oxygen show no evidence that the initial rate of oxidation of part of the *b* or *c* type cytochromes is higher, even within bacteria with a large stoichiometric imbalance of these cytochrome types. Cytochrome a_2 reacts rapidly enough with oxygen to be considered a terminal oxidase, and indirect evidence indicates that cytochrome *o* reacts as rapidly with oxygen as does the mammalian oxidase (pseudo-first order rate constant around 80 sec^{-1} at room temperature).

Hemophilus parainfluenzae synthesize an unusually complex respiratory chain system containing as many as six cytochromes and five flavoprotein dehydrogenases bound to insoluble membranes within the cells (1, 2). Three of the cytochromes will bind carbon monoxide in the ferrous state to form compounds with absorption spectra similar to those which have been characterized as CO compounds of terminal oxidases in other species of bacteria: cytochromes *o*, a_1, and a_2. The bacteria can also reduce nitrate. The total and relative contents of the different cytochromes and flavoproteins are observed to vary widely with changes in growth conditions and time of growth (2, 3). However, the respiration can be very rapid even with an apparent deficiency of one or more cytochromes (4). The dehydrogenase was found to be the rate-limiting step in the respiratory chain system under all conditions tested (2). These characteristics of the system of *H. parainfluenzae* led us to study the oxidation-reduction reactions of the cytochromes with an instrument for rapidly mixing the bacteria with substrate or oxygen to initiate electron transport and recording the ensuing changes in the oxidation state of the cytochromes (5). For these studies bacteria were grown to contain widely different complements of cytochromes.

The reaction with oxygen was found to be rapid, comparable in rate to that of the mammalian oxidase. In addition, some of the dehydrogenases show remarkably high reaction rates. As in a number of other bacteria (6), cytochrome a_2 was confirmed as a terminal oxidase in *H. parainfluenzae*. Surprisingly, cytochrome a_1 does not react rapidly enough on addition of oxygen to establish it as a terminal oxidase; its function in these bacteria remains unknown. Cytochromes of *c* and *b* type act as intermediary electron carriers, and the extent of reduction of these in the aerobic steady state (aerobic in the presence of substrate) depends upon the relative activities of the dehydrogenase and the oxidase (oxidases). On the time scale used the cytochromes were seen to react rapidly and to show no inhomogeneity in the kinetics of change into the steady state.

METHODS

Growth of Bacteria—*H. parainfluenzae*, strain Boss, were grown under three different sets of conditions, as previously described (2); these are listed in Table I, along with the corresponding content of cytochromes. The bacteria were grown at 37°, harvested after 12 hours of growth, and washed with and suspended in 50 mM phosphate buffer, pH 7.6.

Cytochrome Content of Intact Bacteria—Cytochrome content was estimated from the anaerobic (with formate) minus aerobic (no substrate) difference spectrum obtained in the Cary model 14 recording spectrophotometer with the light-scattering attachment, as in previous experiments (4).

Kinetics of Reduction and Oxidation of Cytochromes—The kinetics were followed by observing the rapid changes in absorbance at wave length pairs corresponding to the different cytochromes (given in Reference 4) in the double beam spectropho-

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tometer equipped with the rapid flow device described by Chance and Legallais (5). Electron transport was initiated by the addition of substrate to a washed aerobic suspension of cells which had no endogenous substrate, or of oxygen to an anaerobic suspension of bacteria containing substrate. During the flow 1 ml of substrate or oxygen is added to 80 ml of bacterial suspension; thus a given batch can be used with several additions of oxygen by allowing the cells to become anaerobic between additions. The rate of flow in these experiments was 320 cm per sec and the distance from mixing to the point of spectrophotometric observation was 1.1 cm. The observations were made 3.4 msec after mixing. The traces recorded (Figs. 1 to 5) show the changes in absorbance during the flow and then after the flow stops until the aerobic steady state is reached. In some experiments (Fig. 2), the observation was continued until the bacteria exhausted the oxygen in solution and returned to the anaerobic state.

Pseudo-First Order Rate Constants—Rate constants for the oxidation of the intermediary cytochromes of *b* and *c* type on addition of oxygen were calculated from the extent of oxidation during the flow according to the method described by Chance and Williams (7) (see "Results"). This method cannot be used with cytochrome a_2, since it is essentially completely oxidized during the flow.

RESULTS

Washed cells of *H. parainfluenzae* contain little or no endogenous substrate (4). Thus, in an aerobic suspension all of the cytochromes and flavoprotein dehydrogenases are presumably in the oxidized state. The *top tracing* of Fig. 1 records the change in absorbance at 423 minus 402 mμ (corresponding to the peak of the γ-absorption band of cytochrome c_1 in the reduced minus oxidized difference spectrum (1)) on addition of formate to a concentration of 8.3 mM to a washed aerobic suspension of the bacteria in the flow apparatus. These bacteria were grown anaerobically with nitrate (type III) and show strong absorption peaks for both *b* and *c* type cytochromes in the difference spectra. The trace indicates a rapid small reduction of the cytochrome c_1 during the flow, and then a further reduction after the flow stops to the aerobic steady state level. After the bacteria became anaerobic (not shown), oxygen was added in the flow apparatus to a final concentration of 15 μM, giving the *bottom trace*. In the presence of these concentrations of oxygen and formate the cytochrome *c* was about 40% reduced. The extent of reduction of cytochromes *b* or *c* in the aerobic steady state with all three types of the bacteria (see Table I) was found to be dependent upon the concentration of formate when it was added as substrate; the reduction was greater when saturating concentrations of formate were added than with saturating concentrations of succinate as substrate (Table II). Thus in the presence of 15 μM O_2 the steady state level of reduced cytochromes was dependent upon the level of dehydrogenase present and the concentration of substrate. The amounts of dehydrogenase present depend upon the growth conditions, but there is always greater formate dehydrogenase than succinate dehydrogenase activity (2).

The pseudo-first order rate constants for the oxidation of *b* or *c* type cytochromes on addition of oxygen to anaerobic cells containing succinate were calculated in a manner similar to that used with the system in mammalian mitochondria (7). The formula is:

$$k = \frac{2.3}{t} \log \frac{p_0}{p_0 - p}$$

where *t* is the time after mixing when the oxidation of the cytochromes has proceeded to *p* per cent of its final level of oxidation, p_0. In these experiments *t* was 3.4 msec. Some of these values are listed in Table III.

The tracings of Fig. 1 show no inhomogeneity in the kinetics of oxidation and reduction of the cytochrome c_1 on the time scale used; that is, none of the cytochrome reacts at a rate lower than the rest in the rapid reaction under observation. Fig. 2 pictures the changes on addition of O_2 to an anaerobic suspension of the same kind of bacteria in the presence of 12.5 mM formate, recorded at the γ-absorption peaks of both cytochrome c_1 (*bottom trace*) and *b* (*top trace*) on a slower time scale than that of Fig. 1. The cytochromes are oxidized to the aerobic steady state, and then reduced again as the oxygen concentration falls to zero as a result of the respiration of the bacteria. Again there is no

TABLE I

Growth conditions used for different types of bacteria

The cytochrome content of each of the different kinds of bacteria was assessed from measurements of anaerobic minus aerobic difference spectra made in the Cary spectrophotometer (see "Methods").

Type and growth conditions	Cytochrome content
I. Nitrate in medium, shaken gently during growth, no aeration	High a_2 and *c*; average a_1 and *o*; low *b*
II. No nitrate in medium, aerated strongly during growth	High a_1, a_2, and *b*; little or no *c*
III. Grown anaerobically with nitrate in the medium	Very high *c*; reasonably high *b*; no a_2

TABLE II

Reduction of b or c type cytochromes in aerobic steady state in presence of varying concentrations of formate and succinate

Substrates were added to washed aerobic suspensions of bacteria to give the concentrations indicated and the increase in absorbance in the aerobic steady state was measured at either 430 minus 410 mμ or at 423 minus 402 mμ.

A. Type I bacteria; substrate	Increase in absorbance at 430 minus 410 mμ in aerobic steady state
20 μM formate	0.0044
12.5 mM formate	0.0154
8 mM succinate	0.0038
B. Type II bacteria; substrate	Increase in absorbance at 430 minus 410 mμ in aerobic steady state
50 μM formate	0.00324
12.5 mM formate	0.0081
8 mM succinate	0.0054
C. Type III bacteria; substrate	Increase in absorbance at 423 minus 402 mμ in aerobic steady state
0.05 mM formate	0.0027
12.5 mM formate	0.00486

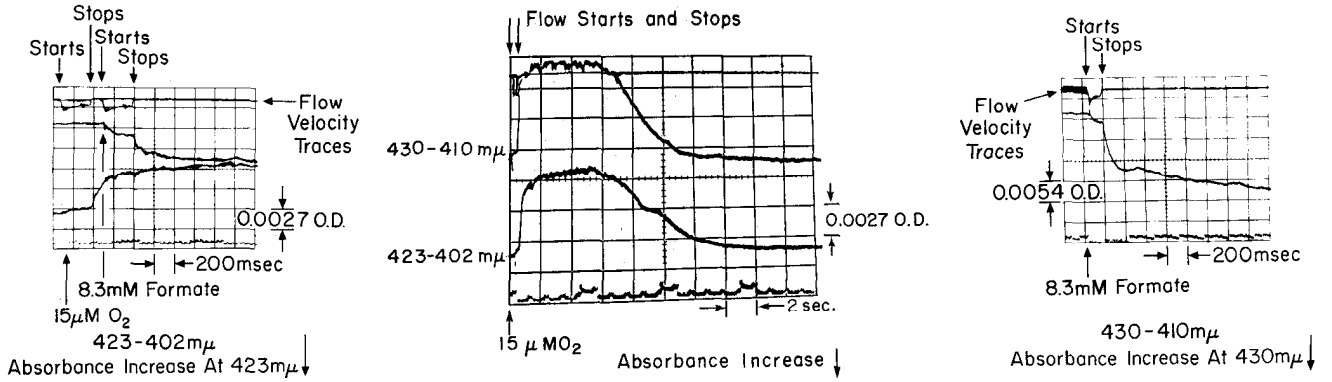

FIG. 1. (*left*) Changes in absorbance at 423 minus 402 mμ on addition of substrate and O_2 to *H. parainfluenzae*, type III. *Top trace* records change on addition of 1 ml of 1 M formate to 80 ml of washed aerobic bacteria (0.10 mg, dry weight, per ml) in 50 mM phosphate buffer, pH 7.6. This shows the change from the aerobic state in the absence of substrate during and after the flow to the aerobic steady state in the presence of 12.5 mM formate. After the bacteria became anaerobic (not shown), 1 ml of buffer saturated with O_2 was added and the *bottom trace* was recorded, showing the change from the anaerobic state containing substrate during and after the flow to the aerobic steady state. A downward deflection of the trace represents an increase of absorbance at 423 mμ. The *first signal* of the flow velocity trace refers to the *bottom trace* and the *second* to the *top trace*.

FIG. 2. (*middle*) Same bacterial suspension as in Fig. 1, containing 12.5 mM formate. *Traces* record effect of addition of 1 ml of buffer saturated with O_2 to the anaerobic bacteria at 423 minus 402 mμ and at 430 minus 410 mμ as the cytochromes are oxidized and then reduced again as the O_2 in solution is exhausted. The time scale is slower than that of Fig. 1.

FIG. 3. (*right*) Change in absorbance at 430 minus 410 mμ on addition of 1 ml of 1 M formate to 80 ml of washed aerobic suspension of type I bacteria containing 0.14 mg, dry weight, per ml. The cytochrome is reduced during and after the flow to the aerobic steady state.

evidence of inhomogeneity of rates of oxidation or reduction. Similarly, there was no evidence of kinetic inhomogeneity observed on addition of oxygen when the changes in absorbance were measured at the α-absorption peaks of the cytochromes. In fact, there was no evidence of biphasic reactions seen on addition of oxygen to anaerobic suspensions of any of the bacteria on the time scales of these experiments. The only evidence of a biphasic reaction was seen when formate was added to a washed aerobic suspension of bacteria which had been grown anaerobically with nitrate. This is illustrated in Fig. 3, where a rapid reduction of about 15% of the cytochrome *b* is recorded during the flow and 56% after the flow; then this is followed by a slower reduction of 29% of the cytochrome.

Fig. 4 records experiments with bacteria grown with gentle shaking with nitrate in the medium (type I); these show high contents of cytochromes a_2 and c_1 in the anaerobic minus aerobic difference spectra, moderate amounts of cytochromes a_1 and *o*, and only a low content of *b* type cytochromes. Recordings were made at two wave length pairs, 423 minus 402 mμ and 430 minus 410 mμ, on addition of oxygen to anaerobic bacteria containing 600 μM formate without (*A*) and with (*B*) the prior addition of 14 μM CO. The presence of CO decreased the extent of oxidation during the flow and the rate of decrease after the flow, the inhibitory effect being slightly more pronounced at 430 minus 410 mμ than at 423 minus 402 mμ.

Fig. 5 pictures the changes in absorbance at several representative wave length pairs at the α-absorption peaks of the cytochromes on addition of oxygen to more concentrated suspensions of bacteria grown with aeration without nitrate in the medium (type II). Anaerobic minus aerobic difference spectra of these bacteria showed high contents of cytochromes *b*, a_1, and a_2, but little cytochrome c_1. The data are summarized in Table IV and indicate the time sequence of reaction of the different pigments with oxygen to be cytochrome $a_2 > b > a_1$, as delineated by

TABLE III

Rate constants for oxidation of c or b type cytochromes on addition of oxygen to anaerobic suspensions of bacteria

Oxygen (15 μM) was added in the flow apparatus to anaerobic suspensions of the three types of bacteria containing 8 mM succinate. The pseudo-first order rate constants were calculated from the percentage oxidation of the *c* or *b* type cytochromes during the flow, as outlined by Chance and Williams (7).

Type bacteria	Wave length of observation	Pseudo-first order rate constant
		sec^{-1}
I	430 minus 410 mμ	66
II	430 minus 410 mμ	112
II	423 minus 402 mμ	142
III	430 minus 410 mμ	78
III	423 minus 402 mμ	88

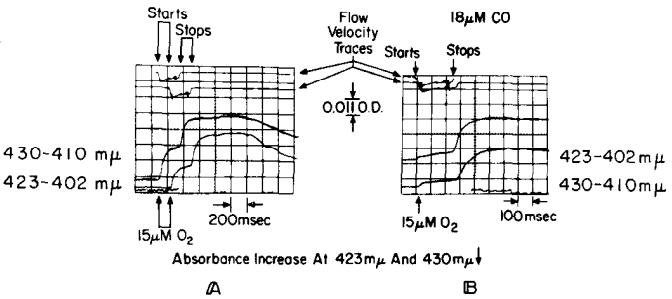

FIG. 4. Change in absorbance on addition of O_2 to an anaerobic suspension of type I bacteria (0.14 mg, dry weight, per ml) containing 600 μM formate without (*A*) and with (*B*) prior addition of CO. The final concentrations of O_2 and CO were 15 and 14 μM, respectively. The change from the anaerobic state during and after the flow to the aerobic steady state is pictured by recordings at 423 minus 402 mμ (*top trace* in *B*, *bottom trace* in *A*) and at 430 minus 410 mμ (*top trace* in *A*, *bottom trace* in *B*). The bacteria became anaerobic between the recordings.

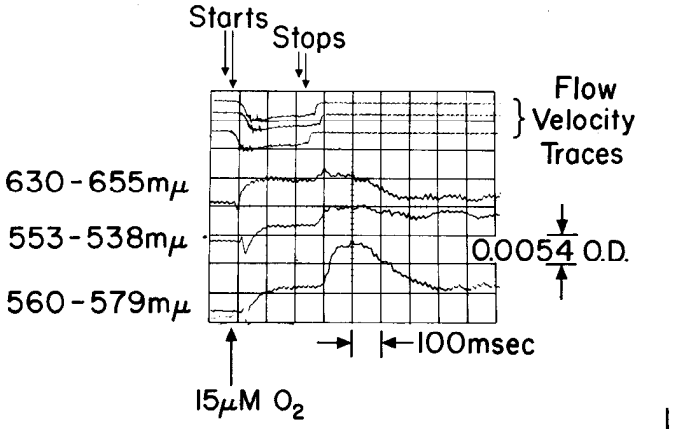

FIG. 5. Absorbance changes on addition of O_2 to an anaerobic suspension of type II bacteria (6.31 mg, dry weight, per ml) containing 8.3 mM succinate. Recordings were made successively at 630 minus 655 mμ, 533 minus 538 mμ, and 560 minus 575 mμ, allowing the suspension to become anaerobic between successive runs. The flow velocity traces refer to the successive runs.

TABLE IV

Changes in absorbance on addition of oxygen to anaerobic bacteria in flow apparatus

The bacteria were grown with aeration without nitrate in the medium; they contained high levels of cytochromes *b*, *o*, a_1, and a_2, but relatively low content of *c* type cytochrome. Oxygen (15 μM) was added to anaerobic suspension of the bacteria containing succinate in the flow apparatus and the changes in absorbance at the different wave length pairs were observed during and after the flow.

Wave length pair of observation	Predominant cytochrome observed	Amount oxidized during flow
		%
559-575	*b* type	33
553-538	*c* type	42
598-575	a_1	Nearly 0
630-655	a_2	100

Chance and Williams (7). Relative rates of reaction are estimated from a comparison of the extent of reaction during the interval of flow with that after the flow stops (7). The data show that cytochrome a_2 reacts very rapidly with oxygen, but that cytochrome a_1 reacts relatively slowly (not shown in Fig. 5). The latter observation was confirmed when similar recordings were made at the γ-absorption peak of cytochrome a_1; it can hardly be considered a rapidly reacting oxidase. The kinetics of the reaction of ferrocytochrome *o* cannot be visualized independently, since both the γ- and α-absorption peaks overlap those of cytochromes *b* and *c*. The changes in absorbance on addition of oxygen up to the aerobic steady state at 630 minus 655, 553 minus 538, and 559 minus 575 mμ were 0.049, 0.0065, and 0.013, or in the ratio 1:1.3:2.7. The corresponding values at these wave length pairs in the anaerobic minus aerobic difference spectrum of type II bacteria with the same substrate (formate) were in the ratio 1:1.9:2.1. In the difference spectrum there is a large absorption peak at 559 mμ and no separate peak at 553 mμ.

DISCUSSION

These experiments show *H. parainfluenzae* to be good material for studies of the rapid reactions of the respiratory chain of intact bacteria with added substrates, since they can be washed free of endogenous substrates; this is difficult to do with most other bacterial species. Also, the relative amounts of the different cytochromes and flavoprotein dehydrogenases can be varied easily by changing growth conditions. However, so many pigments are involved (six cytochromes, five flavoproteins) that quite extensive experimentation may be required to unravel all of the different reaction sequences.

The pseudo-first order rate constants for the oxidation of the intermediary *b* or *c* type cytochromes of *H. parainfluenzae* on addition of oxygen to an anaerobic suspension containing succinate (Table III) compare favorably with those reported by Chance and Williams (7) for the mammalian system. Thus, one or more active oxidases are present. Cytochrome a_2 is shown to react very rapidly with oxygen and thus qualifies as a terminal oxidase, as suggested by its ability to bind CO. Cytochrome a_1 can also combine with CO. However, our experiments do not furnish evidence that it reacts rapidly enough with oxygen to function effectively as a terminal oxidase (Table IV). The combination of a cytochrome with CO cannot be considered definitive evidence that it is a terminal oxidase. A cytochrome a_1 can be the sole terminal oxidase or part of a combination of oxidases in other bacterial species (6). Cytochromes of the a_2 type can also function as oxidases in other bacteria (6), but have not been seen as the sole oxidase in any (8). Since cytochrome a_2 is missing from type III bacteria and cytochrome a_1 is not a rapidly reacting oxidase, the sole oxidase here must be cytochrome *o*, and the data show it to be quite active.

One of the oxidases seems to have a rather high sensitivity to inhibition by CO, as shown by the data of Fig. 4, obtained with type I bacteria which have a high content of cytochrome a_2 and average cytochrome *o*. When CO is present in about equal concentration to that of oxygen (both about 15 μM), the extent of oxidation of the intermediary *b* and *c* type cytochromes during the flow is markedly reduced, giving a several-fold decrease in the calculated first order rate constants. However, increasing the concentration of CO did not have any further inhibitory effect, suggesting that the other oxidase is fairly insensitive.

As in the mammalian system and those of other microorganisms, *b* and *c* type cytochromes act as intermediary electron carriers in the respiratory chain system of *H. parainfluenzae*. The levels of reduction of these cytochromes in the presence of 15 μM O_2 with substrates of different dehydrogenases or with different concentrations of the substrate of one dehydrogenase seem to vary in a straightforward manner with the relative activities of the dehydrogenases (Table II). So far no evidence has been seen which would indicate the presence of multiple independent electron transport chains, but more quantitative data are required to be certain of this.

The membrane-bound formate dehydrogenase of *H. parainfluenzae* has unusually high activity, compared with the dehydrogenases of the mammalian system. When formate is substrate, the cytochrome c_1 can be as much as 40% reduced in the presence of 15 μM O_2. This is in agreement with the observation that the rate of oxidation of formate by O_2 is very high in these bacteria (4). As expected from the measurements of rates of O_2 uptake (4), the intermediary cytochromes were reduced less rapidly via the other membrane-bound dehydrogenases.

There is no evidence that one part of the cytochromes of *H. parainfluenzae* is oxidized at a different rate than the rest in the rapid reactions under observation, even though the different types are present in different stoichiometric ratios. Although there was one instance seen of inhomogeneity of reduction of cytochrome *b* on addition of formate (Fig. 3), one cannot be certain that the final lower rate did not result from the production of other substrates. The ratios at the α-absorption peaks of cytochromes a_2, *c*, and *b* of type II bacteria in the aerobic steady state differ somewhat from these values in the anaerobic minus aerobic difference spectrum (1:1.3:2.7 as compared with 1:1.9:2.1). Thus, it is possible that not all of the cytochromes are participating in the rapid reactions under observation. However, in the mammalian system the *b* type cytochromes are more highly reduced in the aerobic steady state than are the *c* type cytochromes (9).

The difference in reaction rate sometimes observed on addition of O_2 when observations are made at the γ-absorption peak of cytochrome *b* as compared with that of cytochrome *c* is puzzling. For example, in Fig. 1 the rate at 423 minus 402 mμ is greater than that at 430 minus 410 mμ, even though the anaerobic minus aerobic difference spectrum shows no evidence of a separate absorption peak for cytochrome *c* in this region. The difference in rate is even greater in the presence of CO (Fig. 4). Such a difference would not be expected in the reactions of *b* or *c* type cytochromes on the same electron transport chain, judged by observations with the mammalian system (7). However, the overlapping of the γ-absorption bands of reduced cytochromes *b*, *c*, *o*, and the CO compound of cytochrome *o* makes for some difficulty in interpretation. Many more data would be needed with bacteria containing different ratios of the different cytochromes to clarify the situation and also to make certain of the presence of only one electron transport chain. The finding of conditions in which the bacteria synthesize very low cytochrome *o* and high cytochrome a_2 would be a great help in this respect.

The preliminary conclusion from the present experiments is that the data with *H. parainfluenzae* are better explained by the variable three-dimensional array of pigments previously proposed (4) rather than by fixed assemblies of stoichiometric ratios of pigments. However, more extensive quantitative studies are needed on the extent of involvement of the different cytochromes in the rapid reactions following initiation of electron transport on addition of oxygen and of different substrates.

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