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Kinetic Characterization of the Membrane-Bound Cytochromes of *Escherichia coli* Grown Under a Variety of Conditions by Using a Stopped-Flow Dual-Wavelength Spectrophotometer

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A study was made of the rapid oxidation kinetics of the cytochromes of *Escherichia coli*. The *b*-type cytochromes were kinetically heterogeneous, with one species (presumably cytochrome *o*) oxidized so rapidly that it could fully support observed oxidation rates. Cytochrome *d* but not cytochrome a_1 was also kinetically competent to support respiration. However, in cells grown anaerobically in the presence of NO_3^-, cytochrome *d* exhibited slow oxidation kinetics and a red-shift in its reduced-minus-oxidized difference spectrum.

Escherichia coli has the ability to synthesize a variety of cytochrome components, including two *c*-type cytochromes (cytochromes c_{550} and c_{548}), five *b*-type cytochromes [cytochromes b_{556}, b_{558}, b_{562}, $b_{566}^{NO_3^-}$ and *o* (an additional cytochrome b_{556} which binds CO; Castor & Chance (1959)], cytochrome a_1 and cytochrome *d* [for references see Haddock & Schairer (1973)]. The concentration of a particular cytochrome in the cell varies considerably, depending on the growth phase, the terminal electron acceptor, the carbon source for growth and the strain. The various cytochromes have been identified by low-temperature difference spectroscopy and by fourth-order finite difference analysis of such spectra obtained with whole cells and derived subcellular fractions (Shipp, 1972), but comparatively little attention has been directed towards the functional characterization of their physicochemical properties. Such information is obviously important in any attempt to correlate observed changes in the cytochrome components of the cell, after alterations to the growth conditions, with their functional organization in the membrane and metabolic significance to the cell. The object of the present report was to investigate the kinetic behaviour of the various cytochromes during oxidation–reduction reactions by stopped-flow dual-wavelength spectroscopy, and thereby determine their kinetic competence for respiration.

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Materials and Methods

Bacterial strain, growth conditions and preparation of cells and particles

E. coli strain EMG-2 (prototroph) was grown in 8-litre volumes of the mineral salts medium described by Cohen & Rickenberg (1956) containing vitamin-free casamino acids (0.1%, w/v) in a 10-litre fermentor vessel maintained at 37°C. For aerobic growth conditions vigorous stirring was used, the air-flow rate was approx. 5 litres/min, and the medium was supplemented with glycerol (0.5%, w/v); these cells are referred to subsequently as aerobically grown (glycerol) cells. For anaerobic-growth conditions slow stirring, sufficient to prevent sedimentation of the cells, was used and anoxic conditions were maintained by bubbling the cultures with O_2-free N_2 (about 200ml/min, 'White Spot' N_2, 99.9% pure; BOC, London S.W.19, U.K.), which had been sparged through 1 litre of Fieser's solution (Vogel, 1956) and then 1 litre of degassed water to remove all traces of O_2. The growth medium was supplemented with K_2SeO_3 (1 μM) and $(NH_4)_6Mo_7O_{24},4H_2O$ (1 μM) together with xylose (0.5%, w/v) for anaerobically grown (xylose) cells, with glycerol (0.5%, w/v) and potassium fumarate (50mM) for anaerobically grown (glycerol with fumarate) cells and with glycerol (0.5%, w/v) and KNO_3 (1%, w/v) for anaerobically grown (glycerol with nitrate) cells.

In all cases, cells were harvested in the late-exponential phase of growth, when E_{420} (10mm light-path: 1/10 dilution) had reached 0.3–0.5.

Electron-transport particles were prepared as

described previously (Haddock, 1973), except that all procedures were done in a medium containing 50mM-KCl and 50mM-Tes [*N*-tris(hydroxymethyl)-methyl-2-aminoethanesulphonic acid]/KOH (pH7.0) rather than the medium described previously: this medium was used in all the experiments described in the present paper.

Stopped-flow dual-wavelength spectrophotometry

Measurements of the rapid changes in the extent of reduction of cytochromes were made with the stopped-flow dual-wavelength spectrophotometer described in the preceding paper (Garland *et al.*, 1976). Suspensions of bacteria or electron-transport particles, anaerobic as a result of respiration, were rapidly mixed with a terminal acceptor for respiration (usually air-saturated buffer) in a ratio of 17:1 by volume. Operating variables such as the path-length (8 or 10mm), temperature (usually room temperature), response time of the measuring circuit and collection of data by storage oscilloscope or transient recorder are given in the legends to the Figures. The spectrophotometer was used to measure the percentage transmission in the 90–110% T range, except where otherwise stated. Pseudo-first-order rate constants for the more rapid oxidation of cytochromes were calculated from the extent of change observed during the brief steady state of continuous flow occurring between the start and the stop of the flow. The average age of the mixture in the observation chamber during this steady state is approx. 3.3ms at the drive pressure of 280kPa (40lb/in^2) used in this work, and the pseudo-first-order rate constant K was calculated according to the equation

$$K = \frac{2.3}{3.3\times10^{-3}} \log \frac{p_0}{p_0 - p} s^{-1}$$

where p_0 is extinction change corresponding to completed oxidation of the cytochrome and p is the change observed during the flow and corresponding to 3.3ms after mixing of the anaerobic suspension with oxygen (Chance & Williams, 1955; Smith *et al.*, 1970; Chance, 1974). Pseudo-first-order rate constants for more slowly oxidized cytochromes that were effectively unoxidized during the flow were calculated from the half-time ($t_{\frac{1}{2}}$, s) of the course of the extinction change initiated when the flow stopped and the mixed reactants started to age. The formula is

$$K = \frac{0.693}{t_{\frac{1}{2}}} s^{-1}$$

The values given for time-constants refer to the time taken for the measuring circuits to respond to 63% of an instantaneous change. In a number of traces there was an overshoot of the spectrophotometer reading at the onset of flow. This is probably due to slower flow at the onset. The overshoot was therefore disregarded. In all experiments the cells or electron-transport particles were suspended in 50mM-KCl/50mM-Tes buffer, pH7.0, and used for filling the major syringe. Additions from the minor syringe were made in 50mM-KCl/50mM-Tes buffer, pH7.0.

Dual-wavelength spectrophotometric measurements were converted into cytochrome concentrations by the use of appropriate millimolar extinction coefficients (expressed as litre^{-1}·mmol^{-1}·cm^{-1}). For cytochrome *b* we used an ε_{mM} value of 17.5 at 560–575nm (Jones & Redfearn, 1966) and 180 at 430–410nm (Chance, 1957). For cytochrome *d* we used a value of 8.5 for 630–610nm (Jones & Redfearn, 1966) and 19 for 630–655nm (calculated from the data of Kauffman & van Gelder, 1973). We do not know of any report for the extinction coefficients of cytochrome a_1, and have assumed a value for ε_{mM} at 594–610nm of 8.5 as suggested by Meyer & Jones (1973), which is arbitrary but unlikely to be in error by more than twofold. In some experiments we have used wavelength pairs such as 430–460nm for cytochrome *b* and 440–460nm for cytochrome *d* and a_1 when it was desirable to work with fast response times. These experiments gave half-times of oxidation, but the absolute amount of cytochrome under oxidation was determined from measurements made at the α bands with a slower response time.

Other assay techniques

O_2 uptake and protein were measured as described previously (Haddock, 1973). Difference spectra were obtained with a wavelength-scanning spectrophotometer by using low-temperature accessories (Haddock & Garland, 1971); the spectral bandwidth was 1nm, the scanning speed was approx. 5nm/s, the time-constant of the measuring circuit was 0.1s and cuvettes of 0.2cm light-path were used.

Reagents

Vitamin-free casamino acids were from Difco (Detroit, Mich., U.S.A.), D-xylose (grade II) was from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and D-lactate (lithium salt) was from Calbiochem (London W1H 1AS, U.K.). Ethyl hydrogen peroxide (10%, w/w) was from Ferrosan, Malmo, Sweden. All other reagents were from BDH Chemicals (Poole, Dorset, U.K.) and were of the highest available purity.

Results

Spectral contributions from peroxidase (EC 1.11.1.7) or catalase (EC 1.11.1.6)

We attempted to estimate the maximal extent to which changes in the concentrations of enzyme–substrate complexes of catalase or peroxidase might interfere with spectroscopic studies of the oxido-reductions of cytochrome oxidation. The estimate was made by measuring the spectral changes that occurred when ethyl hydrogen peroxide (0.67mM) was added to aerobic or anaerobic suspensions of cells, under conditions otherwise as in Fig. 1. Catalase and peroxidase would both be completely converted into their complex II forms by ethyl hydrogen peroxide, and their concentration can be calculated from the extinction coefficients given by Chance (1952) for these conversions. It was found that ethyl hydrogen peroxide when added to suspensions of *E. coli* did cause small spectral changes in the Soret region, but the extent of these changes was less than 5% of those caused by oxidoreduction of cytochromes as measured in reduced-minus-oxidized difference spectra (Fig. 1). Any spectral effects of ethyl hydrogen peroxide in the α-band region (540–650nm) were undetectable. We conclude that neither catalase nor peroxidase interfered significantly with our measurements of oxidoreductions of cytochromes.

Cytochrome content of cells grown under different conditions

Low-temperature difference spectra obtained with whole cells of *E. coli* grown under three different conditions are shown in Fig. 1. Aerobically grown (glycerol) cells contain three *b*-type cytochromes, of which only two, cytochromes b_{556} and b_{562}, are seen in reduced-minus-oxidized difference spectra (Fig. 1*e*).

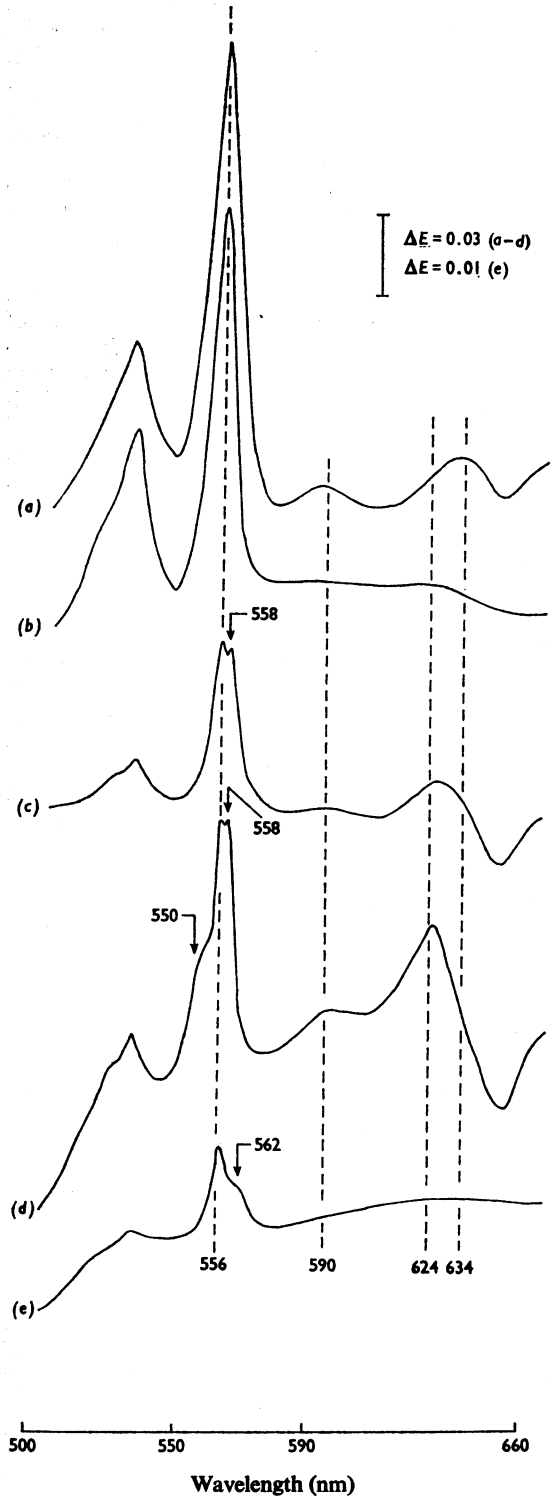
The peak at 556nm is due to two cytochrome components that can be separated by their differential ability to react with CO when in the ferrous state (results not shown). Cytochrome *o* binds CO, contributes 20–40% of the total change at 556nm and acts as the terminal oxidase in cells grown under these conditions. It has been suggested (Fujita, 1966) that cytochrome b_{562} is a soluble protein and not required for membrane-bound electron-transport processes, yet a significant amount is detectable in electron-transport particles (Ashcroft & Haddock, 1975).

Anaerobically grown (xylose) cells and anaerobically grown (glycerol with fumarate) cells (results not given) contain cytochromes c_{550}, b_{556}, b_{558}, a_1 (peak at 590nm) and *d* (peak at 624nm) as shown in Fig. 1(*d*). Two CO-reacting terminal oxidases can be identified in these cells, cytochrome *o* and cytochrome *d* (results not given): the concentration of cytochrome a_1 is low and it is difficult to determine if it binds CO, as demonstrated in certain *Acetobacter* species (Castor & Chance, 1959; Meyer & Jones, 1973). Cytochrome c_{550} and cytochrome c_{548}, if it is present, are reported to be soluble proteins in *E. coli* (Fujita, 1966) and not required for membrane-bound electron-transport processes.

Difference spectra illustrating the cytochrome composition of anaerobically grown (glycerol with nitrate) cells are shown in Figs. 1(*a*)–1(*c*). The following conclusions can be drawn from these data and from CO difference spectra (results not shown). (*a*) Anaerobic growth in the presence of nitrate results in the synthesis of high concentrations of a *b*-type cytochrome with maximum absorption at 556nm (Fig. 1*a*), and, since it is specifically oxidized by nitrate (Fig. 1*b*), this is referred to as cytochrome $b_{556}^{NO_3^-}$ (Ruiz-Herrera & De Moss, 1969). (*b*) Such cells also contain cytochromes b_{556}, b_{558}, a_1 and *d* (Fig. 1*a*) and these cytochromes are specifically oxidized by O_2 but not by nitrate (Fig. 1*c*). (*c*) CO difference spectra indicate that the major oxidase present in these cells is cytochrome *d*, there is only a small amount of cytochrome *o*, and CO does not bind to cytochrome $b_{556}^{NO_3^-}$, in agreement with the observation of Ota *et al.* (1964) that CO does not affect nitrate respiration. (*d*) There is a 10nm red-shift in the absorption maximum of cytochrome *d* in reduced-minus-oxidized difference spectra of anaerobically grown (glycerol with nitrate) cells (compare Figs. 1*a* and 1*d*). Thus anaerobically grown (glycerol with nitrate) cells differ from anaerobically grown (xylose or glycerol with fumarate) cells in possessing a specific cytochrome $b_{556}^{NO_3^-}$ and modified cytochrome *d* and in failing to synthesize detectable amounts of *c*-type cytochromes and possibly cytochrome *o*, though it is difficult to be certain of this last-mentioned difference, since, in these cells, the cytochrome *o* concentration would be less than 5% of the total *b*-type cytochrome concentration.

Kinetics of the oxidation of cytochrome b by oxygen

The rapid oxidation of cytochrome *b* by oxygen in whole cells and electron-transport particles from aerobically grown (glycerol) *E. coli*, and in whole cells of anaerobically grown (glycerol with fumarate) *E. coli*, is shown in Fig. 2. Aerobically grown (glycerol) cells contain cytochrome *b* as their main cytochrome species (Fig. 1*e*) and measurement in the Soret band is free of interference from other cytochromes. Fig. 2(*a*) shows that of the total extent of oxidation of cytochrome *b* resulting from mixing anaerobic cells with oxygen, approx. 50% occurred during the flow and the remainder within 50ms of the cessation of flow. There are two extreme interpretations of this kinetic behaviour. The first



is that there was a single pool of autoxidizable cytochrome *b* that was oxidized with a half-time of 3.3 ms. This would account for the 50% oxidation during the flow, and although oxidation of the remaining 50% should proceed with an identical half-time, the observed rate would be slowed by the 10 ms time-constant that was set in the measuring circuit. The other interpretation is that there are two distinct pools of cytochrome *b*, one mainly oxidized and the other mainly unoxidized during the flow. The latter interpretation is supported by the markedly biphasic kinetics of reduction of cytochrome *b* when the pulse of oxygen is exhausted, as shown in Fig. 2(*c*). Further evidence on this point was sought by using a measuring time-constant of 2.5 ms and, to improve the signal-to-noise ratio, electron-transport particles in place of whole cells. Fig. 2(*b*) shows the kinetics of cytochrome *b* oxidation in electron-transport particles. As with whole cells, 50% of the total oxidation was completed during the flow. The remainder became oxidized on cessation of the flow, and the observed half-time for this phase was about 20–30 ms. The measuring accuracy of the kinetics of this phase was limited by noise, the time-constant of 2.5 ms, and the wavelength chopping frequency of 150 Hz. Nevertheless, it is clear that the half-time of this phase was severalfold greater than 3.3 ms. So it seems that the cytochrome *b* oxidized within 100 ms of mixing anaerobic cells with oxygen consisted of two pools of about equal size. One was oxidized with a half-time of less than 3 ms, the other with a half-time of about 25 ms.

The oxidation kinetics of cytochrome *b* of anaerobically grown (glycerol with fumarate) cells measured in the Soret band are shown in Fig. 2(*d*). About 40% of the total cytochrome *b* oxidation occurring within 0.1 s after mixing anaerobic cells with

Fig. 1. D-*Lactate-reduced-minus-oxidized difference spectra recorded at 77°K obtained with E. coli cells grown under a variety of conditions*

Cells were grown and harvested and difference spectra were recorded as indicated in the Materials and Methods section. Before the samples were frozen in liquid N_2 the cells were reduced in all cases with D-lactate (5 mM) and, where indicated, reoxidized by the addition of either KNO_3 (1 mM) or H_2O_2 (1 mM). The cell types used, the type of difference spectrum recorded and the final protein concentration in the cuvettes, for each trace, were: (*a*) anaerobically grown (glycerol with nitrate), reduced minus H_2O_2-oxidized, 8.5 mg/ml; (*b*) anaerobically grown (glycerol with nitrate), reduced minus KNO_3-oxidized, 8.5 mg/ml; (*c*) anaerobically grown (glycerol with nitrate), KNO_3-oxidized minus H_2O_2-oxidized, 8.5 mg/ml; (*d*) anaerobically grown (xylose), reduced minus H_2O_2-oxidized, 6.0 mg/ml; and (*e*) aerobically grown (glycerol), reduced minus H_2O_2-oxidized, 13.4 mg/ml.

oxygen occurred during the flow. The remaining 60% was oxidized with a half-time of 150ms after the flow had stopped. Similar identification of a very rapidly oxidized cytochrome *b* ($t_{\frac{1}{2}}$<3ms) and of a more slowly oxidized cytochrome *b* ($t_{\frac{1}{2}}$ about 100ms) were also made by measuring the α-band at 575–560nm (Fig. 2*e*). Measurements with a longer-time-course, shown in Fig. 2(*f*), demonstrated a third and even slower phase of cytochrome *b* oxidation that proceeded for up to 5s after the flow had stopped. Its half-time was about 2s. Inhomogeneity of the cytochrome *b* was also shown by the markedly biphasic kinetics of cytochrome *b* reduction that occurred on exhaustion of the oxygen pulse (Fig. 2*f*).

Kinetics of the oxidation of cytochrome d by oxygen in anaerobically grown (glycerol with fumarate) cells

The rapid oxidation by oxygen of cytochrome *d* in suspensions of anaerobically grown (glycerol with fumarate) cells is shown in Fig. 3. The presence of cytochromes a_1, *b* and *d* in those anaerobically grown cells (Fig. 1) required the choice of wavelength pairs that were essentially specific for each of the cytochromes. We first established [using aerobically grown (glycerol) cells, which contain almost exclusively cytochrome *b*] that a wavelength pair of 460–440 (or 465–445)nm was practically free from interference by oxidoreductions of cytochrome *b*. So the rapid decrease in extinction at 440nm with respect to 460nm seen when anaerobically grown (glycerol with fumarate) cells were mixed with oxygen (Fig. 3*a*) can be attributed to cytochrome a_1 or cytochrome *d*, or both. About 67% of the total change at 460–440nm was completed during the flow, and the remaining 33% proceeded with a half-time of about 30ms. It follows that there must be two distinct cytochrome components contributing to the changes at 440–460nm, one fully oxidized during the flow and one oxidized after the flow. Measurements at 655–630nm (Fig. 3*b*) showed that cytochrome *d* was fully oxidized during the flow, and this can be equated with the 67% of the change at 460–440nm that also occurred during the flow. The remaining 33% of the change at 460–440nm that was oxidized with a half-time of 30ms was probably cytochrome a_1, although we were not able to obtain with these cells convincing evidence by measurement at the α-band for cytochrome a_1. However, we were able to

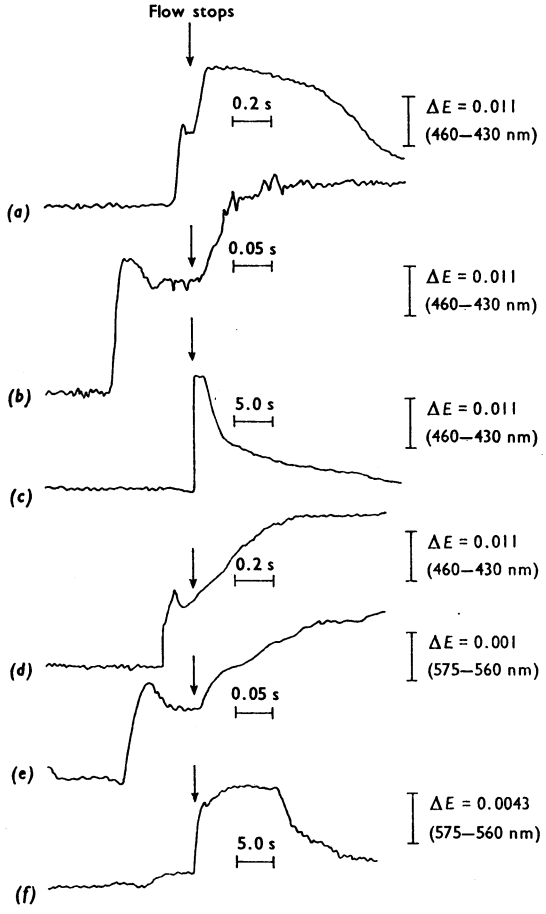


Fig. 2. *Kinetics of cytochrome b oxidation by oxygen in aerobically grown (glycerol) cells and anaerobically grown (glycerol with fumarate) cells*

Dual-wavelength spectrophotometric measurements were stored with a transient recorder, plotted with a pen-recorder, and traced for presentation in the Figure. The six traces shown have been stacked to bring the moment at which the flow stops into line, shown by the vertical arrows. The point at which flow commences can be judged from the fact that flow lasted for approx. 0.2s. The wavelength pair for traces (*a*)–(*d*) was 460–430nm, and for traces (*e*) and (*f*), 575–560nm. Spectrophotometric sensitivity is shown by the vertical bars and the time-sweep by the horizontal bars. Traces (*a*) and (*c*) are for aerobically grown (glycerol) cells, trace (*b*) is for electron-transport particles derived from such cells, and traces (*d*), (*e*) and (*f*) are for anaerobically grown (glycerol with fumarate) cells. In each experiment an upward deflexion of the trace indicates oxidation of cytochrome *b*. The suspension of cells or electron-transport particles in the major syringe also contained 5mM-D-lactate. The minor syringe contained air-saturated buffer, giving an oxygen concentration on mixing of 25ng-atoms/ml. The concentration of protein (mg/ml) in the major syringe was as follows: (*a*) and (*c*) 2.5; (*d*)–(*f*) 4.0. The measuring time constants were 10, 2.5, 10, 10, 50 and 50ms for traces (*a*)–(*f*) respectively. The temperature was 27°C and 0.75ml of mixed reactants were used for each trace. The path-length of the observation chamber was 10mm.

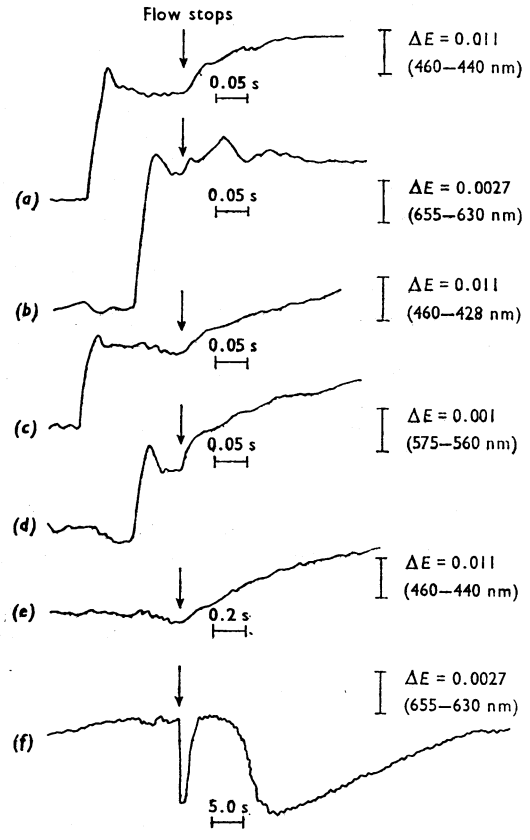


Fig. 3. *Kinetics of the oxidation of cytochrome d by oxygen in anaerobically grown (glycerol with fumarate, or glycerol with nitrate) cells*

Dual-wavelength spectrophotometric measurements were either plotted directly with a pen-recorder for the slowest time-sweep [trace (*f*)], or first stored with a transient recorder for the other traces. As in Fig. 2, the six traces have been stacked in line, with the point at which flow stopped shown by the vertical arrows. Spectrophotometric sensitivities, wavelength pairs and time-sweeps are shown against each trace. In all cases an upward deflexion indicates an increase in extinction of the higher of each wavelength pair referred to the lower of the pair, and is equated with oxidation of the cytochrome studied at that wavelength pair. Anaerobically grown (glycerol with fumarate) cells (4.0mg of protein/ml) were used for traces (*a*)–(*d*), and anaerobically grown (glycerol with nitrate) cells (5.0mg of protein/ml) for traces (*e*) and (*f*). The cell suspensions were supplemented with 5mM-D-lactate. The measuring time constants were (*a*) 10, (*b*) 20, (*c*) 10, (*d*) 50, (*e*) 20 and (*f*) 100ms. The flow volume of mixed reactants was 0.75ml, and the operating temperature 26°C. The path-length of the observation chamber was 10mm.

obtain confirmation of a half-time of about 30ms for cytochrome a_1 measured at the α-band (594–610nm) with anaerobically grown (xylose) cells (see below). Traces (*c*) and (*d*) of Fig. 3 are of the Soret and α-bands of cytochrome *b* of anaerobically grown (glycerol with fumarate) cells, and serve to demonstrate the kinetic differences between cytochromes *d* (and a_1) and *b*. It seems unlikely that cytochrome *b* was interfering with the measurement of cytochrome *d*.

Kinetics of the oxidation of cytochrome d by oxygen in anaerobically grown (glycerol with nitrate) cells

Fig. 3(*e*) shows that the changes at 460–440nm seen on mixing anaerobically grown (glycerol with nitrate) cells with oxygen were slow, there being no change during the flow and a slow oxidation with $t_{\frac{1}{2}}$ of about 0.4s when the flow stopped. Similar kinetics were observed at 655–630nm, at the α-band for cytochrome *d*. When measurement was made over a longer time-course (Fig. 3*f*), most unusual kinetics were observed; after mixing cells with oxygen there was oxidation of cytochrome *d* occurring when the flow stopped, followed by reduction when the oxygen became exhausted after about 7s, followed by a slower oxidation extending over 20s or more back to the original oxidized state.

Kinetics of the oxidation of cytochrome b by oxygen and nitrate in anaerobically grown (glycerol with nitrate) cells

Fig. 4(*a*) shows that oxygen oxidizes cytochrome *b* in anaerobically grown (glycerol with nitrate) cells, and that under the conditions used the reduction of oxygen (125ng-atoms/ml) was completed within a few seconds. When nitrate (100μM) and oxygen were mixed simultaneously with cells (Fig. 4*b*), there was a biphasic response in the oxidation of cytochrome *b*. The duration of the initial extensive oxidation was shown to be independent of the nitrate concentration, dependent on the oxygen concentration, unaffected by the presence of 4μM-NaN_3, and abolished by previously saturating the cell suspension with CO (results not shown). The duration of the second phase of less extensive oxidation of cytochrome *b* was dependent on the nitrate concentration, greatly extended by 4μM-NaN_3, and unaffected by saturation of the cell suspension with CO (Fig. 4*c*). Clearly the first phase of oxidation is due to oxygen, the second to nitrate. It was therefore imperative that attempts to study the rapid kinetics of cytochrome *b* oxidation by nitrate required strictly anoxic conditions or the use of CO-treated cells. Trace (*e*) of Fig. 4 shows that when oxygen was added to anaerobic cells, oxidation of cytochrome *b* did not

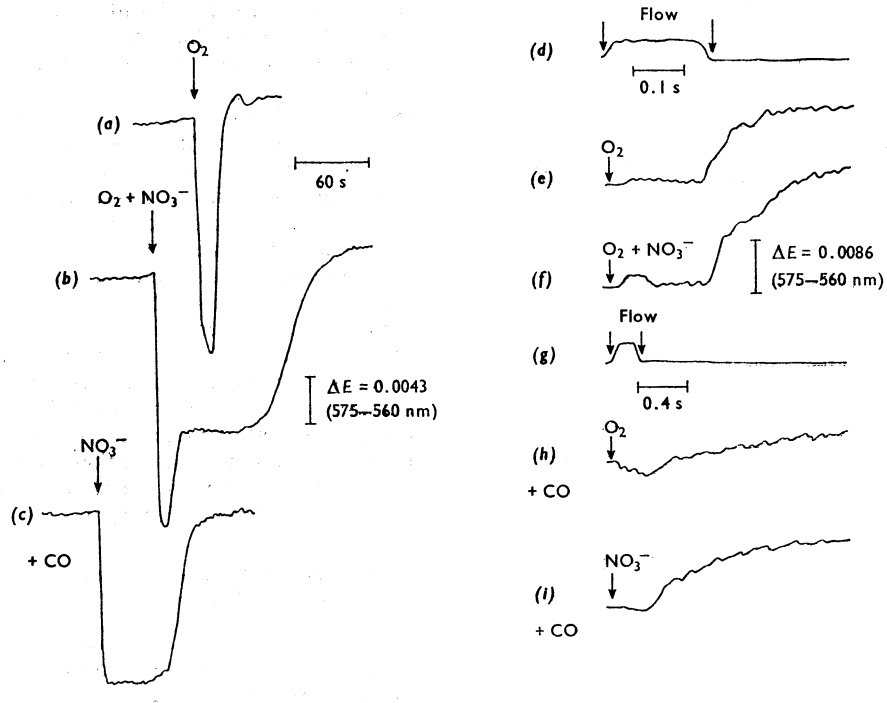


Fig. 4. *Kinetics of cytochrome b oxidation by oxygen or nitrate in anaerobically grown (glycerol with nitrate) cells*

Dual-wavelength spectrophotometric measurements were plotted directly with a pen recorder [traces (*a*), (*b*) and (*c*)], and the early events after rapid mixing were recorded in parallel with a storage oscilloscope [traces (*d*)–(*i*)]. Thus traces (*a*), (*b*) and (*c*) are from the same experiment as traces (*e*), (*f*) and (*i*) respectively. Trace (*d*) is the flow velocity recording for spectrophotometric traces (*e*) and (*f*); trace (*g*) is the flow-velocity trace for spectrophotometric traces (*h*) and (*i*). The time-scales and spectrophotometer sensitivities for the two sets of traces (pen-recorder and storage oscilloscope) are shown by the horizontal and vertical bars. The spectrophotometric sensitivity for the pen recordings is 2.24 times as sensitive as the oscilloscope recordings. The pen recordings are for extinction changes at 560 minus 575nm; cytochrome *b* oxidation is downwards. The oscilloscope recordings are for 575 minus 560nm; cytochrome *b* oxidation is upwards. The time-sweep for traces (*d*)–(*f*) is four times as fast as for traces (*g*)–(*i*). The major syringe contained cells at a protein concentration of 4.1 mg/ml supplemented with 20mM-L-malate (Tris salt), pH 7.0. In the experiments of traces (*c*), (*h*) and (*i*), the cell suspension had been gassed with CO for 10min in the reservoir syringe before transfer to the major syringe. The minor syringe contained: oxygen-saturated buffer for traces (*a*), (*e*) and (*h*); oxygen-saturated buffer containing 1.8mM-KNO_3 for traces (*b*) and (*f*); and nitrogen-saturated buffer containing 1.8mM-KNO_3 for traces (*c*) and (*i*). The presence of CO is indicated by '+CO' for traces (*c*) and (*i*). Mixing with oxygen and/or nitrate is indicated by appropriately labelled arrows at the start of mixing for each spectrophotometric trace. The measuring time-constants were (*a*) and (*e*) 10ms, (*h*) and (*i*) 20ms. The temperature was 29°C and 0.75ml of mixed reactants was used for each trace. The path-length of the observation chamber was 8mm.

effectively start until the flow stopped. Oxidation then proceeded with a half-time of 50ms, without evidence for more than one component. When oxygen and nitrate were added together (Fig. 4*f*) there was again no oxidation during the flow. When the flow stopped, oxidation proceeded slightly more rapidly than with oxygen alone; however, the final extent of oxidation was the same, as shown by comparing traces (*a*) and (*b*). Saturation of the cell suspension with CO greatly decreased the oxidation of cytochrome *b* after addition of oxygen, and permitted observation of the oxidation by nitrate alone (Figs. 4*h* and 4*i*).

Kinetics of the oxidation of cytochromes by oxygen in anaerobically grown (xylose) cells

These cells were of particular interest because we were able to make satisfactory measurements of the kinetics of cytochrome a_1 oxidation. Fig. 5(*a*) shows that on mixing anaerobic cells with oxygen there was a decrease in extinction at 594nm (the α-band peak of

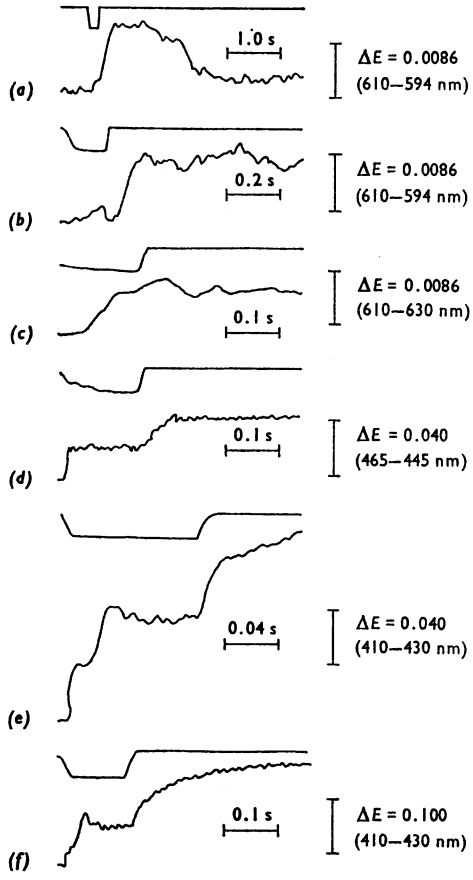


Fig. 5. *Kinetics of cytochrome oxidation by oxygen in anaerobically grown (xylose) cells*

Dual-wavelength spectrophotometric measurements were recorded with a storage oscilloscope. Each trace is presented in the Figure in combination with its corresponding flow-velocity recording and vertical and horizontal bars showing the spectrophotometric sensitivity and time sweep. The cell concentration, as mg of protein/ml, was 6.6 for traces (*a*) and (*b*), 5.1 for (*c*), 3.3 for (*d*) and 6.6 for (*e*) and (*f*). No substrate was added. The minor syringe contained oxygen-saturated buffer. The measuring time-constants in ms were (*a*) 50, (*b*) 20, (*c*) 5, (*d*) 5, (*e*) 2.5 and (*f*) 10. The flow volume of mixed reactants was 0.75ml, and the operating temperature 29°C. The path-length of the observations chamber was 8mm. The spectrophotometer was used in the extinction mode for traces (*d*)–(*f*).

cytochrome a_1), taking 610nm as the reference wavelength. The oxidation was sustained briefly until the oxygen became exhausted. Fig. 5(*b*), in which a faster time-sweep was used, shows that oxidation of cytochrome a_1 started when the flow stopped, and proceeded with a half-time of about 30ms. By contrast the oxidation of cytochrome *d* measured at 630nm with reference to 610nm was completed during the flow (Fig. 5*c*). Measurement in the Soret-band region at 465–445nm showed two components (Fig. 5*d*); one oxidized during the flow and identified with cytochrome *d*, the other oxidized after the flow with a half-time of about 25ms and identified with cytochrome a_1. The oxidation of cytochrome *b* measured at 430–410nm showed three phases; oxidation during the flow, a very rapid oxidation ($t_{\frac{1}{2}} < 10$ms) when the flow stopped, and then a much slower oxidation ($t_{\frac{1}{2}} \simeq 100$ms). The first two of these phases are shown in Fig. 5(*e*), and probably represent a single cytochrome *b* component oxidized with a half-time of 2–3ms. This would account for both the oxidation during the flow and the fast oxidation when the flow stopped. All three phases are shown with the slower time-sweep and lower sensitivity as in Fig. 5(*f*).

Comparison of pseudo-first-order rate constants with steady-state respiration rates

Ideally, the rate of oxidation of a respiratory-chain carrier during the transition from reduced to oxidized in response to oxygen should be sufficient to support the observed rate of electron flow through the respiratory chain. In practice this criterion of kinetic competence may not be fulfilled for one of two reasons. First, the carrier studied may indeed be off the main respiratory pathway, on a side path. Secondly, the measured kinetics of the transition may be slowed because the carrier is reduced by its reductant almost as rapidly as it is oxidized by its oxidant. This problem is particularly severe with whole cell preparations, where the flow of reducing equivalents into the respiratory chain is not easily manipulated. Nevertheless it is still useful to look for kinetic competence in respiratory carriers.

Table 1 summarizes half-times and pseudo-first-order rate constants for oxidation of cytochromes and compares them with steady-state rates of respiration.

Discussion

Identification of kinetically competent terminal oxidases

Results in Table 1 identify cytochrome *d* but not cytochrome a_1 as a kinetically competent terminal oxidase. A similar conclusion for these cytochromes in *Haemophilus parainfluenzae* was reached by Smith *et al.* (1970). A fast-reacting cytochrome *b* oxidized with a half-time of less than 3.3ms was also kinetically competent to support the observed respiration rates in cells grown aerobically with glycerol, and anaerobically with glycerol and fumarate, and was almost competent in cells grown

Table 1. *Summary of pseudo-first-order rate constants for cytochrome oxidation, maximal electron-transfer rates calculated from the rate constants and cytochrome concentration, and the observed electron-transfer rates*

The pseudo-first-order rate constants (K) were calculated from the half-times for oxidation as observed in Figs. 2–5. Where a *b*-type cytochrome showed more than one kinetic component, only the faster phase was taken for calculation. Maximal rates of electron transfer (as nequiv./s per mg of cell protein) were calculated as the product of the pseudo-first-order rate constant for a particular cytochrome and the concentration (nmol/g of protein of that cytochrome determined from the dual-wavelength spectrometric recording of its oxidation. The observed electron transfer rates (as nequiv./s per mg of protein) were calculated from the time elapsing between the moment of initiating oxidation in the stopped-flow spectrophotometer and the onset of anoxia indicated by the start of cytochrome reduction. This is shown in these spectrophotometric traces recorded with a relatively slow time-sweep.

Cytochrome (and oxidant)	Cell growth	Fig.	$t_{\frac{1}{2}}$ (ms)	K (s^{-1})	Electron-transfer rates (equiv./s per mg)	
					Calculated	Observed
b (oxygen)	Aerobic, with glycerol	2(*a*)	<3.3	>210	>16	10
b (oxygen)	Anaerobic, glycerol with fumarate	2(*e*)	<3.3	>210	>5	1.0
b (oxygen)	Anaerobic, glycerol with nitrate	4(*e*)	50	14	2.5	4.0
b (nitrate)	Anaerobic, glycerol with nitrate	4(*i*)	300	2.3	0.5	2.0
b (oxygen)	Anaerobic, with xylose	5(*e*)	<3.3	>210	>14	21
d (oxygen)	Anaerobic, glycerol with fumarate	3(*b*)	<3.3	>210	>26	5
d (oxygen)	Anaerobic, glycerol with nitrate	3(*e*)	0.4	1.7	0.1	4.0
d (oxygen)	Anaerobic, with xylose	5(*c*)	<3.3	>210	>54	21
a_1 (oxygen)	Anaerobic, with xylose	5(*b*)	25	27	3.6	21

anaerobically with xylose (Table 1). This rapidly oxidized cytochrome *b* is presumably the CO-sensitive terminal oxidase, or cytochrome *o* (Castor & Chance, 1959).

Terminal respiratory enzymes of E. coli grown anaerobically with nitrate

Respiration rates with oxygen as the acceptor have, in our experience, varied greatly from batch to batch, by up to tenfold. In the spectrophotometric recordings for the batch strain in Fig. 4, there was no evidence for a very rapidly oxidized cytochrome *b*. However, this has not always been so, and in some batches we have observed oxidation of cytochrome *b* during the 3.3 ms aged flow. This raises the possibility that either cytochrome *o* synthesis is repressed under these growth conditions, or, if its synthesis is constitutive, then cytochrome *o* oxidase activity must be inhibited under these conditions. Further investigation was deferred pending a better knowledge of the factors causing the great variation of oxygen-uptake rate between cell batches. The rate of oxidation of cytochrome *b* by nitrate was about a quarter of the actual rate of electron transfer to nitrate. This does not mean that the cytochrome *b* is therefore off the main respiratory pathway from substrates to nitrate. The most likely explanation is that the cytochrome *b* was being reduced by substrates at a rate not greatly removed from its rate of oxidation by nitrate and nitrate reductase. Chance (1955) made a similar conclusion for the oxidation of cytochromes by nitrate in *Paracoccus denitrificans*. The kinetic behaviour of cytochrome *d* in these cells was curious. The rate of oxidation by oxygen was at least 100-fold slower than that for cytochrome *d* in the other cell types. There was also oscillatory behaviour. These features and the unusual spectrum of cytochrome *d* in cells grown anaerobically with nitrate remain unexplained.

Kinetic heterogeneity of cytochrome b

Not surprisingly, in view of the spectral (Shipp, 1972, Fig. 1) and thermodynamic (Hendler *et al.*, 1975) heterogeneity of cytochrome(s) *b* in *E. coli*, more than one cytochrome *b* species was revealed kinetically. Thus there were at least two species in cells grown aerobically with glycerol, three in cells grown anaerobically with glycerol and fumarate, and two in cells grown anaerobically with xylose. Whether or not these additional cytochromes are on the main respiratory pathway cannot be decided from their rate constants alone, and further information is required on the kinetic reactions of NADH, ubiquinone and menaquinone. The kinetic heterogeneity of the *b* cytochromes of *E. coli* is in marked contrast with *H. parainfluenzae*, where Smith *et al.* (1970) concluded that there was but a single kinetically homogeneous cytochrome *b*.

Ordering of cytochromes in respiratory chains

Although our data demonstrate the kinetic competence of two terminal oxidases, cytochrome *d* and the fast-reacting cytochrome *b*, which is presumably cytochrome *o*, we are unable to describe the point at which electron flow from common substrates divides

into independent pathways leading to the two oxidases when both are present together. The same is true for the pathway leading to nitrate reductase (EC 1.7.99.4). Nevertheless, overlap of pathways at the level of quinone seems likely, and this would be a common point of convergence from substrates and divergence to oxidases. It would be interesting to explore spectrophotometrically the kinetic characteristics of ubiquinone and menaquinone in *E. coli*, and to exploit various mutants deficient in their ability to synthesize quinones (Cox *et al.*, 1970; Newton *et al.*, 1971) and haem (Haddock, 1973), in which the concentration of a particular redox carrier in the membrane, and hence the rate of electron transport, can be manipulated at will.

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