Biochimica et Biophysica Acta, 725 (1983) 168–177
Elsevier

BBA 41388

CYTOCHROMES OF THE TRIMETHYLAMINE *N*-OXIDE ANAEROBIC RESPIRATORY PATHWAY OF *ESCHERICHIA COLI*

PHILIP D. BRAGG and NEIL R. HACKETT

Department of Biochemistry, University of British Columbia, Vancouver, B.C. V6T 1W5 (Canada)

(Received July 8th, 1983)

Key words: Cytochrome; Anaerobiosis; Electron transport; Respiratory chain; Trimethylamine N-oxide; (E. coli)

***Escherichia coli* grown anaerobically with trimethylamine *N*-oxide (TMAO) as a terminal electron acceptor develops a new cytochrome pathway in addition to the aerobic respiratory pathways which are still formed. Formate, NADH, and possibly other substrates derived from glucose, supply electrons to this pathway. Cytochromes with α-absorption peaks at about 548, 552, 554 and 557 nm are rapidly reoxidized by TMAO in a reaction which is not inhibited by 2-*n*-heptyl -4-hydroxyquinone *N*-oxide. $CuSO_4$ inhibits the reoxidation by TMAO of the first two of these cytochromes. This suggests that the pathway of electron transfer leading to the reduction of TMAO is: substrates → cytochromes 548,552 → cytochromes 554,557 → trimethylamine-*N*-oxide reductase → TMAO. These cytochromes, but not those of the aerobic respiratory pathways, are reoxidized by the membrane-impermeant oxidant ammonium persulfate in intact cells. This suggests that the cytochromes of the TMAO reduction pathway and/or trimethylamine-*N*-oxide reductase are situtated at the periplasmic surface of the cytoplasmic membrane of *E. coli*.**

Introduction

Escherichia coli, a facultative anaerobe, can respond to changes in its environment by synthesizing different types of respiratory pathways [1–4]. Under anaerobic conditions fumarate or nitrate can act as terminal electron acceptors. The respiratory chains of the fumarate and nitrate reduction pathways have been well characterized [1,3,4]. Cytochromes of the b type transfer electrons derived from the repiratory substrates to the fumarate or nitrate reductase enzymes responsible for reduction of the terminal electron acceptors. Both reductases are iron-sulfur proteins. Nitrate reductase in addition has a molybenum-containing prosthetic group [4,5].

A less well characterized anaerobic pathway uses TMAO as the terminal electron acceptor. TMAO is reduced to trimethylamine by trimethylamine-*N*-oxide reductase [6]. Trimethylamine-*N*-oxide reductase appears to contain the same type of molybenum-containing prosthetic group as nitrate reductase, since mutants (*chl*A, *chl*B and *chl*D) in which the prosthetic group is not formed have no TMAO reductase activity [7,8]. However, trimethylamine-*N*-oxide reductase and nitrate reductase do not contain the same polypeptides [7,8].

The TMAO reduction system is associated with the cytoplasmic membrane of *E. coli*. Formate and NADH can supply electrons for the reductions [9–11]. On the basis of studies with mutants, ubiquinone and/or menaquinone is a component of the NADH-TMAO reduction pathway [7]. Cytochromes of the *c* and *b* types are present in cells grown anaerobically with TMAO, and appear to undergo oxidation and reduction in the presence of TMAO and a respiratory chain substrate [6,11].

Abbreviations: TMAO, trimethylamine *N*-oxide; HOQNO, 2-(*n*-heptyl)-4-hydroxyquinoline *N*-oxide

0005-2728/83/$03.00 © 1983 Elsevier Science Publishers B.V.

However, the sequence and number of the cytochromes in the respiratory pathways to TMAO have not been clearly demonstrated. Furthermore, a potential problem not considered by earlier workers is the coexistence in membranes of *E. coli* of the cytochromes from several respiratory pathways [12,13]. Since the absorption maximum in the reduced minus oxidized difference spectrum of the various *b*- and *c*-type cytochromes may be similar, particular care is necessary to resolve the cytochromes present in the resulting fused absorption peak.

In this paper we have examined the cytochromes present in cells and membranes of *E. coli* grown anaerobically with TMAO. Several *b*- and *c*-type cytochromes present in the TMAO reduction pathway have been recognized and their sequence in the electron-transfer pathway determined. Cytochromes of the aerobic respiratory pathway were also present in these cells but could be distinguished from those of the TMAO reduction pathway. The cytochromes and/or trimethylamine-*N*-oxide reductase of the latter pathway were associated with the periplasmic surface of the cytoplasmic membrane.

Materials and Methods

Bacterial strains, growth conditions, and preparation of everted membrane vesicles

The following strains of *E. coli* were used in this investigation: HfrH (*thi relA spoT supQ* λ−), LCB517 (F^- *thi leu thr arg his pro purE supE lacY malA xyl ara mtl gal tonA rpsL fdhA*), NH30 (as HfrH but *narI::Tn 10*) [13].

The medium consisted of salts [14] supplemented with thiamine (1 μg/ml), 1 μM ammonium molybdate, 1 μM selenous acid, 12 μM ferric citrate and Bacto-Peptone (Difco Laboratories) (0.5%). Strains LCB517 and NH30 were grown in the presence of adenine (50 μg/ml) and tetracycline (20 μg/ml), respectively. After autoclaving, glucose (1%), $NaHCO_3$ (50 mM) and TMAO (0.5%) were added from sterile solutions. The cells were grown at 37°C as nonstirred standing cultures in 1- or 2-l flasks completely filled with medium.

The cells were harvested by centrifugation at 5000 rpm for 20 min in a JA10 rotor of a Beckman J-21 centrifuge. The cells were washed three times by suspension in 0.9% NaCl and resedimentation as above. For experiments with intact cells, the cells were suspended at a concentration of 1 g/5 ml in 50 mM potassium phosphate, pH 7.0.

Everted membrane vesicles were prepared from cells disrupted in a French press as described previously [12]. The membranes were suspended at a concentrations of about 20 mg protein/ml in 50 mM potassium phosphate, pH 7.0.

Difference spectra and redox titration

Reduced minus oxidized difference spectra were recorded at 77 K in 1 M sucrose in 0.1 M potassium phosphate, pH 7.0. When a substrate (NADH, glucose, formate) was used as the reductant, it was added to 2 ml of cells or membranes suspended in the sucrose/phosphate buffer in a narrow diameter tube. After 10 min at room temperature one-half of the sample was transferred to the sample cuvette of the cryogenic cell and kept for a further 5 min before it was frozen in liquid nitrogen. Care was taken to avoid undue aeration during the transfer of sample to the cuvette. Oxidant (TMAO or ammonium persulfate) was added to the other half of the sample before it was transferred to the reference cuvette of the cryogenic cell. The reference sample was frozen after a further 5 min incubation at room temperature. The samples were immediately frozen if sodium dithionite and H_2O_2 were used as reductant and oxidant. Further details for running the spectra are given in Ref. 11.

Redox titration of the membrane *b*- and *c*-type cytochromes was carried out at pH 7.0 in 0.1 M potassium phosphate, pH 7.0, as described previously [12,13,15].

Determination of protein

Protein was measured by the method of Lowry et al. [16], using bovine serum albumin as a standard.

Results

Cytochromes of cells grown anaerobically with TMAO

The cytochromes of the respiratory chain of *E. coli* grown aerobically show maxima in reduced minus oxidized difference spectra at 556, 558, 562,

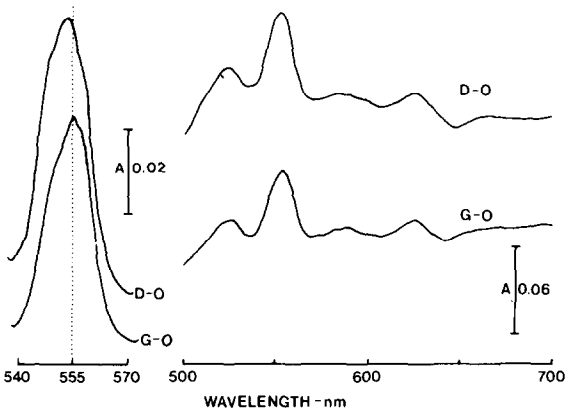

Fig. 1. Dithionite (D − O)- and glucose (G − O)-reduced minus H_2O_2-oxidized difference spectra of intact of cells of NH30 (*narI::Tn* 10) measured at 77 K. The concentration of glucose was 12.5 mM. *A*, absorbance units.

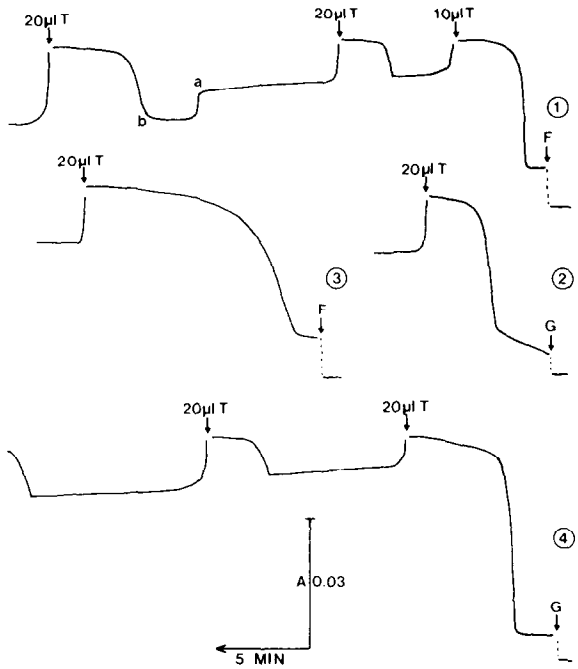

Fig. 2. Reduction of *b*- and *c*-type cytochromes in intact cells of various strains. (1) HfrH, (2,3) NH30 (*narI::Tn* 10), (4) LCB517 (*fdhA*). Cytochrome reduction was measured at room temperature by scanning the absorption of the sample at 555 nm relative to 575 nm with a Perkin-Elmer 356 spectrophotometer operating in the dual-wavelength mode. The cuvette contained 2 ml of cell suspension in 50 mM potassium phosphate, pH 7.0, to which 25 µl of 3% H_2O_2 were added at the commencement of the experiment. The following additions were made: (C) 25 µl of 1 M glucose, (F) 25 µl of 1 M sodium formate, (T) volumes of 0.66 M TMAO as indicated. The traces run from right to left. Phases 'a' and 'b' are discussed in the text. *A*, absorbance units.

590 and 625 nm (with a through at 646 nm) which have been attributed to cytochromes *b*-556, *b*-558, *b*-562, a_1 and *d*, respectively [2,3]. Growth of *E. coli* anaerobically in the presence of nitrate results in the repression of the formation of cytochromes *b*-558, a_1 and *d*, and in the appearance of a new absorption maximum in the difference spectrum at 555.5 nm which has been attributed to the presence of cytochromes b^{fdh} and b^{nr}, the cytochromes associated with formate dehydrogenase and nitrate reductase, respectively [17]. When *E. coli* NH30 was grown anaerobically with TMAO the dithionite-reduced minus H_2O_2-oxidized difference spectrum showed a maximum at 553 nm on which shoulders at 548 and 557 nm were evident (Fig. 1). Cytochromes a_1, *d* and *o* (detected by its carbon monoxide spectrum) [12] were also present. This spectrum was clearly different from those seen in cells grown aerobically or anaerobically with nitrate (or fumarate). When glucose was used as the reductant, the absorption in the difference spectrum was less, and the maximum was shifted to 555 nm. Shoulders at 548, 552 and 557 nm were present (Fig. 1). The same spectrum was seen with formate as reductant. The decrease in cytochrome reduction and the shift in the position of the absorption maximum were due to the lesser ability of glucose and formate to reduce cytochromes absorbing at 548 and 552 nm.

Oxidation of cytochromes by TMAO in intact cells

The reduction and oxidation of the cytochromes contributing to the absorption peak observed at 550–560 nm could be conveniently followed at room temperature by dual-wavelength spectrophotometry in which changes in the absorption at 555 nm were measured relative to the isosbestic wavelength of 575 nm. Since the cytochromes in intact cells were already reduced by endogenous substrates, the experiment was commenced by reoxidizing the cytochromes with a small amount of hydrogen peroxide. As shown in Fig. 2 (curve 1) for wild-type *E. coli* HfrH addition of formate rereduced the cytochromes to an aerobic steady-state level. This was followed after 1 min,

when all dissolved oxygen in the cuvette had been consumed, by further reduction of the cytochromes to their anaerobic steady-state level of reduction. Now, addition of 6.6 μmol TMAO resulted in oxidation of about 22% of the reduced cytochromes. A steady state was established until all of the TMAO had been consumed when rereduction of the cytochromes to the anaerobic steady-state level occurred. If 13.2 μmol TMAO were now added a complex pattern of oxidation and reduction was observed. Initially, TMAO oxidized the cytochromes to the steady-state level of reduction observed previously, but the extent of oxidation gradually increased as formate became depleted. When all the formate had been consumed, there was an abrupt drop to a new steady-state level of cytochrome reduction (point a on Fig. 2, curve 1). This steady state was that which could be maintained by the endogenous substrates in the presence of TMAO. At point 'b' (Fig. 2, curve 1) all of the TMAO had been consumed and endogenous substrates were able to reduce the cytochromes to the anaerobic steady-state level. Now, addition of TMAO reoxidized the cytochromes immediately to the endogenously maintainable steady state. That the changes in the steady-state levels of reduction were due to depletion of TMAO and formate was shown in control experiments using different amounts of these substances. Oxygen present in the added solutions produced only small and transient changes in the reduction state of the cytochromes.

Use of the otherwise isogenic *narI* strain NH30, in which cytochrome b^{nr} is not present [13], showed that this cytochrome is not part of the pathway leading to reduction of TMAO. Addition of glucose or formate to cells of this strain reduced cytochromes which could be reoxidized by TMAO (Fig. 2, curves 2 and 3). Although not shown, the cytochromes were rereduced following consumption of the added TMAO. Since formate as well as glucose was an effective electron donor for the reduction of TMAO in these experiments, it was of interest to determine if glucose had to be converted to formate in order to reduce TMAO. The strain used in the experiment shown in Fig. 2, curve 4, lacked formate dehydrogenase activity and cytochrome b^{fdh} [13]. Addition of formate to these cells did not result in reduction of cytochromes. However, glucose reduced the TMAO-oxidizable cytochromes and cycles of cytochrome oxidation and reduction due to TMAO reduction were observed. Clearly, another substrate as well as formate could provide electrons for the reduction of TMAO.

Identification of cytochromes oxidized by TMAO in intact cells

Using *E. coli* NH30 to avoid possible interference from the presence of cytochrome b^{nr}, it was possible to examine the spectra of the cytochromes oxidized by TMAO in intact cells. The glucose-reduced minus H_2O_2-oxidized difference spectrum (Fig. 3, curve G-O) showed an absorption maximum at 555 nm together with shoulders on this peak at about 548 and 557 nm. Cytochromes a_1 and d were also reduced. The cytochromes oxidized following addition of TMAO in the anaerobic steady state were demonstrated by measuring the difference spectrum between cells reduced by glucose in the presence and absence of TMAO (Fig. 3, curve G − G + T). Little, if any, cytochrome a_1 and d were oxidized by TMAO.

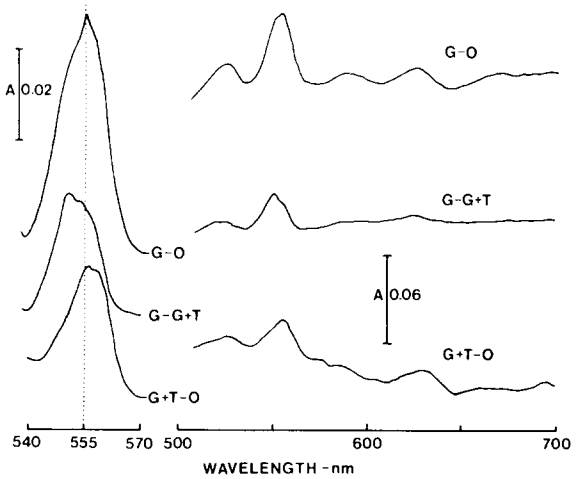

Fig. 3. Reduced minus oxidized difference spectra of intact cells of NH30 (*narI::Tn* 10) measured at 77 K. (G − O) Glucose (12.5 mM)-reduced minus H_2O_2-oxidized spectrum. (G − G + T) The cytochromes were reduced with glucose (12.5 mM) and TMAO (13.2 mM) was added to the reference sample 5 min before freezing. (G + T − O) The cytochromes were reduced with glucose and partially reoxidized by addition of TMAO 5 min before freezing. The difference spectrum was measured relative to a reference sample which contained H_2O_2 in addition. *A*, absorbance units.

However, cytochromes with absorption maxima at 548 and 552 nm (these give a composite peak at 550 nm), 554 and 557 nm were oxidized by TMAO. The cytochromes reduced by glucose, but not re-oxidized by TMAO, had absorption maxima at 556 and 558 nm (Fig. 3, curve G + T − O). Since, cytochromes a_1 and *d* also showed this behavior, it was concluded that those cytochromes not oxidized by TMAO were those of the aerobic respiratory chain. The results suggest that the cytochromes of the two pathways are independent.

Cellular location of cytochromes oxidized by TMAO in intact cells

The cellular location of the cytochromes of the TMAO reduction pathway was demonstrated by using ammonium persulfate as a membrane-impermeant oxidant. Cytochromes reducible by dithionite but oxidizable by TMAO showed an absorption maximum at 550 nm, with a shoulder on the peak at 557 nm. Cytochromes a_1 and *d* were oxidized only slightly by TMAO (Fig. 4, curve D − T). A similar spectrum was obtained using ammonium persulfate as the oxidant, although the contributions of the cytochromes absorbing at 548 and 552 nm to the peak at 550 nm were more evident (Fig. 4, curve D − AP). Cytochromes *b*-556, *b*-558, a_1 and *d* of the aerobic respiratory pathway were not oxidized by TMAO (Fig. 4, curve T − O) or ammonium persulfate (data not shown). Similar results were obtained if glucose or formate were used as reductants of the cytochromes. However, less cytochrome absorbing at 548 and 552 nm was reduced by these substrates.

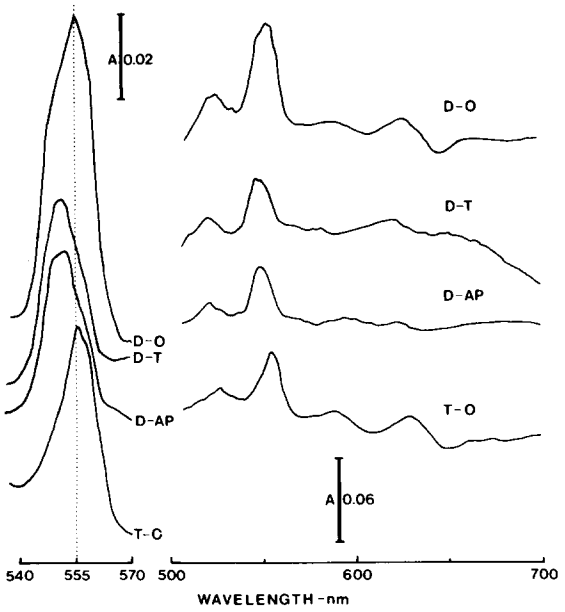

Fig. 4. Reduced minus oxidized difference spectra of intact cells of HfrH measured at 77 K. (D − O) Dithionite-reduced minus H_2O_2-oxidized difference spectrum. (D − T) Dithionite-reduced minus TMAO-oxidized difference spectrum. TMAO (3.3 mM) was added to the reference sample 5 min before freezing. (D − AP) Dithionite-reduced minus ammonium persulfate-oxidized difference spectrum. (T − O) TMAO-oxidized minus H_2O_2-oxidized difference spectrum. TMAO was added to the sample 5 min before freezing. *A*, absorbance units.

These results suggest that the cytochromes and/or the trimethylamine-*N*-oxide reductase of the TMAO reduction pathway are located on the external surface of the cytoplasmic membrane or in the periplasmic space. The reduction of these cytochromes and their oxidation by TMAO were not affected by submitting the cells to the osmotic shock procedure of Abou-Jaoudé et al. [18]. This procedure had been found to release the periplasmic cytochrome *c*-552 of the formate-nitrite reductase system of *E. coli*. Thus, it is likely that the cytochromes of the TMAO reduction pathway are more tightly bound to the cytoplasmic membrane than those of the nitrite reductase pathway.

Oxidation of cytochromes by TMAO in everted membrane vesicles

The presence of endogenous substrates in intact cells complicated studies on the source of electrons for the reduction of TMAO. Everted membrane vesicles were used to overcome this difficulty. The cytochromes of the vesicles were in the oxidized state until a reductant was added.

The reduction and oxidation of the cytochromes contributing to the absorption peak at about 550–560 nm were studied by dual-wavelength spectrophotometry (Fig. 5). Addition of formate to an aerobic suspension of vesicles resulted in a clearly triphasic sequence of cytochrome reduction on anaerobiosis (Fig. 5, curve 1). An initial very rapid phase was followed by a slower phase of reduction to reduce about 50% of the cytochromes which were present. After 3–4 min a third phase of reduction resulting in the reduction of a further 25% of the cytochromes became evident. About

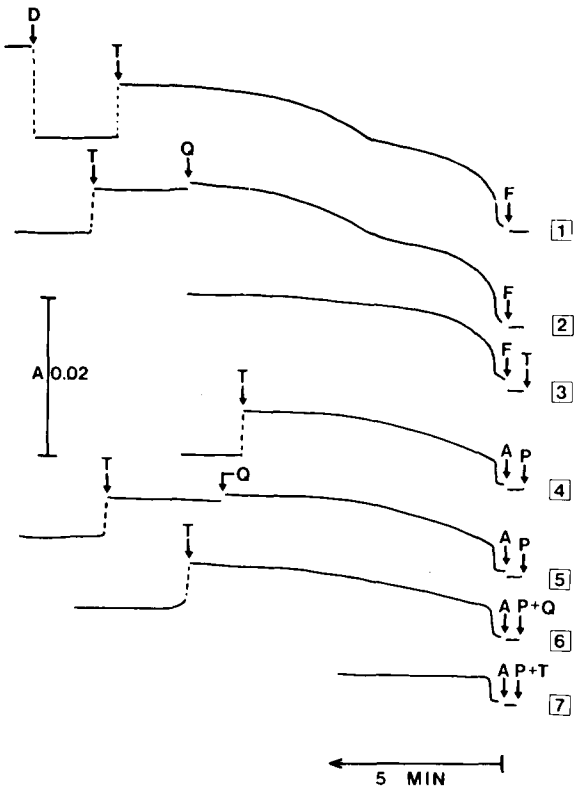

Fig. 5. Reduction of *b*- and *c*-type cytochromes is everted membrane vesicles of NH30 (*narI::Tn* 10). Cytochrome reduction was measured as described in the legend to Fig. 2 except that H_2O_2 was not added. The following additions were made to 2 ml of membrane suspension (20 mg protein) in 0.1 M potassium phosphate, pH 7.0, containing 1 M sucrose: (F) 25 μl of 1 M sodium formate, (T) 25 μl of 0.66 M TMAO, (D) a crystal of sodium dithionite, (Q) 5 μl of 38 mM HOQNO in ethanol, (P) 10 μl of 10 mM phenazine methosulfate, (A) 25 μl of 0.2 M potassium ascorbate, pH 7.0. The traces run from right to left.

25% of the cytochrome was not reduced by formate but could be reduced by dithionite. Addition of 16.5 μmol TMAO resulted in immediate reoxidation of 40% of the cytochrome. This reoxidation was not due to the addition of oxygen, since control experiments showed that this gave only a small and transient change in the reduction state of the cytochromes. The inhibitor HOQNO had only a slight effect on the extent of oxidation produced by TMAO (Fig. 5, curve 2). Addition of HOQNO to the system before formate resulted in a slower rate of reduction of cytochrome in all phases but did not markedly affect the extent or rate of oxidation obtained with TMAO. If TMAO was added prior to the addition of formate then only the first two phases of cytochrome reduction were observed (Fig. 5, curve 3). Similar data were obtained when formate was replaced by NADH (results not shown). As shown below, the cytochrome reduced in the first two phases were those of the aerobic respiratory chain. In membrane vesicles the rate of electron transfer from substrate to the TMAO reduction pathway is slower than that to the aerobic cytochromes. This is shown by the slowness of reduction of the TMAO-oxidizable cytochromes in the third phase and their complete oxidation on addition of TMAO.

Two phases of cytochrome reduction could be recognized when the higher potential electron donor system of ascorbate with phenazine methosulfate was used (Fig. 5, curves 4–7). This system will reduce cytochromes of midpoint redox potential greater than 0 mV. The more slowly reducing cytochromes were reoxidized by TMAO in a reaction which was insensitive to HOQNO. The amount of TMAO-oxidizable cytochrome reduced by ascorbate with phenazine methosulfate was about 85% of the amount of TMAO-oxidizable cytochrome reduced by formate or NADH. This result suggested that the TMAO-oxidizable cytochrome had a midpoint oxidation-reduction potential of 0 mV or greater.

The identity of the cytochromes undergoing oxidation and reduction in the everted membrane vesicles was examined by taking difference spectra (Fig. 6). The cytochromes reduced by formate showed a main absorption peak at 555.5 nm with shoulders at 557.5, 552 and 548 nm (Fig. 6, curve 2). Cytochrome a_1, *o* and *d* were also reduced. Dithionite reduced a larger amount of cytochrome absorbing in the 550 nm region, resulting in a shift in the position of the absorption maximum to 554 nm. As with formate, shoulders on the absorption peak were seen at 548 and 557.5 nm (Fig. 6, curve 1). A difference spectrum between dithionite- and formate-reduced systems showed a maximum at 552.2 nm with a marked shoulder at 548 nm (data not shown) confirming that formate (and NADH) was unable to reduce a substantial amount (25–30%) of cytochromes absorbing at these wavelengths.

The absorption spectrum of the formate-reduci-

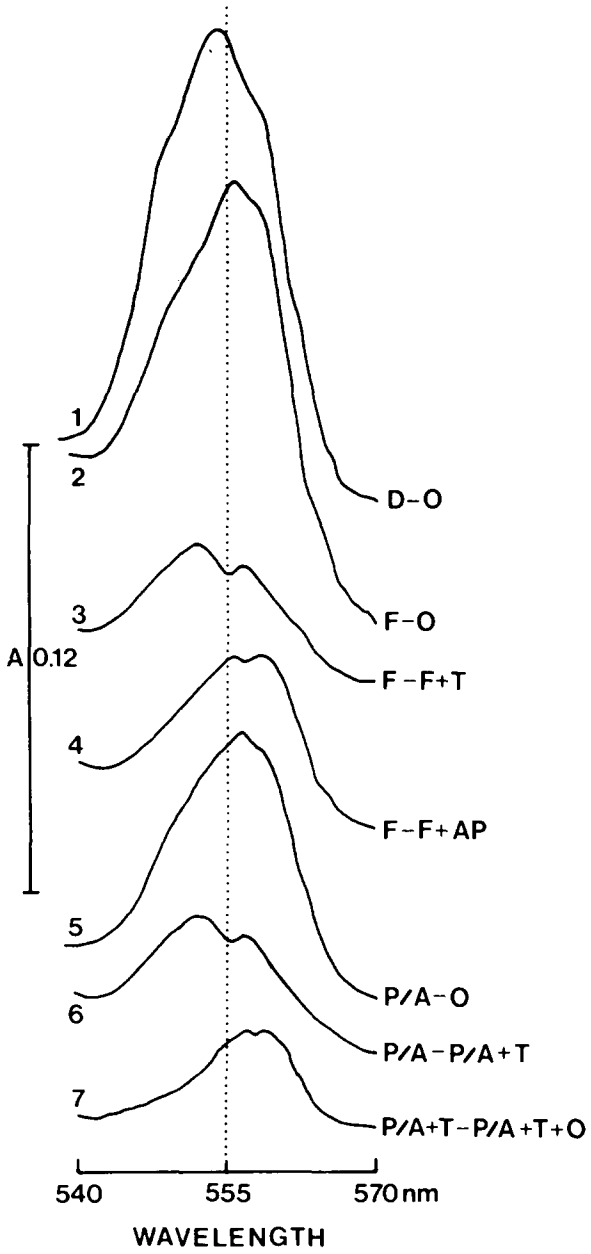

Fig. 6. Reduced minus oxidized difference spectra of *b*- and *c*-type cytochromes in everted membrane vesicles of NH30 (*narI::Tn* 10) measured at 77 K. (D − O) Dithionite-reduced minus H_2O_2-oxidized difference spectrum. (F − O) Formate (12.5 mM)-reduced minus H_2O_2-oxidized difference spectrum. (F − F + T) The cytochromes were reduced with formate and TMAO (16.5 mM) was added to the reference sample 5 min before freezing. (F − F + AP) The cytochromes were reduced with formate and ammonium persulfate was added to the reference sample 5 min before freezing. (P/A − O) Ascorbate (2.5 mM) with phenazine methosulfate (50 μM)-reduced minus H_2O_2-oxidized difference spectrum. (P/A − P/A + T) The cytochromes were reduced with ascorbate and phenazine methosulfate. TMAO was added to the reference sample 5 min before freezing. (P/A + T − P/A + T + O) The cytochromes were reduced with ascorbate and phenazine methosulfate and partially reoxidized by TMAO 5 min before freezing. The reference sample contained H_2O_2 in addition. *A*, absorbance units. Protein concentration, 10 mg/ml.

ble cytochromes oxidized by TMAO showed maxima at 557 and 552 nm with a shoulder at 548 nm (Fig. 6, curve 3). A different spectrum was obtained when the membrane-impermeant oxidant ammonium persulfate was used instead of TMAO (Fig. 6, curve 4). The TMAO-nonoxidizable cytochromes showed maxima at 556 and 558 nm (results not shown). Cytochromes a_1 amd *d* were oxidized by ammonium persulfate but not by TMAO.

Results similar to those above were obtained when ascorbate with phenazine methosulfate was used as the reductant. A reduced minus oxidized difference spectrum showed peaks and shoulders at wavelengths similar to those observed with formate (Fig. 6, curve 5). However, less cytochrome, particularly of components absorbing in the wavelength range 555–560 nm, was reduced by the high-potential reductant. The decrease in reduction of these cytochromes was particularly evident when the formate- and ascorbate-reducible cytochromes not oxidized by TMAO were compared (Fig. 6, curves 4 and 7). The amounts of formate- and ascorbate-reducible cytochromes oxidizable by TMAO were almost identical, confirming their relatively high midpoint potential.

The dual-wavelength and difference spectra studies confirmed the coexistence in everted vesicles of cytochromes of the aerobic (cytochromes *b*-556, *b*-558, a_1 and *d*) and TMAO reduction (cytochromes *c*-548, *c*-552 and *b*-557) pathways. In contrast to intact cells, the cytochromes and/or trimethylamine-*N*-oxide reductase of the TMAO reduction pathway were located on the interior membrane surface of the vesicle and were not accessible to the nonpermeant oxidant ammonium persulfate.

Midpoint oxidation-reduction potentials of cytochromes in membranes from cells grown with TMAO

A redox titration of the cytochromes absorbing in the 550–560 nm range in membranes from *narI*

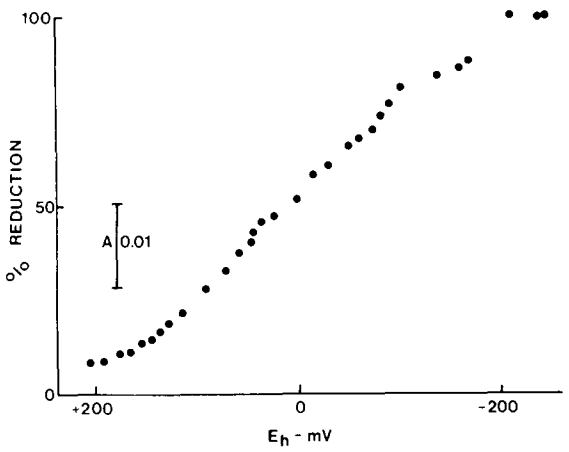

Fig. 7. Redox titration of *b*- and *c*-type cytochromes in everted membranes vesicles of NH30 (*narI::Tn* 10). The % reduction of the cytochromes is plotted against the applied oxidation-reduction potential (E_h). Everted membrane vesicles (10.6 mg/ml) were titrated as described in the text. *A*, absorbance units.

strain NH30 grown anaerobically with TMAO is shown in Fig. 7. Several components with different midpoint potentials were clearly present. Computer-based curve analysis [12,13,15] for up to a minimum of four components did not give a satisfactory fit of the data points. Analysis for more than this number of components is in general of little value and so was not carried out [12,13]. However, it is evident from these results that about one-half of the cytochrome was not reduced at a potential of 0 mV. Part of this cytochrome was undoubtedly cytochrome b^{fdh} ($E_m = -110$ mV) [13]. The remainder may be those cytochromes reducible by dithionite but not by NADH and formate.

Sequence of cytochromes in the TMAO reduction pathway

The effect of 1 mM $CuSO_4$, a sulfhydryl-reacting inhibitor, on the oxidation by TMAO of formate-reduced cytochromes in intact cells of the *narI* strain NH30 is shown in Fig. 8, panel 1. $CuSO_4$ decreased the amount of cytochrome which was oxidized on addition of TMAO. The rate of reduction of TMAO by formate was also diminished by $CuSO_4$ as shown by the increase in time before the anaerobic steady-state level of reduction was reestablished after the addition of TMAO.

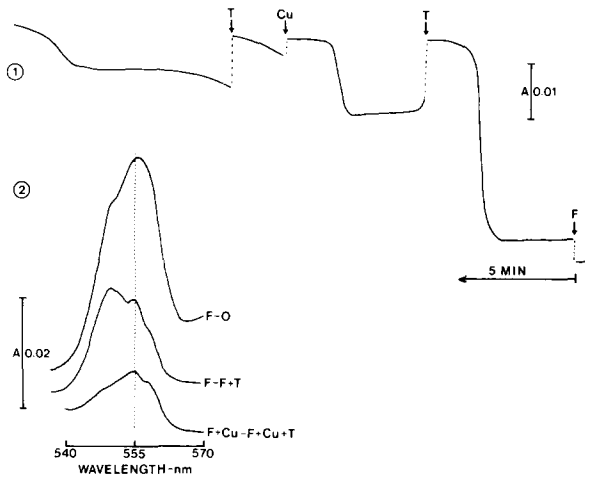

Fig. 8. Effect of $CuSO_4$ on the reduction of *b*- and *c*-type cytochromes in intact cells of NH30 (*narI::Tn* 10). (Panel 1) The reduction of cytochrome was measured as described in the legend to Fig. 2. The following additions were made to 2 ml of cell suspension: (F) 25 μl of 1 M sodium formate, (T) 10 μl of 0.66 M TMAO, (Cu) 50 μl of 40 mM $CuSO_4$. The trace runs from right to left. (Panel 2) Reduced minus oxidized spectra measured at 77 K. (F − O) Formate (12.5 mM)-reduced minus H_2O_2-oxidized. (F − F + T) The cytochromes were reduced with formate. TMAO (16.5 mM) was added to the reference sample 5 min before freezing. (F + Cu − F + Cu + T) The cytochromes were reduced with formate in the presence of 1 mM $CuSO_4$. TMAO was added to the reference sample 5 min before freezing. *A*, absorbance units.

The cytochromes oxidized by TMAO were identified from difference spectra. In the absence of $CuSO_4$ addition of TMAO resulted in the oxidation of cytochromes showing absorption maxima at 550 nm (this is considered to be due to fusion of the absorption peaks of cytochromes absorbing at 548 and 552 nm), 554 and 557 nm (Fig. 8, panel 2, curve F − F + T). In the presence of $CuSO_4$ the cytochromes contributing to the peak at 550 nm were no longer oxidized by TMAO. Those absorbing at 554 and 557 nm were still oxidized (Fig. 8, panel 2, curve F + Cu − F + Cu + T). Thus, $CuSO_4$ must have inhibited electron flow between the two groups of cytochromes on the TMAO reduction pathway.

Discussion

Previous studies have suggested that *b*- and *c*-type cytochromes are involved in the pathway

which supplies elèctrons to trimethylamine-*N*-oxide reductase for the reduction of TMAO to trimethylamine [6,10,11]. However, the number of the individual cytochromes and their sequence were not determined. Furthermore, the possibility that some of the cytochromes observed might be components of other respiratory pathways was not considered.

Reduced minus oxidized difference spectra, measured at 77 K, of cells and membranes of the *narI* strain NH30, used because it lacks cytochrome b^{nr} [13], showed a broad α-absorption peak in the 545–565 nm wavelength region. The maximum absorption was at 552–554 nm. This suggested that substantial amounts of *c*-type cytochromes, which generally absorb in the range of 548–554 nm [19], were present. The exact position of the maximum varied with the reductant used and also to some extent, from preparation to preparation. This was considered to be due to variations in the relative amounts of the several components whose presence was indicated by shoulders on the main absorption peak. The peak extended into the 555–565 nm range showing that *b*-type cytochromes were present. Cytochromes *o*, a_1 and *d* were also found in the cells grown anaerobically on TMAO. These cytochromes are components of the aerobic respiratory pathways [2,3]. Thus, *b*-type cytochromes of these pathways were likely contributing to the α-absorption peak observed at 545–565 nm. The multiple nature of the components contributing to this peak was also shown by redox titration. The presence of at least four cytochromes was indicated by these data.

We were able to recognize three groups of cytochromes in membranes from cells grown anaerobically on TMAO. About 15–25% of the cytochrome in intact cells and membranes was not reducible by substrate (glucose, formate, NADH) but could be reduced by dithionite. This cytochrome was characterized by absorption maxima at 548 and 552 nm, and so is likely to be *c*-type cytochrome. There are several possible explanations for the lack of reduction of this cytochrome by substrate. However, it is unlikely that the redox potential of this cytochrome is too low, since all of the cytochrome in isolated membranes was reduced at a potential of -200 mV (Fig. 7). The redox potentials of the $NAD^+/NADH$ and CO_2/formate couples are -320 and -420 mV, respectively. It may be that this cytochrome is part of another system or that it has not been assembled into a functional respiratory chain.

The second group of cytochromes appeared to be components of the aerobic respiratory pathways. They constituted about 50% of the total cytochromes present, and were rapidly reduced in membranes by formate and NADH. They were revealed when the cytochromes of the TMAO reduction pathway had been oxidized by TMAO. Absorption peaks in reduced minus oxidized difference spectra characteristic of cytochromes *b*-556, *b*-558, a_1 and *d* were observed. Cytochrome b^{fdh} should also be present although its absorption peak at 555 nm [5,13] was masked by those of the other cytochromes.

The third groups of cytochromes were those considered to be in the TMAO reduction pathway, since they were rapidly reoxidized by TMAO. Although they were rapidly reduced by formate and by metabolites of glucose in intact cells, they were reduced more slowly in membranes than those of the aerobic pathways. This has aided in their identification. Reoxidation of reduced cytochromes by TMAO showed that a *b*-type cytochrome with an absorption maximum at 557 nm was component of the TMAO reduction pathway. In intact cells reoxidation by TMAO also revealed an absorption peak at 550 nm. In membranes, this was at 552 nm, with a shoulder on the peak at 548 nm. This suggests that two *c*-type cytochromes, absorbing maximally at about 548 and 552 nm, were contributing to absorption changes in this region. Shoulders at these wavelengths on the main absorption peak, which were detected when different substrates were used, support this explanation. Changes in the relative amounts or extent of reduction of these cytochromes would be responsible for the observed changes in the wavelength of maximum absorption. TMAO oxidation experiments with membranes, or with intact cells in the presence of $CuSO_4$, showed that a further *b*(?)-type cytochrome, with an α-absorption band at 554 nm (Figs. 3 and 8), was present in the TMAO reduction pathway. It should be noted that the wavelengths assigned to the α-absorption bands of the cytochromes of this pathway must be considered tentative because of the great degree of overlapping of the absorption bands. Moreover, it is

possible that cytochromes with a double α-band, like cytochrome b_5 [20], may be present.

$CuSO_4$ inhibited the reduction of TMAO by formate. A site of inhibition in the TMAO reduction pathway prevented the reoxidation of cytochromes *c*-548 and *c*-552 by TMAO. Cytochromes *b*(?)-554 and *b*-557 were reoxidized. This result permits the assignment of a sequence of electron transfer in the TMAO reduction pathway as follows:

$$\text{Substrate} \rightarrow \begin{matrix} c\text{-}548 \\ c\text{-}552 \end{matrix} \xrightarrow[CuSO_4]{} b(?)\text{-}554\ b\text{-}557 \rightarrow \begin{matrix} \text{trimethylamine-} \\ N\text{-oxide} \\ \text{reductase} \end{matrix} \rightarrow \text{TMAO}$$

The immediate source of electrons for the cytochromes of this pathway is probably ubiquinone or menaquinone, since a mutant lacking these quinones had lost NADH-trimethylamine-*N*-oxide reductase activity [7]. The quinones may provide a branch point between the aerobic respiratory pathways and the TMAO reduction pathway, since NADH and formate supply electrons to both pathways.

The NADH- and formate-trimethylamine-*N*-oxide reductase activities in membrane preparations were lower than the NADH and formate oxidase activities. This contrasts with the active and comparable rates of reduction of TMAO and oxygen by glucose and formate in intact cells. Either a 'factor' required by the pathway has been depleted by disruption of the cells or else the linkage between the dehydrogenase and the cytochromes of the TMAO reduction pathway has been affected. There is not apparent loss of cytochrome on disruption.

The use of ammonium persulfate as a membrane-impermeant oxidant revealed a feature of the organization of the components of the TMAO reduction pathway. Cytochromes of this pathway were reoxidized by persulfate in intact cells whereas those of the aerobic respiratory pathways remained unaffected. The opposite behavior was seen with everted membrane vesicles. On the basis of studies on the organization of the cytochromes of the respiratory chain in mammalian mitochondria [21], it is unlikely that all of the cytochromes of the aerobic pathways would be directly accessible to persulfate on the inner surface of the cytoplasmic membrane. Thus, it is probable that the observed oxidation was caused by reaction of this oxidant with the terminal oxidase cytochrome *d* (and probably *o*), and with trimethylamine-*N*-oxide reductase. This result indicates that the active site of cytochrome *d* (and probably of *o*) is at the cytoplasmic surface of the cell membrane whereas that of trimethylamine-*N*-oxide reductase faces the periplasm.

Acknowledgements

This work was supported by a grant from the Medical Research Council of Canada. N.R.H. is a Commonwealth Scholar.

References

1 Bragg, P.D. (1980) in Diversity of Bacterial Respiratory Systems (Knowles, C.J., ed.), Vol. 1, pp. 115–136, CRC Press, Boca Raton, FL
2 Bragg, P.D. (1979) in Membrane proteins in energy tranduction (Capaldi, R.A., ed.), pp. 341–449, Marcel Dekker, New York
3 Haddock, B.A. and Jones, C.W. (1977) Bacteriol. Rev. 41, 47–99
4 Stouthamer, A.H., Van 't Riet, J. and Oltmann, L.F. (1980) in Diversity of Bacterial Respiratory Systems (Knowles, C.J., ed.), Vol. 2, pp. 19–48, CRC Press, Boca Raton, FL
5 Enoch, H.G. and Lester, R.L. (1975) J. Biol. Chem. 250, 6693–6705
6 Ishimoto, M. and Shimokawa, O. (1978) Z. Allg. Mikrobiol. 18, 173–181
7 Cox, J.C. and Knight, R. (1981) FEMS Microbiol. Lett. 12, 249–252
8 Takagi, M., Tsuchiya, T. and Ishimoto, M. (1981) J. Bacteriol. 148, 762–768
9 Cox, J.C., Madigan, M.T., Favinger, J.L. and Gest, H. (1980) Arch. Biochem. Biophys. 204, 10–17
10 Sakaguchi, M. and Kawai, A. (1977) Bull. Jap. Soc. Sci. Fish. 43, 437–442
11 Sakuguchi, M. and Kawai, A. (1978) Bull. Jap. Soc. Sci. Fish. 44, 511–516
12 Hackett, N.R. and Bragg, P.D. (1983) J. Bacteriol. 154, 708–718
13 Hackett, N.R. and Bragg, P.D. (1983) J. Bacteriol. 154, 719–727
14 Davis, B.D. and Mingioli, E.S. (1950) J. Bacteriol. 60, 17–28
15 Hackett, N.R. and Bragg, P.D. (1982) FEMS Microbiol. Lett. 13, 213–217
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
17 Ruiz-Herrera, J. and DeMoss, J. (1969) J. Bacteriol. 99, 720–729
18 Abou-Jaoudè, A., Pascal, M.-C. and Chippaux, M. (1979) Eur. J. Biochem. 95, 315–321
19 Lemberg, R. and Barrett, J. (1973) Cytochromes, Academic Press, New York
20 Bois-Poltoratsky, R. and Ehrenberg, A. (1967) Eur. J. Biochem. 2, 361–365
21 Capaldi, R.A. (1982) Biochim. Biophys. Acta 694, 291–306