# 大腸菌によるトリメチルアミンN-オキサイドの還元にたいするチトクロムの関与



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# The Participation of Cytochromes in the Reduction of Trimethylamine *N*-oxide by *Escherichia coli*

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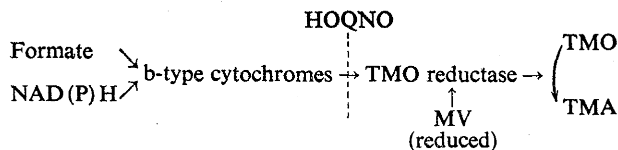
The participation of cytochromes of the membrane preparation from *Escherichia coli* in the reduction of trimethylamine *N*-oxide (TMO) was investigated spectrophotometrically.

Two physiological reductants, formate and NADH, could reduce the cytochromes in the membrane preparation. The difference spectra of the cytochromes reduced by both the reductants were entirely the same as the spectrum recorded with $Na_2S_2O_4$ as a reductant; all these spectra had 3 major peaks: at 555 nm, 526 nm, and 425–426 nm. The low-temperature spectrum obtained at liquid nitrogen temperature (77K) revealed that the cytochromes are composed of multiple components. The formate- and NADH-reduced cytochromes were gradually reoxidized by TMO, suggesting the participation of the cytochromes in TMO reduction. 2-Heptyl-4-hydroxyquinoline *N*-oxide (HOQNO) partially inhibited the reoxidation by TMO. In connection with this HOQNO-inhibition, different types of cytochrome components are discussed.

In the previous paper[1], it was suggested that the membrane fraction but not the supernatant fraction obtained by centrifugation of *Escherichia coli* extracts at $48,000 \times g$ for 30 min was capable of utilizing formate and NAD(P)H as major electron donors for the reduction of trimethylamine N-oxide (TMO) to trimethylamine (TMA). 2-Heptyl-4-hydroxyquinoline *N*-oxide (HOQNO) markedly inhibited the reducing activity of the membrane fraction. This indicated the apparent participation of b-type cytochromes in the electron transport system. HOQNO, however, failed to inhibit TMO reduction linked to reduced methylviologen (MV). In view of these evidence we proposed the following scheme for a possible mechanism of the reduction of TMO:




The reductase in the above scheme was also found, being membrane-bound in its nature[2].

Apart from exact identification of types of the cytochromes involved in this system, the present investigation was undertaken to show more direct evidence on the participation of cytochromes bound to *E. coli* membranes in the reduction of TMO.

## Materials and Methods

### *Growth of Organism and Preparation of Membrane Fragments*

The strain of *Escherichia coli*, growth condition, and subsequent spheroplast formation from cells induced with TMO were all the same as reported previously[1]. Membrane fragments were collected in exactly the same manner as also described previously[1], and suspended in 0.1 M Tris-HCl buffer containing 5 mM $MgCl_2$ and 50 mM potassium phosphate (pH 7.2). This was referred to as a membrane preparation, unless otherwise stated. Cells induced with nitrate were obtained on the culture medium containing 2.0 g sodium nitrate per liter instead of TMO and the membrane preparation was obtained in the same manner.

### *Spectrophotometry*

For reduction of cytochromes, 3 m*l* of the membrane preparation was placed in the main compartment of a Thunberg-type cuvette (light path, 10 mm). In the side arm was taken liquid paraffin with a volume to be layered as high as 3–4 cm over the content of the main compartment. After replacement of the atmosphere by purified

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argon (99.99%), the liquid paraffin was transfered to the main compartment by tipping the side arm and then the mixture was preincubated for 30 min at 37°C. Either 10 μmol sodium formate or 7 μmol NADH dissolved in 75 μl of the buffer was added through the liquid paraffin layer in the open cuvette, followed by incubation at room temperature (25°C) for 25–60 min to complete the reduction of cytochromes in the membrane preparation. For full reduction of the cytochromes, a few grains of $Na_2S_2O_4$ were added directly to the membrane preparation in an open cuvette without containing liquid paraffin. In this case no incubation was carried out and the $Na_2S_2O_4$-reduced cytochromes were brought immediately to spectrum recording.

Difference spectra were recorded at room temperature by placing the membrane preparation thus reduced in the sample cuvette and that oxidized with either air or TMO in the reference cuvette.

For measurement of low-temperature difference spectra at liquid nitrogen temperature (77K), the membrane fragments collected by centrifugation at 48,000×g for 30 min were suspended in 50 mM potassium phosphate buffer (pH 7.2), mixed with an equal volume of glycerol. The difference spectrum was recorded with the mixture reduced with $Na_2S_2O_4$ against that oxidized by air. Cuvette used had 2 mm-light path length.

*Determination of Protein*

Contents of protein were estimated by the method of LOWRY *et al.*[8] with bovine serum albumin as a standard.

## Results and Discussion

*Reduction of Cytochromes in the Membrane Preparation*

In the presence of the membrane fraction but not the supernatant fraction, formate and NAD(P)H were effective electron donors to the TMO-reducing system, as reported previously[1]. These physiological reductants as well as a nonphysiological reductant $Na_2S_2O_4$ were added to the membrane preparation to see whether they can actually reduce cytochromes. Fig. 1 illustrates reduced minus oxidized difference spectra, indicating that the reduced cytochromes had 3 peaks with maxima at 555 nm, 527 nm, and 425–426 nm. All the spectra obtained with the three reductants were same each other except that the maximum of the last peak of NADH-reduced cytochromes was at 425 nm. The extent of the reduction estimated from peak height ($\Delta A_{555-575}$ and $\Delta A_{555-540}$) was about 70% for these physiological reductants of that obtained by the addition of $Na_2S_2O_4$.

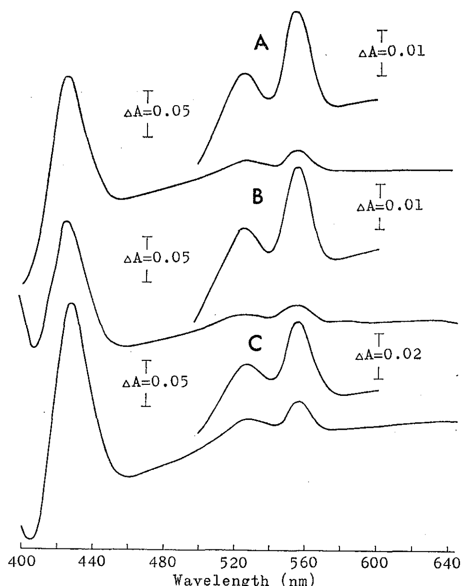


**Fig. 1.** Difference spectra of the cytochromes in the membrane preparation reduced by formate, NADH, and $Na_2S_2O_4$.

Formate and NADH were added to the membrane preparation (1.5 mg protein/ml) and incubated for 60 min and 25 min, respectively, to reduce the cytochromes. In the case of reduction by $Na_2S_2O_4$, no incubation was performed. Upper traces indicate the expansion of lower traces from 500 nm to 600 nm. (A), formate; (B), NADH; (C), $Na_2S_2O_4$.

In the spectra for all the reductants tested, no prominent peaks were detected at 590–600 nm and 630–634 nm corresponding to the α peaks of cytochrome $a_1$ and d[4,5], respectively. The difference spectra of the membrane preparation had the maximum at 555 nm of lower wavelength than the maximum values reported in *E. coli* membrane preparations[5,6]. The reported values were about 560 nm, which is due to the α peak of cytochrome $b_1$. One might suspect that the maximum with the lower wavelength resulted from contamination with soluble cytochrome c. This possibility, however, could be excluded by washing the spheroplasts and the membrane preparation with 0.75 M sucrose-0.1 M Tris buffer (pH 7.2) containing 0.3 M NaCl. The resulting difference spectra measured with similar amounts of the membrane preparation was completely the same as given in Fig. 1. This

indicates that there was no significant amount of soluble cytochrome c in the membrane preparation. The 555 nm peaks of all the spectra recorded with those reductants have apparently no shoulder on either the short or long wavelength flank. Low-temperature spectra, however, will allow shoulders to be more distinct by sharpening the associated peaks and roughs of the cytochromes. Fig. 2 shows a low-temperature spectrum of the cytochromes in the membrane preparation, indicating the 3 peaks with maxima at 551 nm, 523.5 nm, and 527 nm. The 551 nm peak displayed a somewhat peculiar shape: there were at least 3 shoulders detected at 549.5 nm, 552.5 nm and 558 nm. These shoulders suggest the existence of multiple components of the cytochromes. The spectral properties of the components greatly differ from those of cytochrome components which were reported by SHIPP[7]. He found that membrane fragments from aerobically grown *E. coli* had 548 nm and 552 nm components both of which may have been due to a c-type cytochrome; 556 nm, 559 nm, and 565 nm components to a b-type cytochrome. Also a- and d-type cytochromes had been found.

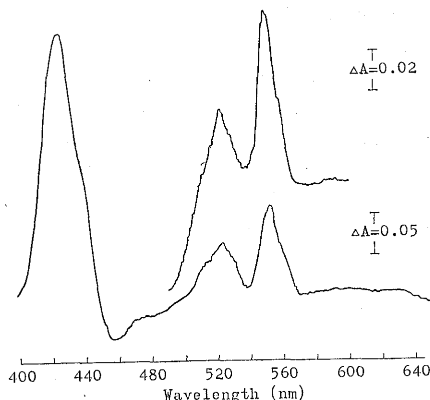


**Fig. 2.** Low-temperature difference spectrum of the cytochromes in the membrane preparation.

The membrane fragments were suspended in 50% glycerol and 50 mM of the phosphate buffer (pH 7.2) (2.5 mg protein/m*l*).

Room- and low-temperature difference spectra were recorded with the membrane preparation from nitrate-induced cells for comparison. Generally, nitrate-induced *E. coli* contains cytochrome $b_1$ in membranes[8]. Fig. 3 is the spectrum obtained at room temperature, having the peaks at 560 nm, 530 nm, and 430 nm. The 560 nm peak perfectly consists with the $\alpha$ peak of cytochrome $b_1$[9]. The low-temperature spectrum from the same origin is given in Fig. 4, where the peaks were found at 556 nm, 527 nm, and 429 nm. The peak at 556 nm was steep and almost symmetrical, in striking contrast to the comparable peak at 551 nm shown in Fig. 2, and seems to reflect the single component of cytochrome $b_1$ in the present preparation. In nitrate reduction by *E. coli* this cytochrome is implicated in both formate dehydrogenase and nitrate reductase with two different redox poten-

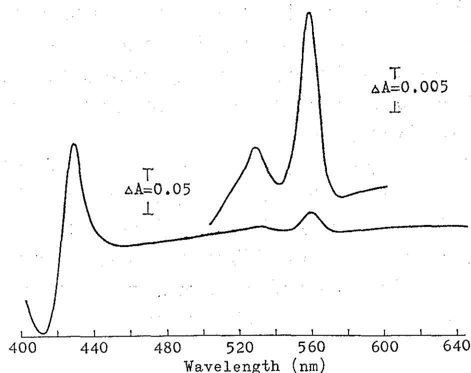


**Fig. 3.** Difference spectrum of the cytochrome in the membrane preparation from nitrate-induced cells.

The membrane preparation from nitrate-induced cells (1.3 mg protein/m*l*) was reduced with $Na_2S_2O_4$ and the spectrum was recorded against the air-oxidized sample.

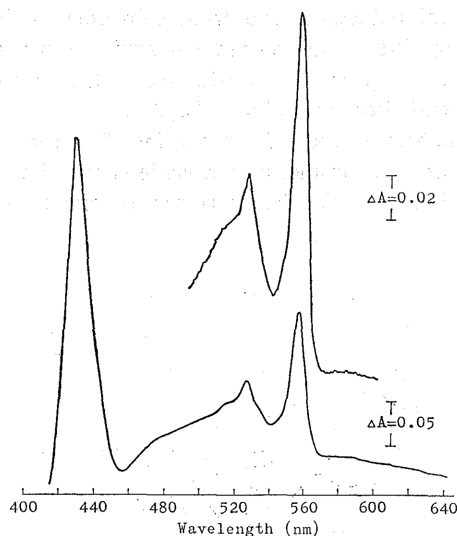


**Fig. 4.** Low-temperature difference spectrum of the cytochromes in the membrane preparation from nitrate-induced cells.

The membrane fragments were suspended in 50% glycerol and 50 mM of the phosphate buffer (pH 7.2) (3.1 mg protein/m*l*).

tials[10-12]. In the low-temperature spectrum of Fig. 2, we did not observe the presence of major components which would exactly correspond to the 556 nm peak ($\alpha$ peak) of cytochrome $b_1$ within experimental error.

These findings strongly support the idea that the cytochromes in the membrane preparation from cells induced by TMO are different from cytochrome $b_1$. The possibility, however, can not be excluded that a part of the cytochrome components belongs to a b-type cytochrome but not $b_1$.

*Reoxidation of the Formate- and NADH-Reduced Cytochromes by TMO*

Formate and NADH could effectively reduce $^{14}$C-TMO in the presence of the membrane fraction, as reported previously[1]. If some cytochromes are associated with this TMO reduction, the phenomenon can be interpreted as resulting from reduction of the cytochromes by the above physiological reductants and from subsequent oxidation by TMO. In order to verify this assumption, changes in spectra of the reduced cytochromes were followed after the addition of TMO.

Fig. 5A shows a typical recording of the formate-reduced cytochromes at various intervals after the addition of TMO. The spectrum obtained before the addition (0 min) had the same peaks with the maxima at 555 nm and 526 nm as shown in Fig. 1A. Shortly after the addition (3 min), both the peaks were lowered considerably. The maximum slightly shifted from 555 nm to 556 nm, displaying a sloping shoulder at 561–562 nm, and again shifted to 561–562 nm with the shoulder at about 556 nm at 30 min. Finally, the peak disappeared at 60 min. This suggests reoxidation of the reduced cytochromes by TMO in the presence of the membrane preparation. When added the buffer instead of TMO, no reoxidation of the cytochromes was detected even at 67 min, as given in Fig. 5B. Fig. 6A and 6B show the same set of the experiment using NADH as the reductant. The spectral change was essentially the same as observed for formate after the addition of TMO and no change could also be detected for the buffer.

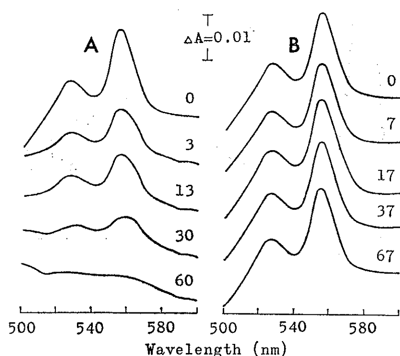


**Fig. 5.** Changes in difference spectra of the formate-reduced cytochromes with and without the addition of TMO.

Formate was added to the membrane preparation (1.3 mg protein/m*l*) and incubated for 45 min. Soon after performed the initial recording of difference spectra (0 min), either TMO (20 $\mu$mol in 50 $\mu l$ of the buffer) or the buffer (50 $\mu l$) was immediately added through the liquid paraffin layer. The spectra were recorded against the preparation oxidized by TMO. The time intervals indicate the time (min) elepsed from addition of TMO or the buffer. (A), TMO; (B), buffer.

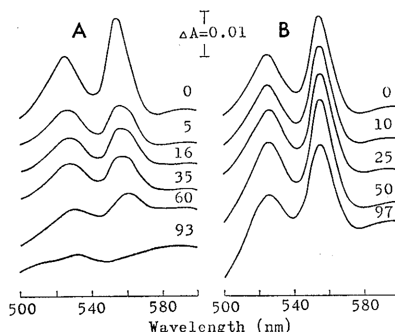


**Fig. 6.** Changes in difference spectra of the NADH-reduced cytochromes with and without the addition of TMO. NADH was added to the membrane preparation (1.4 mg protein/m*l*) and incubated for 20 min. The subsequent procedure was the same as described in Fig. 5. (A), TMO; (B), buffer.

All these facts suggest that the reduced form of the cytochromes was produced by formate and NADH, then reoxidized by TMO. This process no doubt indicates the participation of the cytochromes in TMO reduction as a member of the electron transport chain.

The 560 nm peak in the spectra shown by nitrate-induced cell membrane preparation (Fig. 3) shifted by 4 nm to lower wavelengths at the low temperature (Fig. 4). Therefore, the 556 nm and 561–562 nm peak which transiently appeared in the course of the reoxidation by TMO (30 min), seem to correspond to the 552.5 nm and 558 nm components, respectively, in the low-temperature spectrum. Again, both the 552.5 nm and 558 nm components could be differentiated

from cytochrome $b_1$ which had the $\alpha$ peak at 556nm Each reduced component was reoxidized by TMO sooner or later, presumably depending on its specific redox potential.

*Effect of HOQNO on the Reoxidation of the Reduced Cytochromes by TMO*

HOQNO produced the inhibitory effect on the reduction of TMO, particularly when formate was used as the electron donor[1]. The effect was examined spectrophotometrically to provide additional evidence on the block of electron transfer to TMO. Fig. 7A and 7B illustrate recordings of

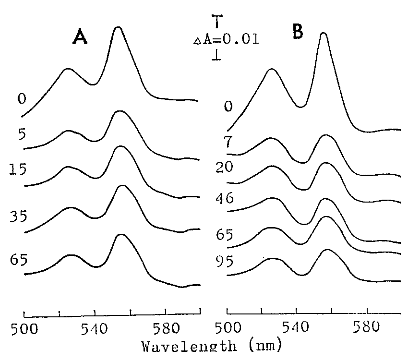


**Fig. 7.** Inhibition by HOQNO of oxidation of the formate- and NADH-reduced cytochromes in the membrane preparation. The membrane preparation (3 m*l*) was incubated for 10 min at 37°C in the presence of HOQNO (0.35 $\mu$mol in 50 $\mu l$ ethanol). The subsequent procedures for reduction of the cytochromes with formate or NADH and oxidation with TMO were indicated in Fig. 5A. (A), formate as the reductant added to the membrane preparation (1.2 mg protein/m*l*); (B), NADH as the reductant added to the preparation (1.4 mg protein/m*l*).

various time intervals following the addition of TMO to the formate- and NADH-reduced cytochromes, respectively, in the presence of HOQNO. The spectra taken before the addition (0 min) were wholly the same as seen in the corresponding spectra of Fig. 5A and 6A where HOQNO was absent. Incidentally, the peak height ($\Delta A_{555-575}$ and $\Delta A_{555-540}$) in the presence of HOQNO was about 10% lower than that in the absence, indicating that the inhibitor could very weakly inhibit the reduction of the cytochromes. The spectra recorded at 5 min (Fig. 7A) and 7 min (Fig. 7B) had the transfigured peaks with the maximum at about 556 nm and with the shoulder at 561–562 nm. This shape of the peaks was the same as displayed already at 3 min in Fig. 5A and at 5 min in Fig. 6A. Thereafter, no more change occurred in the spectra with the prolonged incubation. This observation suggests that one portion of the cytochrome components was reoxidized rapidly but another portion remaining reduced. This is the inhibition caused by HOQNO, which in turn blocks the electron transfer from formate and NADH to TMO, actually as reported in the previous paper[1].

It is of interest to describe further the cytochrome components remaining reduced, and also those reoxidized rapidly on the addition of TMO. The inhibition site for HOQNO currently recognized is at cytochrome b level, likewise for antimycin A[13,14]. Cox *et al.*[15], however, placed the site at ubiquinone levels situated both before and after cytochrome $b_1$ in aerobic electron transfer of *E. coli.* If ubiquinone (or other quinones[16]) functions also in the electron transport system of TMO reduction, the virtual inhibition site would be placed at the quinone(s) after cytochrome $b_1$, since in *E. coli* membranes this cytochrome is the only known cytochrome before the quinone positions[15]. It is evident, however, that the remaining components were distinguishable in the spectral properties from cytochrome $b_1$, as described above. On the basis of these arguments, the remaining components seem to be a b-type cytochrome other than $b_1$ and hence HOQNO acts either at or after the component.

The cytochrome component oxidized rapidly by TMO may probably be a c-type because it was beyond the HOQNO-inhibition (Fig. 7A and 7B). In our preliminary experiment, pyridine hemochrome spectra of an acid-acetone-insoluble fraction of the membrane preparation indicated that cells induced with TMO contain certain amounts of heme c bound to membranes. Further investigations on this aspect are required particularly in relation to TMO reductase which is most likely close to the component in the electron transport chain.

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