Nitrate, but not silver, ions induce spectral changes in *Escherichia coli* cytochrome *d*

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The absorbance maximum (630 nm) of reduced cytochrome *d* in *Escherichia coli* membrane particles was diminished by 160 μM $AgNO_3$ or $NaNO_3$ and accompanied by the formation of a species with an absorption maximum at 640–645 nm. Nitrite, trioxodinitrate and nitric oxide elicited qualitatively similar, but faster, changes in the spectrum of cytochrome *d*, suggesting that formation of a nitrosyl complex may be involved in all cases. In direct contrast to an earlier report, silver ions (160 μM) were without effect on the α-bands of reduced cytochromes *d*, *b* or a_1.

Cytochrome d Escherichia coli *Silver* *Nitrate* *Nitrite* *Trioxodinitrate*

1. INTRODUCTION

Silver ions inhibit substrate oxidation [1] and solute transport [2] by *Escherichia coli* as well as growth of other bacteria (e.g., [3]). Authors in [4] demonstrated inhibition by 86 μM $AgNO_3$ of the aerobic oxidation of endogenous and various added substrates by intact *E. coli*, and of NADH by membranes derived therefrom. Low concentrations (13 μM) of $AgNO_3$, but not $NaNO_3$, induced oxidation of cytochrome *d*, indicating a site of action near the substrate side of the oxidase, whereas 26 and 66 μM $AgNO_3$ also elicited oxidation of 'flavoprotein' and cytochrome *b*. At 159 μM, $AgNO_3$ caused a gradual disappearance (over 20 min) of the peak attributed to reduced cytochrome *d* and a slower rise in absorbance at longer wavelengths. They postulated that Ag^+ convert cytochrome *d* to an intermediate form that does not absorb strongly between 610 and 670 nm.

Here, we present evidence that the spectral changes of cytochrome *d* elicited by $AgNO_3$ are due not to silver ions but to nitrate and that similar changes are seen on reaction of cytochrome *d* with other nitrogen-containing compounds.

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2. MATERIALS AND METHODS

2.1. *Preparation of cells and membranes*

Escherichia coli K12, strain A1002 (NCIB 11825) was grown in the medium used in [5] except that the succinate concentration was 20 mM. Cultures (20 l) were grown in 20 l vessels, sparged with air at 2 $l \cdot min^{-1}$ and slowly stirred. Such growth was O_2-limited [6], giving cell yields after about 24 h equivalent to an apparent absorbance (1-cm path) of 0.2–0.5 (600 nm; Pye Unicam SP6-550 UV/VIS spectrophotometer). Membrane particles were prepared from washed cells [6]. The first pellet obtained by high speed centrifugation was resuspended and homogenised in 50 mM Tris–SO_4^{2-} (pH 8.0) to obviate precipitation of AgCl, then recentrifuged and suspended in the same buffer.

2.2. *Spectrophotometry*

A Johnson Foundation dual-wavelength spectrophotometer (DBS-3) was used [7], but employ-

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ing two J-Y H-20V concave holographic grating monochromators (200 mm focal length, dispersion 4 nm · mm^{-1}) and a Hamamatsu R-928 multi-alkali type photomultiplier. The reference wavelength was fixed at 608 nm, while the measuring beam scanned from 500–700 nm. Slit widths were 1 mm.

Membrane suspension (3 ml) was reduced with $Na_2S_2O_4$ or 3.3 mM succinate (adjusted to pH 7.2) in a 1-cm pathlength cuvette fitted with a rubber suba-seal to exclude air. Aqueous solutions of $AgNO_3$, $NaNO_3$, KNO_3, Ag_2SO_4 or $NaNO_2$ were purged with N_2 for >20 min to expel air. Sodium trioxodinitrate was prepared at about 0.2 M in 0.1 M NaOH, and also purged with N_2 to prevent decomposition by oxidation. The solution was used within 90 min of preparation. Addition of small volumes of 0.1 M NaOH to the experimental buffer (50 mM Tris–SO_4, pH 8) was without effect on the buffer pH. The purity of trioxodinitrate, prepared as in [8], was checked from its UV spectrum and measurement of its rate of decomposition at pH 8 and 19°C (measured $k_1 = 5.0 \times 10^{-4}$ s^{-1}, literature value, 4.5×10^{-4} s^{-1}).

3. RESULTS AND DISCUSSION

The effects of adding 160 μM $AgNO_3$ to the reduced form of cytochrome *d* are shown in fig.1a and are similar to those observed in [4]. There is a loss of absorbance of the reduced form at 630 nm, a shift in the apparent absorbance maximum to about 636 nm and an increase in absorbance (relative to the reference sample) at wavelengths above 640 nm. There was little change at 560 nm (cytochrome *b*) or 595 nm (cytochrome a_1). Fig.1b shows the similarity of the spectral changes observed on repeating the experiment with 160 μM $NaNO_3$. In a control experiment in which no additions were made, the spectrum was unchanged over 1 h, indicating that adventitious oxygen did not cause the changes seen in fig.1. The changes could not have been caused by alteration in the redox state of the 'reference sample' since the spectrophotometer allowed this spectrum to be stored in a digital memory. Addition of $AgNO_3$, KNO_3 or $NaNO_3$ (all 160 μM) to membranes reduced with either 3.3 mM succinate or $Na_2S_2O_4$ all gave results similar to those in fig.1, whereas 160 μM Ag^+ as Ag_2SO_4 was without effect.

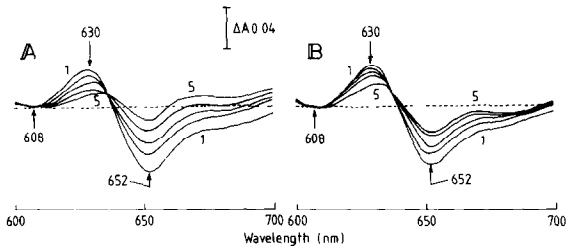

Fig.1. Spectral changes of cytochrome *d* in *E. coli* membranes elicited by 160 μM $AgNO_3$ (A) or $NaNO_3$ (B). A succinate-reduced minus oxidized spectrum was scanned immediately before addition of the salt (scan 1) and then at intervals of 5, 10, 20 and 30 min (scans 2–5). The directions of change in successive scans were increases in absorbance above about 640 nm and decreases below 640 nm. In (a) and (b), the dashed line is the oxidized minus oxidized baseline.

Similar results were obtained using glycylglycine–KOH buffer (pH 8) as in [4], but we utilised Tris buffer to reduce the risk of precipitation of silver compounds.

Although the spectral properties of *Pseudomonas* cytochrome *d*, are pH-dependent [9], the small pH changes produced by the various additions (final pH in the range 7.12–8.0) had no effect on the positions of the cytochrome *d* absorbance bands.

Absolute spectra of the reduced, reduced plus NO_3^-, and oxygenated forms were obtained using milk as a suitably opaque and scattering reference sample, which had no absorbance maxima in the 500–700 nm region. Such absolute spectra showed peak positions at 630, 641–645, and 650 nm. The broadness and position of the band of the (reduced plus NO_3^-) species casts doubt on the simple interpretation of the difference spectra in [4] where it was suggested that the diminution of the 630 nm peak, without a corresponding increase at 650 nm (attributed by them to the oxidized species), was evidence of the formation of an 'invisible' form of cytochrome *d* (see, however [10]).

A preliminary study of the effects of other nitrogen compounds on cytochrome *d* was undertaken. Thus 1.1 mM NO_2^-, 1.1 mM NO_3^- and $N_2O_3^{2-}$ (1.9 or 0.39 mM) caused diminution of the reduced cytochrome *d* peak (630–608 nm) at rates (20°C; $10^{-3} \times \Delta A \cdot min^{-1}$) of 12.4, 1.76, 47.7 and 8.53, respectively, and the spectral changes were all qualitatively similar. The slower rate of reaction with nitrate may reflect a rate-determining reduc-

tion to nitrite catalyzed by a nitrate reductase. A sample bubbled slowly for 1 min with NO (previously passed through NaOH to remove NO_2, and then dried by passing over silica) showed 'immediate' (within 1 scan, about 2 min) formation of the 'end point' elicited by the other, more slowly reacting ligands. Thus, reaction with all 4 compounds may lead to the formation of the same product, probably a nitrosyl complex as proposed in [11].

The more rapid reaction of trioxodinitrate is of interest as this compound has long been postulated to be an intermediate in microbial denitrification (see, however, [12]). It is unlikely though, that the membrane particles catalyze reduction of the intact trioxodinitrate anion. Comparison of the rate of disappearance of reduced cytochrome *d* with the rate of self-decomposition of the trioxodinitrate which was 10–50-fold greater [8,13] suggests that reaction occurs between cytochrome *d* and nitroxyl ion (NO^-), produced in the self decomposition of trioxodinitrate.

$$HN_2O_3^- \longrightarrow HNO + NO_2^-;\ HNO \longrightarrow H^+ + NO^-$$

A competing reaction would involve production of nitrous oxide via dimerisation of HNO. Reaction of NO^- with reduced cytochrome *d* would give an Fe-nitrosyl species with the nitrosyl present formally as NO^-. It is known [14] that the nitrosyl complex $[Ni(CN)_3NO]^{2-}$ is formed from the decomposition of trioxodinitrate in the presence of $[Ni(CN)_4]^{2-}$ by direct displacement of CN^- by NO^-. Facile formation of the cytochrome *d* complex in the presence of decomposing trioxodinitrate supports its formulation as the nitrosyl species.

This work also suggests an explanation of the unusual spectral properties of cytochrome *d* noted in [15]. In reduced minus oxidised difference spectra of cells grown anaerobically with glycerol and nitrate, the absorption maximum of cytochrome *d* was shifted 10 nm to the red by comparison with the spectrum of cells grown anaerobically with fumarate as oxidant. In the nitrate-grown cells, the trough at about 650 nm was also weak, although this was not described. It seems likely that these spectral differences were due to the formation of a nitrosyl species resulting from the reaction with the oxidase of nitrite (formed by the inducible nitrate reductase from nitrate), or more reduced nitrogen-containing compounds formed subsequently.

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