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Kinetics of the reactions of trioxodinitrate and nitrite ions with cytochrome *d* in *Escherichia coli*

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The rate of reaction of trioxodinitrate with reduced cytochrome oxidase *d* in membrane particles from *Escherichia coli* at pH 7 and 25 °C depends linearly upon $[HN_2O_3^-]$ over the concentration range studied (up to 0.05 mM) and is also first-order in cytochrome *d*. The known rate of decomposition of trioxodinitrate to give NO^- and NO_2^- is about 4.5-times faster than the rate of reaction of reduced cytochrome *d* with trioxodinitrate, implying that cytochrome *d* reacts directly with NO^-, with a trapping ratio of between 0.20 and 0.25, rather than with trioxodinitrate. The implications of the facile formation of the NO^--nitrosyl complex of cytochrome *d* for the mechanism of denitrification are discussed with particular reference to the mechanism of N–N bond formation. The reaction of reduced cytochrome *d* with nitrite (a decomposition product of trioxodinitrate) under these conditions is much slower than that with trioxodinitrate. The kinetics show a biphasic dependence of initial rate upon nitrite concentration. The rate data at low $[NO_2^-]$ are consistent with saturation of a high affinity site for nitrite, having $V_{max} = 4.29 \cdot 10^{-9}$ M s^{-1} and $K_m = 0.034$ mM. The existence of two binding sites for nitrite is consistent with the suggestion that the cytochrome *bd* complex contains two cytochrome *d* haems.

Introduction

The process of denitrification involves the bacterial reduction of nitrite and the release of nitrous oxide or, more usually, dinitrogen into the atmosphere [1–3] (Eqn. 1). Step A is

$$NO_3^- \xrightarrow{A} NO_2^- \xrightarrow{B} N_2O \xrightarrow{C} N_2 \qquad (1)$$

catalyzed by nitrate reductase, a molybdoenzyme [4], while Zumft and his co-workers have shown that nitrous oxide reductase (step C) is a copper enzyme [5]. Two types of nitrite reductase have been identified in denitrifying bacteria. One contains copper (for example in *Alcaligenes faecalis* [6] and *Achromobacter cycloclastes* [7]) and the other contains *c*- and d_1-type cytochromes. The cd_1-type nitrite reductase is found in *Pseudomonas aeruginosa* and *Paracoccus denitrificans* and is also able to reduce dioxygen to water, although this is not its normal physiological function. The mechanism of formation of the N–N bond in nitrous oxide on reduction of nitrite has been much discussed. One proposal invokes the dimerization of a nitrogen species in the formal oxidation state for nitrogen of +I (HNO) [8,9], although the possibility of this pathway has been eliminated for the nitrite reductase from *Pseudomonas stutzeri* [10]. A second proposal [11] involves the nucleophilic attack of nitrite on an enzyme-bound nitrosyl intermediate, E-(NO^+), formed from nitrite by a dehydration reaction: the bound N_2O_3 species is then reduced via two two-electron steps to $N_2O_3^{2-}$ and N_2O. Support for such a sequential mechanism for reduction of nitrite comes from studies using ^{15}N and ^{18}O labels [12], although these studies are also consistent with an alternative sequential mechanism involving the attack of nitrite upon a metal-bound NO^- nitrosyl group to give coordinated trioxodinitrate ($N_2O_3^{2-}$) directly. Such a reaction between NO^- and NO_2^- occurs in aqueous solution, as decomposition of trioxodinitrate to give HNO and nitrite can be reversed by added nitrite, a reaction that has been confirmed by the use of labelled

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nitrite [13–15]. Garber et al. [15] have argued against a role for trioxodinitrate in denitrification, but their arguments are not conclusive.

We now describe a study of the reaction of trioxodinitrate ion with reduced cytochrome oxidase *d* in membrane particles from *Escherichia coli*. While not a denitrifying organism, *Escherichia coli* has several pathways for the reduction of nitrite [16] which are distinct from the route involving cytochrome *d*. It should be noted, however, that there are similarities between the cytochrome oxidase *bd* complex of *Escherichia coli* and the cd_1-type nitrite reductases of *Paracoccus denitrificans* and *Pseudomonas denitrificans*, which are able to reduce dioxygen to water.

The stoicheiometry of the cytochrome *bd* complex is still uncertain. There is general agreement [17] that the complex contains one *b*-558 and one *b*-595 haem, but earlier evidence for the presence of two cytochrome *d* haems per complex [18] has now been questioned [19] on the basis of ESR studies which have been interpreted in terms of only one cytochrome *d* haem per complex. Both cytochromes *b*-595 and *d* involve 5-coordinate iron centres.

The decomposition of trioxodinitrate under the pH conditions used in the present study is well understood and involves the heterolysis of the $HN_2O_3^-$ anion to give HNO and nitrite ion [13,14]. It is possible that trioxodinitrate ion and its decomposition products may both react with cytochrome *d*, but this should be resolved by simple rate comparisons. For this reason, we have also investigated the kinetics of reaction of nitrite ion with reduced cytochrome *d* under the same conditions.

Rothery et al. have shown that nitrite reacts with both haem *b*-595 and cytochrome *d* components of the cytochrome oxidase *bd* complex of *E. coli* [20], although the reaction with cytochrome *d* appears to be some 10-times faster. Hubbard et al. have also reported on the reaction of cytochrome *d* in membranes from *E. coli* with nitrogen oxoanions [21].

Materials and Methods

Chemicals

All chemicals were the best grade available, usually AnalaR. Sodium trioxodinitrate was prepared by the reaction of propyl nitrate with hydroxylamine [22]. The product was recrystallised from 0.1 M sodium hydroxide solution. Purity of the product was confirmed by infrared spectroscopy [23], the molar absorptivity at 248 nm in 0.1 M NaOH solution (8300 $M^{-1} \cdot cm^{-1}$, identical to the literature value [22]) and by measurement of the first-order rate constant for the self-decomposition at pH 7 and 25°C (within 3% of the literature value [24]).

Growth of oxygen-limited Escherichia coli

Escherichia coli K12 (strain A1002), an amino acid auxotroph requiring isoleucine, valine and methionine, was grown in the medium of Scott and Poole [25], containing (mM): K_2HPO_4 (23.0), KH_2PO_4 (7.30), NH_4Cl (18.7), sodium succinate (20.0), $CaCl_2 \cdot 2H_2O$ (0.1), K_2SO_4 (14.9), isoleucine (0.17), valine (0.15), methionine (0.13) plus casamino acids (1.0 $g \cdot l^{-1}$) and trace element solution (10 $ml \cdot l^{-1}$). A 1 M solution of $MgCl_2 \cdot 6H_2O$ was sterilised separately, and after cooling added to the medium (1 $ml \cdot l^{-1}$). Two 20 litre batches of medium were each inoculated with 50 ml of a starter culture grown aerobically for 24 h at 37°C. Air was bubbled into the cultures at 4 $l \cdot min^{-1}$ and the culture stirred slowly, sufficient to prevent the sedimentation of the cells. Cultures were harvested after about 24 h, when the apparent absorbance of the culture at 600 nm was in the range 0.2–0.4, by centrifugation in a Alpha-Laval continuous flow centrifuge.

Preparation of membrane particles

Harvested cells were washed once with 50 mM Tris-sulphate buffer at pH 8, and broken by sonication using an MSE 150 W sonicator for five 30 s periods, with 15 s intervals between each sonication to prevent overheating. Cell debris was removed by centrifugation at 19000 $\times g$ for 15 min at 4°C. The supernatant solution was centrifuged at 225000 $\times g$ for 60 min at 4°C. The resulting pellet of membrane particles was washed by suspension in buffer, and recentrifuged under the same conditions. Membrane pellets were stored at −20°C.

Membranes were resuspended in buffer as required, and reduced by treatment with 6.6 mM succinate for 10 min at room temperature. Concentrations of cytochrome *d* were estimated using an extinction coefficient of 8.4 $mM^{-1} \cdot cm^{-1}$ for $A_{615}-A_{630}$ [26]. A value of 8.5 $mM^{-1} \cdot cm^{-1}$ has been quoted by Haddock and Schairer [27].

Kinetic measurements on the reaction of cytochrome d with trioxodinitrate and nitrite

The reaction was followed in septum-capped cuvettes, in the temperature-controlled cell compartment of a Johnson Foundation dual-wavelength spectrophotometer (DBS-3). Measured volumes (2.5 ml) of membrane suspension in buffer of pH 7.0 were either reduced by addition of 4.0 mmol of succinate or oxygenated by shaking. The reduced-minus-oxygenated spectrum was plotted by subtraction of the individual spectra, obtained in the dual-wavelength scanning mode, and recorded in the digital memories of the spectrophotometer. The reaction of reduced cytochrome *d* was followed in the dual wavelength mode by measuring the absorbance difference $A_{630}-A_{608}$. This pair of wavelengths was chosen rather than the $A_{630}-A_{615}$ pair, as 608 nm is an isosbestic point for oxidised and reduced

cytochrome *d*. The differential extinction coefficient for $A_{630}-A_{608}$ was estimated to be 11.6 $mM^{-1} \cdot cm^{-1}$ from data given by Kita et al. [26]. Argon was bubbled through the cell for 5 min to remove air from the head space, and an appropriate volume of sodium trioxodinitrate (in 0.1 M NaOH) or sodium nitrite solution was then injected to initiate the reaction. The cell was momentarily inverted to mix the solutions and the reaction was followed by monitoring the absorbance difference $A_{630}-A_{608}$. The time between mixing and the initiation of measurement of the absorbance difference was between 3 and 5 sec. Slopes of the absorbance versus time plots were measured to give initial rates of reaction of cytochrome *d*.

Results

The well-characterised reduced–oxidised absorption spectrum (Fig. 1A) of membranes containing high levels of the cytochrome *bd* complex provides a basis for studying the effects of added trioxodinitrate or nitrite on cytochrome *d*. The reduced form of cytochrome *d* gives rise to the 630 nm peak, while the oxygenated form is reflected in the 650 nm trough. The minor peak at 595 nm is due to a high-spin cytochrome *b*-595 which is part of the oxidase complex, and which also makes a major contribution to the shoulder at 442 nm. The peaks at 430 and 560 nm are due to the overlapping signals of several *b*-type cytochromes [17,28].

Rothery et al. [20] have already reported that addition of nitrite results in the appearance (relative to the reduced state) of a peak near 660 nm and troughs near 430 and 630 nm. Significantly, changes in the 600–700 nm region were complete within the time taken to record the full spectrum at the nitrite concentrations used. We now report analogous experiments with trioxodinitrate which elicit spectral changes broadly similar to those caused by nitrite, except that the Soret region shows more detail in that the Soret trough is split with minima at 428 and 442 nm.

These changes in the reduced-minus-oxidised difference spectrum are similar to those reported by Meyer [29] for the reaction of nitrite and NO with reduced cytochrome *d* in membranes, and which were attributed by him to the formation of a nitrosyl complex of the cytochrome. Studies cited by Rothery et al. [20] show that paramagnetic species formed concurrently with the optical changes during the reaction of nitrite with cytochrome *d* have ESR signals characteristic of those produced by nitrosylhaems. This suggestion has also been put forward for nitrite reductases from other organisms [30,31]. Nitrosyl complexes of cytochrome *d* have been postulated to be formed during the reaction of cytochrome *d* with hydroxylamine [32].

Reaction with trioxodinitrate

Kinetic measurements were carried out at pH 7 with [cytochrome *d*] = 2.46 μM and an excess of trioxodinitrate. Trioxodinitrate is present as $HN_2O_3^-$ under these conditions of pH [13]. The rates of reaction of reduced cytochrome *d* with trioxodinitrate were considerably greater than those found with nitrite (next sec-

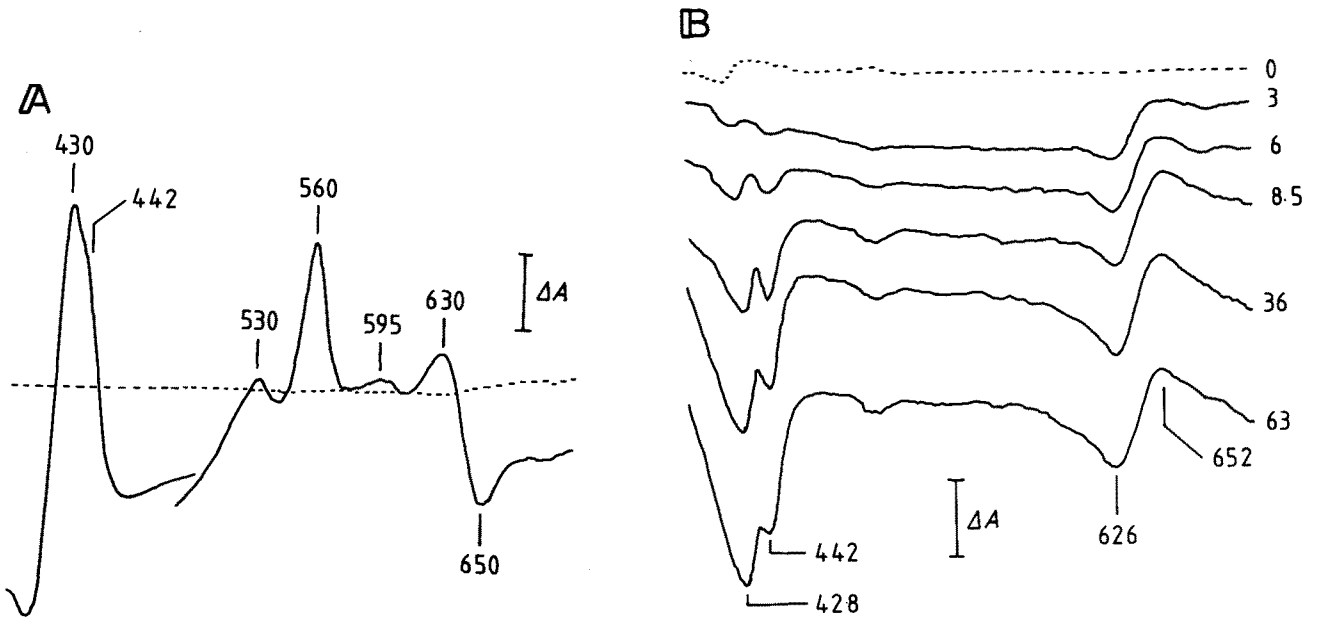

Fig. 1. Room temperature difference spectra of *E. coli* membranes. (A) Reduced (6.7 mM succinate for 10 min) minus oxidised (shaken) spectrum. The absorbance bar represents ΔA of 0.08 below about 500 nm and of 0.02 at higher wavelengths. The dashed line is the oxidised minus oxidised baseline. In (B) is shown the spectral changes elicited by adding 0.01 mM trioxodinitrate in 0.01 M NaOH. The top scan is the baseline (reduced minus reduced), immediately prior to adding trioxodinitrate. Scans were recorded subsequently at the time intervals shown at the right (min). The lightpath was 1 cm, the spectral bandwidth was 4 nm and the scanning speed 2.9 nm/s. Peaks and troughs are labelled in nm.

tion), reactions reaching completion within 1–2 min. To demonstrate that the reaction being followed at $A_{630}-A_{608}$ was due to the reaction with cytochrome *d* and not with a *b*-type cytochrome, spectra were recorded at intervals following the addition of the ligand to a sample of succinate-reduced membranes (Fig. 1B). The changes between 600 and 700 nm were complete within 3 to 6 min of initiating the reaction, whereas changes in the Soret region were not complete within 1 h. Cytochrome *d* is believed to have only minimal absorbance in this region, the major contribution being from *b*-type cytochromes, as noted above. Thus reaction between trioxodinitrate with cytochrome *b*-595 is not significant over the time-scale of the reaction with cytochrome *d*.

Table I shows the effect of a 5-fold variation in trioxodinitrate concentration on the rate of reaction with cytochrome *d*. As shown in Fig. 2, the rate increases linearly with $[HN_2O_3^-]$ over the entire concentration range used. Table I also contains details of kinetics runs for which the concentration of cytochrome *d* was varied; the rate is approximately linearly dependent upon [cytochrome *d*], showing the kinetics to be of first-order with respect to cytochrome *d*.

The rates of decomposition of the monoprotonated trioxodinitrate anion under these conditions are also given in Table I. The rate of formation of NO^- from trioxodinitrate is about 4.5 times greater than the rate of reaction of reduced cytochrome *d*. The ratio of the amount of NO^- reacting with cytochrome *d* to the amount produced by self decomposition of $HN_2O_3^-$ (the trapping ratio) is shown in Table I for each experiment. In the set of experiments where the concentration of cytochrome *d* was increased at constant $[HN_2O_3^-]$ the trapping ratio (as expected) increases with cytochrome

TABLE I

Reaction of cytochrome d with trioxodinitrate at 25°C and pH 7

All rates are in units of 10^{-9} M·s^{-1}.

$[N_2O_3^{2-}]$ (mM)	[Cyt *d*] (μM)	Initial rate	Initial [a] rate	d[NO^-]/d*t* [b]	Trapping ratio
0.0100	2.55	1.44	1.39	6.75	0.21
0.0122	1.94	1.39	1.76	8.30	0.17
0.0124	2.23	1.86	2.05	8.43	0.25
0.0150	2.46	2.38	2.38	10.2	0.23
0.0150	2.46	2.40	2.40	10.2	0.23
0.0250	2.55	3.92	3.78	16.9	0.23
0.0360	2.38	5.04	5.21	24.4	0.21
0.0400	2.55	5.77	5.57	27.2	0.20
0.0500	2.55	7.55	7.28	34.0	0.22
0.0124	0.700	0.770		8.43	0.13
0.0124	1.41	1.45		8.43	0.18
0.0124	2.23	1.86		8.43	0.25

[a] Rates corrected to [cyt *d*] = 2.46 μM.

[b] Initial rate of decomposition of $HN_2O_3^-$, calculated from data in Ref. 24.

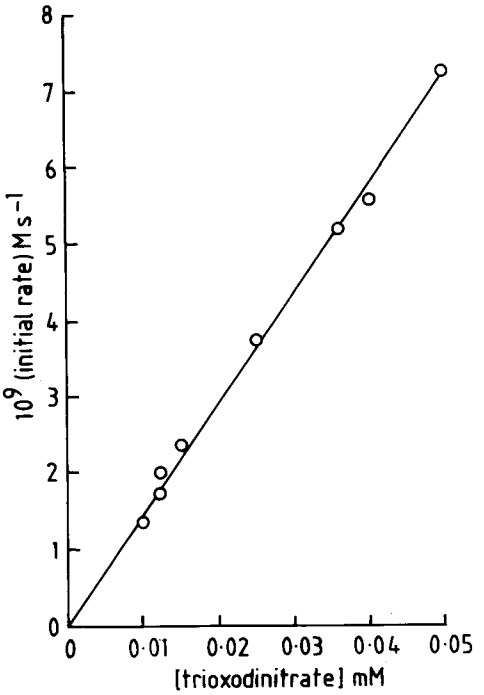

Fig. 2. Dependence of initial rate of reaction on trioxodinitrate concentration at 25° C, [cyt *d*] = 2.46 μM and pH 7.

d concentration. At sufficiently high [cytochrome *d*] all the NO^- would presumably be trapped.

It is clear, therefore, that trioxodinitrate does not react directly with cytochrome *d* under the conditions of these experiments, and that reaction occurs with the products of the self-decomposition reaction (NO^- and NO_2^-). A comparison of the rates of reaction of cytochrome *d* with trioxodinitrate and nitrite (Tables I and II), bearing in mind the lower concentrations of trioxodinitrate, eliminates the possibility that the nitrite fragment formed during the decomposition of trioxodinitrate is responsible for reaction with cytochrome *d*. The reaction must therefore involve NO^- (Eqns. 2–4).

$$HN_2O_3^- \rightarrow H^+ + NO^- + NO_2^- \quad (2)$$

$$NO^- + \text{cyt } d \rightarrow \text{products} \quad (3)$$

$$2H^+ + 2NO^- \rightarrow N_2O + H_2O \quad (4)$$

Reaction with nitrite

Table II gives the rates of reaction of reduced cytochrome *d* with nitrite over the concentration range 0.01 to 1.04 mM nitrite at [cytochrome *d*] = 2.38 μM and pH 7.0. Reaction rates increase with nitrite concentration but Fig. 3 shows the dependence of rate upon nitrite concentration to be biphasic. One explanation of these results is that a binding site for nitrite is becoming saturated at low nitrite concentration, with binding to a

TABLE 2

Reaction of cytochrome d with nitrite at 25°C and pH 7

$[NO_2^-]$ (mM)	[Cyt *d*] (μM)	(initial rate)/($\times 10^{-9}$ M·s^{-1})
0.010	2.38	0.986
0.021	2.38	1.68
0.104	2.32	3.14 [a]
0.130	2.38	3.40
0.175	2.38	3.78
0.510	2.32	6.46 [a]
1.04	2.32	11.5 [a]

[a] Rates corrected to [cyt *d*] = 2.38 μM.

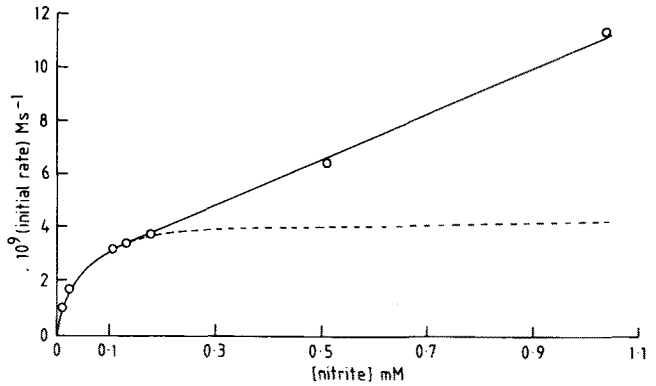

Fig. 3. Dependence of initial rate of reaction on nitrite concentration at 25°C, [cyt *d*] = 2.38 μM and pH 7 (dashed line shows values of initial rate calculated using K_m = 0.034 mM).

second site of lower affinity becoming significant at higher $[NO_2^-]$.

The rate data at low $[NO_2^-]$ are consistent with saturation of the binding site by nitrite. A Lineweaver-Burk plot (Fig. 4) for the data at low nitrite concentrations gives a straight line, values of the intercept and slope allowing the calculation of $V_{max} = 4.29 \cdot 10^{-9}$ M · s^{-1} and $K_m = 0.034$ mM. Similar K_m values for nitrite have been determined in other studies. The denitrification of nitrite by *Pseudomonas stutzeri* follows Michaelis-Menten kinetics with K_m values of 0.204 [11] and 0.160 mM [33], while a K_m value of 0.053 mM has been reported for nitrite binding during reduction by *Pseudomonas* cytochrome cd_1 [32].

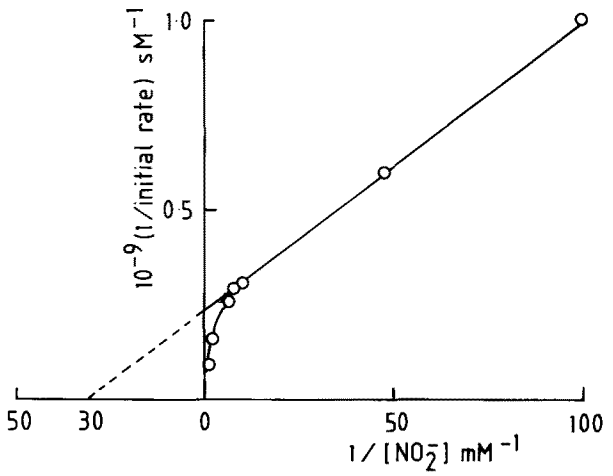

Fig. 4. Double-reciprocal plot of initial rate of reaction against nitrite concentration.

Values of the expected initial rates of reaction of cytochrome *d* and nitrite have been calculated at nitrite concentrations up to 1.04 mM using K_m = 0.034 mM, and are shown in Fig. 3. This confirms the biphasic character of the reaction of nitrite with reduced cytochrome *d*. The limited results available at higher nitrite concentrations are also shown in Fig. 4. Calculations based on the two highest nitrite concentrations give values of K_m of 3 mM and of V_{max} of $50 \cdot 10^{-9}$ M · s^{-1} for reaction at the low-affinity site.

Identity of the product formed between cytochrome d and the nitrogen oxoanions

We have attempted unsuccessfully to demonstrate the presence of the nitrosyl group in the reaction product by both FTIR and resonance Raman spectroscopy. Since such studies should indicate the type of nitrosyl group present, which may be an NO^- rather than NO^+ nitrosyl in view of the observed rapid formation of the complex with free NO^-, this effort is continuing.

Discussion

The present work has shown the absence of direct reduction of trioxodinitrate by reduced cytochrome *d*. It is evident that reaction occurs between reduced cytochrome *d* and the products of decomposition of trioxodinitrate. The rapid rate of this reaction compared with that between nitrite and cytochrome *d* suggests that the reactive species is NO^-. It is clear that trioxodinitrate is an important reagent for the generation of free NO^-, and that high quality kinetic data may be obtained by the careful control of reaction conditions. However, the fact that NO^- reacts directly with reduced cytochrome *d* (to give the Fe(II)(NO^-) nitrosyl species) has important consequences. This nitrosyl species appears to be identical to that obtained by reduction of nitrite with cytochrome *d* in membrane particles, as demonstrated by electronic spectroscopy [21], suggesting that both species are NO^- nitrosyls. In discussing the pathways available for the formation of the N–N bond in denitrification, Weeg-Aerssens et al. [12] have argued for a reaction sequence involving the nucleophilic addition of nitrite to an NO^+ nitrosyl group to give coordinated N_2O_3, which then undergoes successive two-electron reductions to give N_2O. They note that an alternative sequential mechanism, also consistent with their data, is the reaction of Fe(II)(NO^-) with nitrite to produce coordinated trioxodinitrate, a reaction known to occur in aqueous solution [13–15]. Weeg-Aerssens et al. suggested, on the basis of reduc-

tion potentials, that such an Fe(II) intermediate is unlikely to be formed. The results presented here indicate that such a species is formed in the reaction of cytochrome *d* with trioxodinitrate and, by implication, in the reaction with nitrite. This lends support to the suggestion that N–N bond formation in denitrification could involve the formation of trioxodinitrate via reaction of an Fe(II) NO^- species with nitrite.

The results on the reaction with nitrite are of interest, although initially they were obtained only for comparison with the kinetics of the trioxodinitrate-cytochrome *d* reaction. Rothery et al. have claimed [20] that the reaction was of first order with respect to nitrite under all conditions examined. However, the biphasic dependence of reaction rate upon nitrite observed in the present work would only be observed in studies at nitrite concentrations lower than those used by Rothery et al. The present results indicate that there are two sites for nitrite binding at the cytochrome *d* complex, having K_m values of 0.034 mM and about 3 mM. One interpretation of this observation is that the cytochrome *d* complex contains 2 mol of cytochrome *d*, which interact cooperatively with respect to binding of nitrite. This conclusion is of interest in view of the current uncertainty concerning the stoichiometry of the cytochrome *d* complex, although other explanations may also be available for the biphasic dependence of the initial rate of reaction upon nitrite.

Acknowledgements

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