Sulphide-linked Nitrite Reductase from *Thiobacillus denitrificans* with Cytochrome Oxidase Activity: Purification and Properties

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A membrane-bound, sulphide-linked nitrite reductase from *Thiobacillus denitrificans* was solubilized and after further purification its properties were examined. The purified enzyme, mol. wt 120000, contained cytochromes *c* and *d* in the ratio of 1:1. Both cytochromes were reduced by sulphide and re-oxidized with nitrite or air. Oxidation by nitrite resulted in the appearance of an absorption peak at 572 nm. The kinetics of the reduction of the enzyme with sulphide indicated that cytochrome *c* was reduced before cytochrome *d*. The redox potential of cytochrome *d* was 22 mV more positive than that of cytochrome *c*. Cytochromes *c* and *d* were dissociated from the purified enzyme by treatment with sodium dodecyl sulphate. The purified nitrite reductase also had cytochrome oxidase activity and both the activities were stimulated by cytochrome *c*-551 isolated from *T. denitrificans*. Reduced cytochrome *c*-551 was an effective electron donor for the purified enzyme with either nitrite or air as the terminal electron acceptor. Neither cytochrome *c*-554 (also isolated from *T. denitrificans*) nor mammalian cytochrome *c* was effective as reductant for the enzyme. NO and N₂O were identified as the products of nitrite reduction by the purified sulphide-linked nitrite reductase.

INTRODUCTION

Thiobacillus denitrificans, a facultative anaerobe, grows chemolithotrophically on thio-sulphate by cleaving it to sulphide and sulphite with a thiol-requiring enzyme (Aminuddin, 1974; Aleem, 1977). The products, sulphide and sulphite, are oxidized to sulphate. Under anaerobic conditions, the oxidation of sulphide and sulphite is linked to the reduction of nitrite and nitrate respectively (Adams, Warnes & Nicholas, 1971; Aminuddin & Nicholas, 1973, 1974*a*). A sulphite-linked nitrate reductase has been partially purified and some of its properties described (Sawhney & Nicholas, 1977). Peeters & Aleem (1970) proposed that the oxidation of sulphide, either aerobically or anaerobically, was coupled to the respiratory chain at the flavin level. In crude extracts of *T. denitrificans*, Aminuddin & Nicholas (1974*a*) have observed that cytochromes *c* and *d* are reduced by sulphide and re-oxidized by nitrite or air. However, the properties of this sulphide-linked nitrite reductase have not been determined.

In this paper the solubilization, purification and properties of a sulphide-linked nitrite reductase are described. The purified enzyme contains a cytochrome *cd* complex which is resolved into two cytochromes by treatment with sodium dodecyl sulphate. A scheme for electron transfer from sulphide via cytochrome *c*-551 and cytochrome *cd* to either nitrite or oxygen is presented.

METHODS

Culture and harvest of the organism. *Thiobacillus denitrificans* (9547, NCIB strain AB5) was grown and harvested as described by Aminuddin & Nicholas (1974b). The harvested cells were washed twice with 0.025 M-Tris/HCl buffer, pH 7.5, to remove nitrite.

Preparation of acetone-dried cells. The washed cells, suspended in 0.025 M-Tris/HCl buffer, pH 7.5 (500 mg wet wt ml⁻¹), were added slowly to 10 vol. cold acetone at -15 °C with continuous stirring. After 10 min, the mixture was filtered quickly through a Buchner funnel using Whatman no. 41 paper. The cells, after washing twice with 3 vol. cold acetone, were dried under a stream of N₂ at 4 °C. They were further dried under vacuum in a desiccator over 18 M-H₂SO₄ and then stored under vacuum at 4 °C.

Preparation of cell-free extracts. The acetone-dried cells were ground to a fine powder in a pestle and mortar and suspended (10 %, w/v) in 0.025 M-Tris/HCl buffer, pH 7.5. To the suspension, 5 mg DNAase was added with continuous stirring for 4 h at 4 °C. It was then filtered through two layers of cheesecloth and passed through a chilled French pressure cell at 140 MPa. The crude homogenate was centrifuged at 18000 g for 45 min and the supernatant fraction (S₁₈) obtained was used for the purification of nitrite reductase.

Molecular weight of nitrite reductase. The molecular weight of the enzyme was determined by gel filtration through a Sephadex G-150 column (2.5 × 50 cm) as described by Andrews (1964). The column was calibrated with a mixture of the following marker proteins of known molecular weights: γ-globulin (160000), bovine serum albumin (68000), ovalbumin (45000) and myoglobin (17800). The void volume (V₀) of the column was determined with Blue Dextran. The elution volume (V_e) of each protein and also of the nitrite reductase was measured. The molecular weight of the enzyme was calculated using the equation of Squire (1964):

$$M^{\frac{1}{2}} = 151[1.47 - (V_e/V_0)^{\frac{1}{2}}]$$

Estimation of copper. Purified nitrite reductase (1 ml samples containing 12.5 mg protein) was dried in acid-washed test tubes by heating slowly over a Bunsen burner, and its copper content was determined by atomic absorption spectrometry according to the procedure of Nambiar (1976).

Midpoint potential. The difference in midpoint potential of cytochromes *c* and *d* was determined from the equilibrium constant for reduction of the enzyme by the following equation of Shimada & Oriei (1976):

$$\text{Equilibrium constant, } K = \frac{(c^{3+})(d^{2+})}{(c^{2+})(d^{3+})}$$

$$E_{m,\text{cyt.}d} - E_{m,\text{cyt.}c} = 0.06 \log K$$

Spectrophotometry. The absorption spectra of the samples were determined in a recording spectrophotometer (model MP-50L; Shimadzu, Kyoto, Japan). For measurements under anaerobic conditions, Thunberg cuvettes (1 cm) were employed. The cuvettes were evacuated thoroughly and then flushed with N₂; this procedure was repeated twice.

The concentration of the enzyme in purified preparations was calculated from its green haem *d* content. A sample of the purified enzyme was made 25 % (v/v) and 0.1 M with respect to pyridine and NaOH respectively, and was dispensed into two 1 cm glass cuvettes. A crystal of K₃Fe(CN)₆ was added to the reference cuvette to oxidize the green haem. The ΔA₆₁₈₋₆₅₀ between the reference and sample cuvettes was determined on a double-beam recording spectrophotometer and the amount of green haem was calculated using the absorption coefficient of 19.6 mm⁻¹ cm⁻¹ (Newton, 1969).

Dissociation of cytochrome c and d components. The purified nitrite reductase was treated with 1 to 1.5 mg sodium dodecyl sulphate (SDS) (mg protein)⁻¹ at 30 °C for 30 min and then loaded on to a Sephadex G-100 column (2.5 × 45 cm) which had been previously equilibrated with 0.025 M-Tris/HCl buffer, pH 7.5; the column was developed with the same buffer.

Assay of nitrite reductase. Nitrite reductase activity was assayed in Warburg flasks containing (final volume 1 ml): 0.025 M-Tris/HCl buffer, pH 7.5; 1 μmol NaNO₂; and 0.1 ml enzyme. The flasks were evacuated and flushed with high-purity N₂. After 5 min preincubation at 30 °C, 2 μmol Na₂S (freshly prepared solution) was injected from an air-tight syringe into the side-arm of the flask through a Subaseal. The reaction was started by mixing the contents and terminated after 30 min by adding 1 ml 10 % (w/v) zinc acetate in 10 % (v/v) ethanol. After centrifuging at 2000 g for 5 min, nitrite was determined in a sample of the supernatant fraction by the method of Hewitt & Nicholas (1964).

Assay of cytochrome oxidase. This activity was followed by measuring the rate of oxygen uptake in a Beckman oxygen electrode (model 39065) fitted with an adaptor box and recorder. Into the reaction vessel were dispensed 1.8 ml 0.025 M-Tris/HCl buffer, pH 7.5, and 0.1 ml purified enzyme, and these were

Table 1. *Purification of nitrite reductase*

Total activity is expressed as $\mu\text{mol NO}_2^-$ reduced (30 min) $^{-1}$, and specific activity as $\mu\text{mol NO}_2^-$ reduced (30 min) $^{-1}$ (mg protein) $^{-1}$.

Fraction	Total activity	Specific activity	% Recovery of activity	Relative purity
S ₁₈ . Supernatant fraction after centrifuging the disrupted acetone-dried cells at 18000 <i>g</i> for 45 min	507.5	0.05	100	1
S ₁₀₀ . Supernatant fraction after centrifuging S ₁₈ at 100000 <i>g</i> for 90 min	384.0	0.08	75	1.5
(NH ₄) ₂ SO ₄ fraction. Precipitate of S ₁₀₀ obtained between 40 and 95 % saturation (NH ₄) ₂ SO ₄ , dissolved in 0.025 M-Tris/HCl buffer, pH 7.5	184.8	0.11	36	1.9
Combined fractions from DEAE-cellulose column eluted with 0.025 M-Tris/HCl buffer, pH 7.5	173.8	0.71	34	12.8
Combined fractions from Sephadex G-100 column eluted with 0.025 M-Tris/HCl buffer, pH 7.5	132.3	1.12	26	20.4

equilibrated at 30 °C for 5 min. The electron donor (either sulphide or reduced *T. denitrificans* cytochrome *c*-551) was then injected from a microsyringe. The enzyme activity was expressed as nmol oxygen utilized min $^{-1}$ (mg protein) $^{-1}$. Reduced cytochrome *c* was prepared by adding excess Na₂S₂O₄ and then dialysing for 12 h against 0.025 M-Tris/HCl buffer, pH 7.5, to remove the reductant.

Mass spectrometry. The gaseous products of purified nitrite reductase activity were identified in an A.E.I. MS-2 mass spectrometer using ¹⁵N-labelled nitrite. The incubation mixture in Warburg flasks contained (final volume 1 ml): 10 $\mu\text{mol Na}^{15}\text{NO}_2$ (27 atom % excess); 0.1 M-Tris/HCl buffer, pH 7.5; and 0.2 ml purified enzyme containing 2.5 mg protein (specific activity 1.12). The flasks were evacuated with a vacuum pump and then flushed with He. After 5 min preincubation at 30 °C, the reaction was started by injecting 10 $\mu\text{mol Na}_2\text{S}$ (freshly prepared solution) through a Subaseal fitted to the side-arm. The reaction was terminated by injecting 0.25 ml 10 M-KOH into the reaction mixture. The gases formed were determined in the mass spectrometer.

Protein determination. The micro-biuret method of Itzhaki & Gill (1964) was used for estimating protein with bovine serum albumin as a standard.

RESULTS

Purification of nitrite reductase

All procedures during purification of the enzyme were carried out at 4 °C. The cell-free extract (S₁₈, Table 1) was centrifuged at 100000 *g* for 90 min. To this supernatant (S₁₀₀, Table 1) solid (NH₄)₂SO₄ was added gradually with stirring, and the precipitate obtained between 40 and 95 % saturation was collected by centrifuging at 10000 *g* for 20 min. The pellet was re-dissolved in a minimum volume of 0.025 M-Tris/HCl buffer, pH 7.5, and dialysed against double-distilled water overnight. This fraction was loaded on to a DEAE-cellulose column (DE32; 2.5 × 26 cm) previously equilibrated with 0.025 M-Tris/HCl buffer, pH 7.5. Under these conditions, nitrite reductase was not adsorbed and was eluted with the equilibrating buffer in one column volume. Cytochrome *c*-554, which was retained on the column, was eluted with 0.025 M-Tris/HCl, pH 7.5, containing 0.5 M-KCl. This cytochrome had absorption bands at 408 and 525 nm in its oxidized state and at 416, 524 and 554 nm when reduced with Na₂S₂O₄. The fractions containing nitrite reductase were pooled and concentrated by ultrafiltration under N₂ using a PM-10 membrane (Amicon, Massachusetts, U.S.A.). The concentrated fraction was then loaded on to a Sephadex G-100 column (2.5 × 45 cm) equilibrated with 0.025 M-Tris/HCl buffer, pH 7.5. The same buffer was used to elute the sample and 3 ml fractions were collected. Nitrite reductase was recovered after

Fig. 1. Elution of nitrite reductase from Sephadex G-100. Combined fractions from a DEAE-cellulose column were concentrated and loaded on to a Sephadex G-100 column equilibrated with 0.025 M-Tris/HCl buffer, pH 7.5. ●, A_{280} ; ○, A_{405} ; □, nitrite reductase activity, expressed as nmol NO_2^- reduced (30 min) $^{-1}$.

the void volume in fractions 17 to 29 (Fig. 1); following fractions contained a cytochrome which had absorption maxima at 408 and 525 nm in the oxidized form and at 412, 521 and 551 nm in the reduced form.

The purification procedure for nitrite reductase from *T. denitrificans* is summarized in Table 1.

Some properties of the purified enzyme

Absorption spectra. The oxidized enzyme had maxima at 405, 525 and 645 nm and after reduction with $\text{Na}_2\text{S}_2\text{O}_4$, at 418, 468, 523, 549 to 554, 615 and 695 nm (Fig. 2). When reduced anaerobically with sulphide, the physiological reductant for the enzyme, it had absorption bands at 418, 462, 523, 549 to 554, 610 and 664 to 667 nm (Fig. 3a). The ratio of A_{462}/A_{610} for the sulphide-reduced enzyme was three times greater than for the $\text{Na}_2\text{S}_2\text{O}_4$ -reduced enzyme. The absorption bands at 418, 523 and 549 to 554 nm are associated with cytochrome *c* and those at 462, 610 and 664 to 667 nm with cytochrome *d*.

The absorption spectrum of cytochrome *d*, but not that of cytochrome *c*, was pH-dependent. On decreasing the pH from 8.0 to 5.5, the ratio of A_{462}/A_{610} in the reduced enzyme decreased from 1.7 to 0.42.

Reaction with pyridine. The purified enzyme in alkaline pyridine and $\text{Na}_2\text{S}_2\text{O}_4$ had a sharp α -band at 549 nm instead of a bifurcated peak at 549 to 554 nm and the ratio of reduced α /reduced β increased from 1.0 to 2.4. The cytochrome *d* was autoreducible in alkaline pyridine and the α -peak, shifted to 620 nm, was also sharpened considerably.

Reaction with cyanide. When the enzyme was reduced with sulphide in the presence of cyanide, the normal γ -peak of reduced cytochrome *d* was abolished, two bands appeared at 443 and 482 nm and the α -peak was shifted to 627 nm. The absorbance at 523 nm was greatly decreased and the 554 nm component of the bifurcated α -band became more pronounced.

Reaction with nitrite. When nitrite was added to the sulphide-reduced enzyme, cytochromes *c* and *d* both became oxidized (Fig. 3b). This effect was specific for nitrite since nitrate had no effect. On adding nitrite to the reduced enzyme, the absorption peak at 462 nm disappeared immediately followed by the one at 610 nm and subsequently the bands associated

Fig. 2. Absorption spectra of purified nitrite reductase (specific activity 1.12): ---, 6 μ M-nitrite reductase in 43 mM-Tris/HCl buffer, pH 7.5; —, after reduction with $\text{Na}_2\text{S}_2\text{O}_4$.

Fig. 3. Difference spectra of purified nitrite reductase (specific activity 1.12): (a) reduced with sulphide; (b) after addition of NO_2^- to the reduced enzyme. (a) 6 μ M-nitrite reductase in 43 mM-Tris/HCl buffer, pH 7.5, (final volume 2 ml) was dispensed into a Thunberg cuvette and 5 mg Na_2S was placed in the side-arm. The cuvette was evacuated and flushed twice with high-purity N_2 . The reaction was started by tipping Na_2S into the cuvette. After 10 min, when the reduction was complete, the spectrum was recorded against a reference sample without Na_2S . (b) Difference spectrum recorded 10 min after adding 0.1 M- NaNO_2 to (a). (c) No difference line.

Table 2. Ratio of haem *d* to *c* in the purified nitrite reductase

The concentration of cytochrome *d* was calculated as described in Methods, and that of cytochrome *c* was estimated from the difference in absorption of the oxidized and reduced band at 550 nm, using an absorption coefficient of $30.2 \text{ mm}^{-1} \text{ cm}^{-1}$ (Horio *et al.*, 1961).

Preparation no.	Haem <i>c</i> (μ M)	Haem <i>d</i> (μ M)	$\frac{\text{Haem } d}{\text{Haem } c}$
1	104	110	1.05
2	92	96	1.04

with oxidized cytochrome *c* appeared. The oxidation of the cytochrome components of the enzyme was accompanied by the appearance of a peak at 572 nm.

Cytochromes *c* and *d* were present in the ratio of 1:1 (Table 2). The sequence of reduction of cytochromes *c* and *d* on addition of sulphide to the enzyme was determined by monitoring the percentage reduction of these cytochromes at 554 and 610 nm respectively. As shown in Fig. 4, cytochrome *c* was reduced to a greater extent than cytochrome *d* during the initial

Table 3. Comparison of nitrite reductase and cytochrome oxidase activities of purified nitrite reductase

The nitrite reductase and cytochrome oxidase activities of purified enzyme (specific activity 1.12) were determined as described in Methods with either S^{2-} or $Na_2S_2O_4$ -reduced *T. denitrificans* cytochrome *c*-551 as electron donor. In one experiment in which S^{2-} was used as the reductant, the assay mixture also contained *T. denitrificans* cytochrome *c*-551 ($2\ \mu M$). Nitrite reductase activity is expressed as $nmol\ NO_2^-$ reduced $min^{-1}\ (mg\ protein)^{-1}$, and cytochrome oxidase activity as $nmol\ O_2$ utilized $min^{-1}\ (mg\ protein)^{-1}$.

Electron donor	Addition to the assay mixture	Nitrite reductase activity	Cytochrome oxidase activity
Sulphide ($2\ \mu mol$)	None	35	18
Sulphide ($2\ \mu mol$)	Cytochrome <i>c</i> -551 ($2\ \mu M$)	107	45
Reduced cytochrome <i>c</i> -551 ($20\ nmol$)	None	21	20
Reduced cytochrome <i>c</i> -551 ($53\ nmol$)	None	59	53

Fig. 4

Fig. 5

Fig. 4. Sequence of reduction of cytochromes *c* and *d* of purified nitrite reductase. Details of the reaction mixture and experimental conditions were as described in the legend to Fig. 3 except that the rates of reduction of cytochromes *c* and *d* were recorded immediately after adding sulphide from the side-arm of the Thunberg cuvette. The percentage reduction was calculated on the basis of the maximum reduction of the cytochromes obtained, namely $2.5\ \mu M$ -cytochrome *c* and $2.3\ \mu M$ -cytochrome *d*. $\circ$, ΔA_{554} ; $\bullet$, ΔA_{610} .

Fig. 5. The nitrogenous gases produced from $^{15}NO_2^-$ by purified nitrite reductase. The reaction mixture (see Methods) was incubated for various times. Each reaction was terminated by adding $0.25\ ml\ 10\ M\ KOH$ and the ^{15}N -labelled gases thus formed were identified in a mass spectrometer: $\circ$, NO (mass 31); $\bullet$, N_2O (mass 45); $\triangle$, N_2 (mass 29).

stages of reduction. On adding nitrite to the reduced enzyme, cytochrome *d* was oxidized more rapidly than cytochrome *c*.

The molecular weight of the nitrite reductase was found to be about 120000 as determined by gel filtration through Sephadex G-150. The difference in midpoint potential of cytochromes *d* and *c* was calculated from Fig. 4 as described in Methods. The redox potential of cytochrome *d* was 22 mV more positive than that of cytochrome *c*. The copper content of the purified nitrite reductase was less than $0.06\ ng\ atom\ mg^{-1}$.

Enzyme activity. The purified nitrite reductase also showed cytochrome oxidase activity since oxygen uptake was observed on adding sulphide (Table 3). Oxygen uptake and nitrite reduction increased threefold on adding *T. denitrificans* cytochrome *c*-551 to the reaction mixture. In contrast, *T. denitrificans* cytochrome *c*-554 and mammalian cytochrome *c* had no effect. Reduced *T. denitrificans* cytochrome *c*-551 was an effective electron donor for both nitrite reduction and oxygen utilization; activity was proportional to the amount of cytochrome *c*-551 added. The stoichiometry of the oxidation of cytochrome *c*-551 and

Fig. 6. Elution of cytochromes *c* and *d* on a Sephadex G-100 column. Purified nitrite reductase was treated with SDS at 30 °C for 30 min, loaded on to a Sephadex G-100 column and eluted with 0.025 M-Tris/HCl buffer, pH 7.5, at room temperature: ○, A_{405} ; ●, A_{280} .

Fig. 7. Absorption spectra of cytochrome *d*, obtained after treatment of the purified nitrite reductase with SDS: ---, oxidized; —, reduced with Na₂S₂O₄.

nitrite reduction was 1:1 over the range 10 to 100 nmol. This bacterial cytochrome *c* was much more effective than the mammalian cytochrome *c* in reducing nitrite.

Products of nitrite reduction. From $^{15}\text{NO}_2^-$, the purified nitrite reductase produced both NO (mass 31) and N₂O (mass 45) (Fig. 5). During the first 30 min of incubation N₂O was formed in relatively larger amounts than NO.

Dissociation of cytochromes *c* and *d*. Cytochromes *c* and *d* were dissociated from the enzyme by treatment with SDS. Between 1 and 1.5 mg SDS (mg protein)⁻¹ was required to effect dissociation. The absorption spectrum of the SDS-treated enzyme on reduction with sulphide showed a single peak at 549 nm in place of a bifurcated α -peak (cytochrome *c*), whereas the absorption bands due to reduced cytochrome *d* were not apparent. When the SDS-treated enzyme was chromatographed on Sephadex G-100, the pink cytochrome *c* component migrated through the column much faster than did the green cytochrome *d* (Fig. 6). The A_{280} of these two fractions suggested that the bulk of the protein was associated with cytochrome *c* fraction.

The oxidized and Na₂S₂O₄-reduced spectra of the separated cytochrome *c* fraction were similar to those of the cytochrome *c* of the undissociated enzyme, except that on reduction with Na₂S₂O₄, the bifurcation of the α -band at 549 to 554 nm was not so marked but this became apparent on adding nitrite but not nitrate. This effect was not observed for the *T. denitrificans* cytochrome *c*-551.

The cytochrome *d* of the dissociated enzyme had absorption bands in the oxidized form at 405 and 680 nm and a shoulder near 480 nm. On reduction with $\text{Na}_2\text{S}_2\text{O}_4$, two broad bands at 460 and 610 nm were produced (Fig. 7). Reduced cytochrome *d* was rapidly oxidized in air.

Remixing cytochromes *c* and *d* did not restore either nitrite reductase or oxygen uptake activity.

DISCUSSION

Nitrite reductase from *T. denitrificans* is associated with membranes and attempts to solubilize it with detergents, lipases and proteolytic enzymes inactivated the enzyme. However, activity was removed from membranes by acetone treatment of the cells. The purified enzyme showed both nitrite reductase and cytochrome oxidase activities, suggesting that, as in other denitrifying bacteria, e.g. *Pseudomonas aeruginosa* (Yamanaka & Okunuki, 1963*a*), *Micrococcus denitrificans* (Lam & Nicholas, 1969; Newton, 1969), these activities are associated with the same protein which functions with either NO_2^- or O_2 as a terminal electron acceptor. Since in the present investigations the bacteria were grown anaerobically with nitrate, the physiological function of this enzyme was for nitrite reduction. Moreover, the specific activity of nitrite reductase was twice that of cytochrome oxidase.

The difference spectra of purified nitrite reductase from *T. denitrificans* showed typical cytochromes *c* and *d* bands. Nitrite reductase from *P. aeruginosa* (Horio *et al.*, 1961), which was initially studied as cytochrome oxidase but was referred to later as nitrite reductase (Yamanaka & Okunuki, 1974), and those from *M. denitrificans* (Lam & Nicholas, 1969; Newton, 1969) and *Alcaligenes faecalis* (Iwasaki & Matsubara, 1971) also contained haem *c* and *d*. In the present investigations haem *c* and *d* were shown to be in the ratio of 1:1 as was also reported for nitrite reductase from *P. aeruginosa* (Horio *et al.*, 1961) and *A. faecalis* (Iwasaki & Matsubara, 1971). The molecular weight of the *T. denitrificans* nitrite reductase was 120000, in agreement with the enzyme from *M. denitrificans* (Newton, 1969) and *P. aeruginosa* (Gudat, Singh & Wharton, 1973). Values of 90000 and 85000 have also been reported for the *P. aeruginosa* enzyme (Horio *et al.*, 1961; Newton, 1969). As for nitrite reductases from *M. denitrificans* and *P. aeruginosa*, copper was not detected in the purified nitrite reductase from *T. denitrificans*.

Sulphide, the physiological reductant of this enzyme, reduced both cytochromes *c* and *d*. The sequence of the reduction of the *cd* complex by sulphide indicated that initially cytochrome *c* was reduced, followed by the reduction of cytochrome *d*. On adding nitrite to the sulphide-reduced enzyme, cytochrome *d* was oxidized before cytochrome *c*. Wharton, Gudat & Gibson (1973) and Shimada & Orie (1976) observed similar patterns for the reduction of cytochromes *c* and *d* of nitrite reductase from *P. aeruginosa* using different electron donors, but the sequence of oxidation of reduced cytochromes by nitrite has not been reported earlier. The more positive redox-potential of cytochrome *d* than that of cytochrome *c*, reported here, supports the suggested pattern of electron transport.

During the oxidation of reduced cytochromes by NO_2^- , but not O_2 , a shoulder appeared in the spectrum at 572 nm. This may be associated with the formation of a complex between the enzyme and the product of nitrite reduction. Yamanaka & Okunuki (1963*b*) have observed a similar spectral change on oxidation of the enzyme with NO_2^- or on bubbling NO through the reduced enzyme. The effect of NO on the spectrum of a nitrite reductase from *P. aeruginosa* has also been observed by Rowe *et al.* (1977).

During the purification of nitrite reductase from *T. denitrificans*, cytochrome *c*-551 and cytochrome *c*-554 were isolated. The stimulation of both nitrite reductase and cytochrome oxidase activities by catalytic amounts of cytochrome *c*-551, but not by cytochrome *c*-554 or mammalian cytochrome *c*, indicates that it is an electron carrier linking sulphide to the cytochrome *cd* complex. This suggestion was further strengthened by the observation that reduced cytochrome *c*-551 acts as a reductant for the purified enzyme with either NO_2^- or

O₂ as a terminal acceptor and one mole of NO₂⁻ or O₂ was reduced per mole of cytochrome *c*-551 oxidized. Cytochromes *c*-551 from *M. denitrificans* and *P. aeruginosa* have also been reported to serve as electron donors for nitrite reduction and cytochrome oxidase (Horio *et al.*, 1961; Newton, 1969).

The products of ¹⁵NO₂⁻ reduction by the purified enzyme were primarily ¹⁵NO and ¹⁵N₂O. Purified nitrite reductases from *P. aeruginosa* (Walker & Nicholas, 1961), *P. denitrificans* (Radcliffe & Nicholas, 1968), *M. denitrificans* (Lam & Nicholas, 1969) and *P. perfectomarinus* (Cox, Payne & Dervartanian, 1971) produced only NO from NO₂⁻. A highly purified nitrite reductase from *A. faecalis* (Matsubara & Iwasaki, 1972) produced both NO and N₂O as detected by gas chromatography. It was suggested that this nitrite reductase can reduce NO to N₂O in agreement with the results reported here for *T. denitrificans*.

Yamanaka & Okunuki (1963*c*) dissociated the nitrite reductase from *P. aeruginosa* into haem *d* and protein-containing cytochrome *c* components by acid-acetone treatment; cytochrome *d* was extracted into acetone and cytochrome *c* was precipitated. In the present investigations purified nitrite reductase from *T. denitrificans* was resolved into cytochrome *c* and haem *d* by SDS treatment and gel filtration on Sephadex G-100. Neither of these components alone nor when mixed together showed either nitrite reductase or cytochrome oxidase activity. Kijimoto (1968) reported that a cytochrome *d* fraction, obtained by SDS treatment of a nitrite reductase from *P. aeruginosa* and subsequently separated by sucrose density gradient centrifugation, had nitrite reductase activity. Since this cytochrome *d* contained appreciable amounts of cytochrome *c* as well, it is doubtful whether cytochrome *d* alone can function as a nitrite reductase without the cytochrome *c* part of the complex.

A possible mechanism for electron transfer for the sulphide-linked nitrite reductase in *T. denitrificans* is:

The overall nitrate and nitrite reductases may be represented as follows:

REFERENCES

- ADAMS, C. A., WARNES, G. M. & NICHOLAS, D. J. D. (1971). A sulphite-dependent nitrate reductase from *Thiobacillus denitrificans*. *Biochimica et biophysica acta* **235**, 398-406.
- ALEEM, M. I. H. (1977). Energy coupling in chemolithotrophic bacteria. *Symposia of the Society for General Microbiology* **27**, 351-381.
- AMINUDDIN, M. (1974). *Inorganic sulphur compounds oxidation and denitrification in Thiobacillus denitrificans*. Ph.D. thesis, University of Adelaide.
- AMINUDDIN, M. & NICHOLAS, D. J. D. (1973). Sulphide oxidation linked to the reduction of nitrate and nitrite in *Thiobacillus denitrificans*. *Biochimica et biophysica acta* **325**, 81-93.
- AMINUDDIN, M. & NICHOLAS, D. J. D. (1974*a*). Electron transfer during sulphide and sulphite oxidation in *Thiobacillus denitrificans*. *Journal of General Microbiology* **82**, 115-123.
- AMINUDDIN, M. & NICHOLAS, D. J. D. (1974*b*). An AMP-independent sulphite oxidase from *Thiobacillus denitrificans*: purification and properties. *Journal of General Microbiology* **82**, 103-113.
- ANDREWS, P. (1964). Estimation of molecular weights of proteins by Sephadex gel-filtration. *Biochemical Journal* **91**, 222-233.

- COX JR, C. D., PAYNE, W. J. & DERVARTANIAN, D. V. (1971). Electron paramagnetic resonance studies on the nature of hemoproteins in nitrite and nitric oxide reduction. *Biochimica et biophysica acta* **253**, 290–294.
- GUDAT, J. C., SINGH, J. & WHARTON, D. C. (1973). Cytochrome oxidase from *Pseudomonas aeruginosa*. 1. Purification and some properties. *Biochimica et biophysica acta* **292**, 376–390.
- HEWITT, E. J. & NICHOLAS, D. J. D. (1964). Enzymes of inorganic nitrogen metabolism. In *Modern Methods of Plant Analysis*, vol. 7, pp. 67–172. Edited by Y. F. Linskens, B. D. Sanwal and M. V. Tracey. Berlin: Springer Verlag.
- HORIO, T., HIGASHI, T., YAMANAKA, T., MATSUBARA, H. & OKUNUKI, K. (1961). Purification and properties of cytochrome oxidase from *Pseudomonas aeruginosa*. *Journal of Biological Chemistry* **236**, 944–951.
- ITZHAKI, R. F. & GILL, D. M. (1964). A micro-biuret method for estimating proteins. *Analytical Biochemistry* **9**, 401–410.
- IWASAKI, H. & MATSUBARA, T. (1971). Cytochrome *c*-557 (551) and cytochrome *cd* of *Alcaligenes faecalis*. *Journal of Biochemistry* **69**, 847–857.
- KUJIMOTO, S. (1968). Studies on *Pseudomonas* cytochrome oxidase. II. Split of *Pseudomonas* cytochrome oxidase into haem *c*- and haem *d*-components. *Annual Reports of Biological Works, Faculty of Science, Osaka University* **16**, 19–33.
- LAM, Y. & NICHOLAS, D. J. D. (1969). A nitrite reductase with cytochrome oxidase activity from *Micrococcus denitrificans*. *Biochimica et biophysica acta* **180**, 459–472.
- MATSUBARA, T. & IWASAKI, H. (1972). Nitric oxide-reducing activity of *Alcaligenes faecalis* cytochrome *cd*. *Journal of Biochemistry* **72**, 57–64.
- NAMBIAR, E. K. S. (1976). Genetic differences in the copper nutrition of cereals. II. Genotypic differences in response to copper in relation to copper, nitrogen and other mineral contents of plants. *Australian Journal of Agricultural Research* **27**, 465–477.
- NEWTON, N. (1969). The two-haem nitrite reductase of *Micrococcus denitrificans*. *Biochimica et biophysica acta* **185**, 316–331.
- PEETERS, T. & ALEEM, M. I. H. (1970). Oxidation of sulphur compounds and electron transport in *Thiobacillus denitrificans*. *Archiv für Mikrobiologie* **71**, 319–330.
- RADCLIFFE, B. & NICHOLAS, D. J. D. (1968). Some properties of a nitrite reductase from *Pseudomonas denitrificans*. *Biochimica et biophysica acta* **153**, 545–554.
- ROWE, J. J., SHERR, B. F., PAYNE, W. J. & EAGON, R. G. (1977). A unique nitric oxide-binding complex formed by denitrifying *Pseudomonas aeruginosa*. *Biochemical and Biophysical Research Communications* **77**, 253–258.
- SAWHNEY, V. & NICHOLAS, D. J. D. (1977). Sulphite- and NADH-dependent nitrate reductase from *Thiobacillus denitrificans*. *Journal of General Microbiology* **100**, 49–58.
- SHIMADA, H. & ORII, Y. (1976). Oxidation-reduction behaviour of the heme *c* and heme *d* moieties of *Pseudomonas aeruginosa* nitrite reductase and formation of oxygenated intermediate at heme *d*. *Journal of Biochemistry* **80**, 135–140.
- SQUIRE, P. G. (1964). A relationship between the molecular weights of macromolecules and their elution volumes based on a model for Sephadex gel filtration. *Archives of Biochemistry and Biophysics* **107**, 471–478.
- WALKER, G. C. & NICHOLAS, D. J. D. (1961). Nitrite reductase from *Pseudomonas aeruginosa*. *Biochimica et biophysica acta* **49**, 350–360.
- WHARTON, D. C., GUDAT, J. C. & GIBSON, Q. H. (1973). Cytochrome oxidase from *Pseudomonas aeruginosa*. II. Reaction with copper protein. *Biochimica et biophysica acta* **292**, 611–620.
- YAMANAKA, T. & OKUNUKI, K. (1963*a*). Crystalline *Pseudomonas* cytochrome oxidase. I. Enzymic properties with special reference to the biological specificity. *Biochimica et biophysica acta* **67**, 379–393.
- YAMANAKA, T. & OKUNUKI, K. (1963*b*). Crystalline *Pseudomonas* cytochrome oxidase. II. Spectral properties of the enzyme. *Biochimica et biophysica acta* **67**, 394–406.
- YAMANAKA, T. & OKUNUKI, K. (1963*c*). Crystalline *Pseudomonas* cytochrome oxidase. III. Properties of the prosthetic groups. *Biochimica et biophysica acta* **67**, 407–416.
- YAMANAKA, T. & OKUNUKI, K. (1974). Cytochromes. In *Microbial Iron Metabolism*, pp. 349–400. Edited by J. B. Neilands. New York: Academic Press.