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THE TWO-HAEM NITRITE REDUCTASE OF *MICROCOCCUS DENITRIFICANS*

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SUMMARY

1 This work characterises a two-haem cytochrome isolated from *Micrococcus denitrificans* The ratio of the absorbance of the protein band at 280 nm to that of the Soret band at 408 nm of the purest preparation was 0 75, the molecular weight was 120 000 and the isoelectric point was pH 3 85 (0°) In addition to the major component I, there are two slightly less acidic minor components Component I and Components II + III have been separated and compared spectroscopically

2 The two-haem cytochrome contains a *c*-type and an a_2 (*d*)-like haem The *c*-haem must be bound to the protein in an unusual manner, being responsible for the double α-band and the low α/β band ratio in the reduced native cytochrome, in alkaline pyridine a normal *c*-type haemochrome is formed, with a single sharp band at 550 nm The *d*-like (green) haem reacts under different conditions with CO, pyridine, imidazole, CN^-, N_3^- and NO_2^-, it can be removed from the protein by mild procedures and is not identical with haem *d*

3 This cytochrome is present only in cells grown anaerobically in the presence of NO_3^- and it has both nitrite reductase and cytochrome *c* oxidase activities when assayed with reduced Micrococcus cytochrome *c* as substrate

4 *Pseudomonas aeruginosa* cytochrome oxidase/nitrite reductase was prepared for comparison ($A_{280\ nm}/A_{410\ nm}$ = 0 80) It is a less acidic (isoelectric point 7 1) and smaller (mol wt 85 000) protein, but contains the same two haem groups as the Micrococcus enzyme Unlike the Micrococcus enzyme, it has only one component

INTRODUCTION

A soluble haemoprotein with a complex spectrum was first observed in *Micrococcus denitrificans* during the preparation of cytochrome *c* (ref 1) It was partially purified and briefly described by NEWTON[2] as an oxido-reductase containing a *c*-type haem and a green haem This new haemoprotein closely resembled in its spectral

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properties the oxido-nitrite reductase of *Pseudomonas aeruginosa* (EC 1 9 3 2) studied by YAMANAKA[3], who classified the two different haem groups as haem *c* and haem a_2, the latter is now known as haem *d* (ref 4)

The purpose of the research described in this communication was to further purify and characterise the Micrococcus haemoprotein, to establish the conditions under which it occurs and to compare it closely with the Pseudomonas protein Both were extensively purified, their enzymic properties and absorption spectra were very similar, but they had widely differing molecular weights and isoelectric points The Micrococcus enzyme contains several components, but the Pseudomonas enzyme only one Preliminary evidence shows that the green haem is not identical with the haem *d* of *Aerobacter aerogenes*[5]

MATERIALS AND METHODS

Organisms Two strains of *M denitrificans* were used ATCC 13543 and NCIB 8944. They were grown in the yeast–peptone–nitrate medium of VERNON[6] The strain of *P aeruginosa* used was obtained from Dr T YAMANAKA, Osaka, and was grown on the bouillon–peptone–nitrate medium described by HORIO[7]. In small-scale experiments the organisms were grown in closed 600-ml blood bank bottles, and for larger preparations 10-l bottles with a trap allowing for gas escape were used The Pseudomonas was grown for 1–2 days at 37°, and the Micrococcus for 4–5 days at 30°. The organisms were harvested at 4°, washed with 0 01 M potassium phosphate buffer (pH 6 0) and suspended to about 25% (wet wt /v) in 0.1 M phosphate buffer (pH 6 0) for immediate use or for storage at −20°

Preparation and assay of crude extracts The suspension of cells was passed twice through a modified French press[8] at a pressure of 8 tons/inch^2 The viscous suspension of disrupted cells was incubated with deoxyribonuclease (Calbiochem B Grade, 0 1 mg per 10 ml suspension), (5–10 min) and then centrifuged for 30 min at 18 000 × *g* to sediment cell debris and poly-β-hydroxybutyrate granules The resulting opalescent supernatant could be used for direct spectroscopic assay of the enzyme, or could be further clarified by centrifugation for 1 h at 144 000 × *g*, or for 30 min at 10 000 × *g* following addition of $(NH_4)_2SO_4$ to 30% satn

A spectrophotometric assay, based on the 618-nm absorption band of the pyridine haemochromogen formed from the green haem, was devised to estimate the amount of enzyme present a sample of extract was made 25% (v/v) with respect to pyridine and 0 1 M with respect to NaOH and divided between two cuvettes To the reference cuvette was added a drop of dilute $K_3Fe(CN)_6$ solution to oxidise the green haem, which is autoreducible in the presence of pyridine and alkali The ΔA (618—650 nm) between the two cuvettes was read using the 0 1 *A* slidewire of a Cary 14R Spectrophotometer A $\Delta\varepsilon$ (618—650 nm) of 19 6 $mM^{-1} \cdot cm^{-1}$, calculated from the data of YAMANAKA[3] was used for estimation of the green haem

Preparation of Sephadex, DEAE-cellulose, CM-cellulose and hydroxylapatite Sephadex G-100, G-150 and G-200 (Pharmacia) were used for purification procedures and molecular weight determinations, and Sephadex G-15 for desalting procedures For molecular weight determinations, a 78 cm × 5 cm column of Sephadex G-150 was was calibrated with a mixture of marker proteins of known molecular weights in 0 1 M phosphate buffer (pH 6 0) horse heart cytochrome *c* (mol wt 12 270, Sigma,

Type VI), horse heart myoglobin (mol wt 17 800, B.D H), ovalbumin (mol wt. 45 000, Sigma Grade V), crystalline bovine serum albumin (mol wt 67 000, Armour) and γ-globulin (mol wt 160 000, Cohn FII, Sigma) Void volume, V_0 was determined using a Blue Dextran (Pharmacia) marker, total volume, V_t was calculated from the dimensions of the column, elution volume, V_e was measured for each marker and unknown. The K_{av}, $(V_e - V_0)/(V_t - V_0)$ for each protein was plotted against the logarithm of its molecular weight, and from this straight line plot the molecular weights of unknowns were determined

DEAE-cellulose (Cellex-D, Calbiochem) was used for large-scale (batchwise) ion-exchange chromatography It was washed in succession in 0 5 M HCl, water, 0 5 M NaOH, water and then equilibrated with 0 2 M phosphate buffer (pH 6 0) DEAE-cellulose DE-52 (microgranular, Whatman) was used at later stages of the purification as it was found to have greater resolving power and was simply equilibrated with 0 1 M phosphate buffer (pH 6 0) CM-cellulose (CM-52, microgranular, Whatman) was also used without washing and was equilibrated in 0.01 M phosphate buffer (pH 6 0).

Hydroxylapatite was prepared as described by SIEGELMAN *et al* [9] and equilibrated with 0 005 M phosphate buffer (pH 6 0)

Electrophoresis Electrophoresis on cellulose acetate was performed using 1 inch $\times$ 6 75 inch strips of Sepraphore III (Gelman Instrument Co , Ann Arbor, Mich) in a Shandon type electrophoresis apparatus at room temperature for 1–2 h at 200 V The strips were then sliced longitudinally, one half being treated with a protein stain of 0 5% (w/v) Coomassie Blue (Imperial Chemical Industries) in 50% methanol, 10% glacial acetic acid (v/v) and the other half with a haem-specific stain (0 5% benzidine in 70% ethanol (pH 5 2) containing 0 003% H_2O_2)

Polyacrylamide-gel electrophoresis was carried out in small glass tubes using 7 5% acrylamide gel polymerised with 0 08% persulfate at 20° for 30 min The sample, at low ionic strength, was mixed with sucrose and 10-μl aliquots were layered on top of the gels After 2 h electrophoresis at room temperature at a current of 2 mA per tube, the gel was extruded from the tube and fixed in 12 5% trichloroacetic acid One of a duplicate pair was stained in Coomassie blue in 12 5% trichloroacetic acid[10] and the other in aqueous benzidine–H_2O_2 (pH 5 2)

pH-focussing A 450 ml pH-focussing column (L K B , Sweden) was used to determine isoelectric points as described by VESTERBERG AND SVENSSON[11] Ampholine buffers (L K.B) covering the pH ranges 3–5 and 4–6 were used with the heavy anode solution placed at the bottom of the column The column was jacketed and kept at 4 $\pm$ 0 2° during the approx 48-h runs. During the first 5 h the power was increased stepwise to 3 0 W, and then maintained at this level until equilibrium had been reached. The column was then drained and 5–10-ml fractions collected and stored on ice The pH of these fractions was measured at 0° using a Radiometer pH meter type pHM-25SE standardised at 0°, and their spectra (400–500 nm) read at 20°

Spectrophotometry A Cary Model 14R spectrophotometer equipped with 650 W tungsten–iodine light source, alternative 0–1, 1–2 A or 0–0 1, 0 1–0 2 A slidewires, and Model 1462 scattered transmission accessory with Dumont 7664 photomultiplier was used for recording absolute and difference spectra For measurements at −196° the "double-freeze" technique[12] was used, and dilutions were made in phosphate buffers containing 50% glycerol The cuvette assembly (2-mm light path) was cooled

by liquid N_2 contained in an oval vessel hollowed out of polyurethane and fitted with evacuated double glass windows

Nitrite reductase and cytochrome oxidase assays Micrococcus cytochrome *c* prepared as described by SMITH *et al.*[1] was reduced with dithionite and passed through a Sephadex G-15 column equilibrated with N_2-saturated buffer to remove excess reductant It was either used immediately, or flushed with N_2 and stored at −20° The cytochrome oxidase assay was carried out in open cuvettes using O_2-saturated reagents, and the nitrite reductase assay in Thunberg cuvettes under N_2, using N_2-saturated reagents containing 0 002 M $NaNO_2$ Both assays were performed at 25° in 0 05 M phosphate buffer (pH 6 5) and the rate of disappearance of the 550-nm band of this reduced cytochrome *c* (final concn 25 μM) was recorded following the addition of the enzyme preparation

RESULTS

Conditions for production of green haemoprotein

To find the best source of the protein and its possible function, growth experiments were undertaken using *M denitrificans* ATCC 13543 Table I shows the effect of various culture conditions on the growth of the organism and the production of the green haem At a concentration of 2%, NO_2^- was completely inhibitory to growth whether aerobic or anaerobic, $NO_3^- + Cu^{2+}$ was also inhibitory, but only under anaerobic conditions There was no significant growth anaerobically in the absence of NO_3^-, but NO_3^- was not necessary when O_2 was available as electron acceptor. The green haem was only detectable under conditions of anaerobic growth in the presence of NO_3^-

TABLE I

PRODUCTION OF GREEN HAEM UNDER DIFFERENT CONDITIONS

Organisms were grown and the haem content assayed as described in MATERIALS AND METHODS. KNO_3 and KNO_2 concentrations were 2% and $CuSO_4\ 5H_2O$ was 0 001%

Culture conditions	*Yield of cells (g dry wt)*	*Yield of green haem (nmoles/g dry wt)*
M denitrificans ATCC 13543		
Anaerobic	0 07*	31 9
Anaerobic + NO_3^-	0 42	38 0
Anaerobic + NO_2^-	0	—
Anaerobic + NO_3^- + Cu^{2+}	0	—
Aerobic	0 52	0
Aerobic + NO_3^-	0 44	0
Aerobic + NO_2^-	0	—
Aerobic + NO_3^- + Cu^{2+}	0 54	0
M denitrificans NCIB 8944		
Anaerobic + NO_3^-	0 45	34 2
P aeruginosa		
Anaerobic + NO_3^-	0 35	96 7

* This growth was supported by the small quantity of NO_3^- introduced with the inoculum.

Included in Table I are the yields obtained using *M denitrificans* NCIB 8944 and *P aeruginosa*, both grown anaerobically with NO_3^- There is no significant difference between the two strains of *M denitrificans*, but *P aeruginosa*, although a less-prolific grower, produces 3 times as much green haem per g dry wt of cells In subsequent experiments with *M denitrificans* the ATCC strain was used

The production of the haem was followed over a period of 7 days, it was detectable from the earliest stages of growth and, in fact, the yield per g dry wt cells was somewhat higher after 1–2 days than later

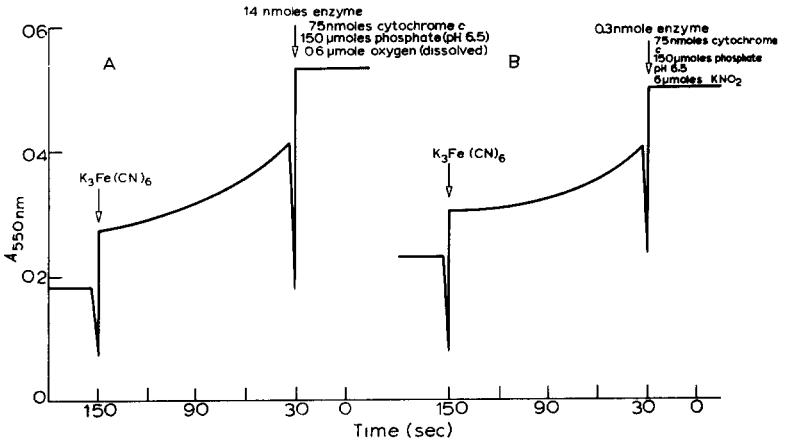

Fig 1 Cytochrome oxidase (A) and nitrite reductase (B) assays The cytochrome oxidase assay was carried out in an open cuvette, and the nitrite reductase assay in a Thunberg cuvette as described in MATERIALS AND METHODS Final volume in each assay was 3 0 ml

Enzymic properties

Fig I shows typical cytochrome *c* oxidase and nitrite reductase assays using the purified protein (see next section) with Micrococcus cytochrome *c* as substrate The affinity for NO_2^- appeared to be greater than that for O_2 under the chosen conditions of assay the calculated turnovers were approx 50 and 250 µmoles cytochrome *c* per µmole green haem per min for the oxidase and nitrite reductase activities respectively. The cytochrome oxidase activity obeyed first order kinetics described by SMITH AND CONRAD for mammalian cytochrome oxidase[13], and likewise was inhibited by CN^- and CO, but the kinetics of nitrite reduction appeared more complex and were not studied in detail

Purification of the cytochrome and related studies

Crude cell-free extracts from 10-l cultures (see MATERIALS AND METHODS) were fractionated with solid $(NH_4)_2SO_4$ and the fraction precipitated between 40 and 95% satn was dialysed against 0 2 M phosphate (pH 6 0) This fraction was then treated batchwise with DEAE-cellulose until none of the cytochrome remained unadsorbed At this salt concentration most of the cytochrome *c* remained in the supernatant, but when required could be adsorbed by lowering the buffer concentration to 0 1 M The adsorbed nitrite reductase was eluted with 1 0 M phosphate buffer and readsorbed from 0 1 M phosphate buffer on a column of DEAE-cellulose DE-52, followed by elution

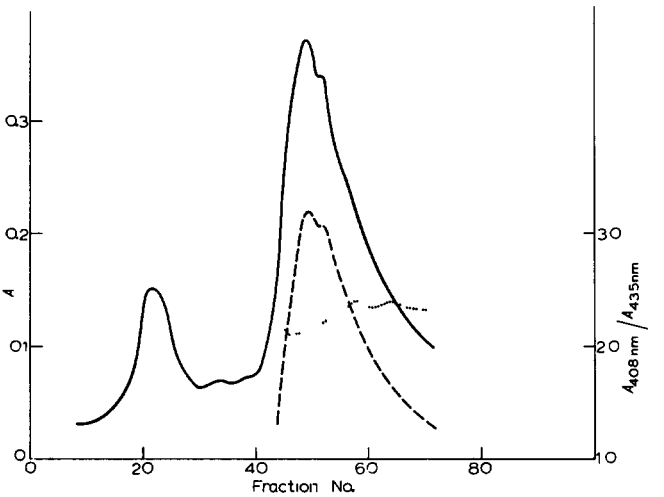

Fig 2 Elution profile of Micrococcus nitrite reductase from a DEAE-cellulose DE-52 column (1 5 cm × 25 cm) using a linear gradient of 0 2–0 6 M phosphate buffer (pH 6 0) ———, 280-nm absorption due to protein, — — —, 408-nm absorption of Soret band maximum, · , ratio of $A_{408\,nm}/A_{435\,nm}$

with a linear gradient of 0 2–0 6 M phosphate (pH 6 0) It is apparent from the elution profile in Fig 2 that there is more than one component present The leading and the tail fractions differed slightly in spectra, most notably, the tail fractions had a much less distinct shoulder at 435 nm on their 408-nm Soret band (see plot of the $A_{408\,nm}/A_{435\,nm}$ ratios) Cellulose acetate electrophoresis showed only one haemoprotein band in leading fractions but two or three bands in later ones Gel filtration on Sephadex G-100, G-150 or G-200 at this stage removed considerable protein impurity but the

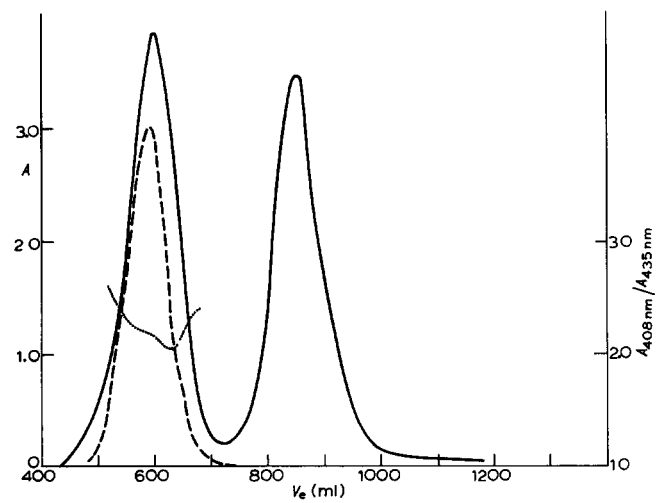

Fig 3. Elution curve of Micrococcus nitrite reductase on a Sephadex G-100 column (5 cm × 78 cm) in 0 1 M phosphate buffer (pH 6 0) ———, 280-nm absorption due to protein, — — —, 408-nm absorption of Soret band maximum, , ratio of $A_{408\,nm}/A_{435\,nm}$

different haemoprotein components did not separate, there was only one symmetrical band of haemoprotein, as measured by the 408-nm absorption (Fig 3), again the $A_{408\ nm}/A_{435\ nm}$ ratios of the fractions differed throughout the zone and the leading fractions had a pinker tinge than the tailing greener fractions

A major component (I) was separated from two minor components (II and III) by subsequent chromatography on hydroxylapatite, but Components II and III were inseparable (see Fig 4) Fraction I had an $A_{280\ nm}/A_{408\ nm}$ ratio of 0 77 and cellulose acetate or polyacrylamide-gel electrophoresis revealed that it was only about 1% contaminated with Components II and III Fraction II contained approximately equal amounts of all three components and Fraction III contained approximately equal amounts of Components II and III Fractions II and III had higher $A_{280\ nm}/A_{408\ nm}$ ratios than Fraction I, and in addition to the protein bands corresponding to the haem components, each contained several other protein bands, more readily apparent on polyacrylamide gel than on cellulose acetate Components I, II and III are named in order of decreasing electrophoretic mobility towards the anode (0 37, 0 34 and 0 32 $cm^2\ V^{-1}\ h^{-1}$ on cellulose acetate strips in 0 05 M Tris (pH 7 5) at 20°) As in the DEAE-cellulose DE-52 elution profile, the early fraction had lower $A_{408\ nm}/A_{435\ nm}$ ratios than the later ones

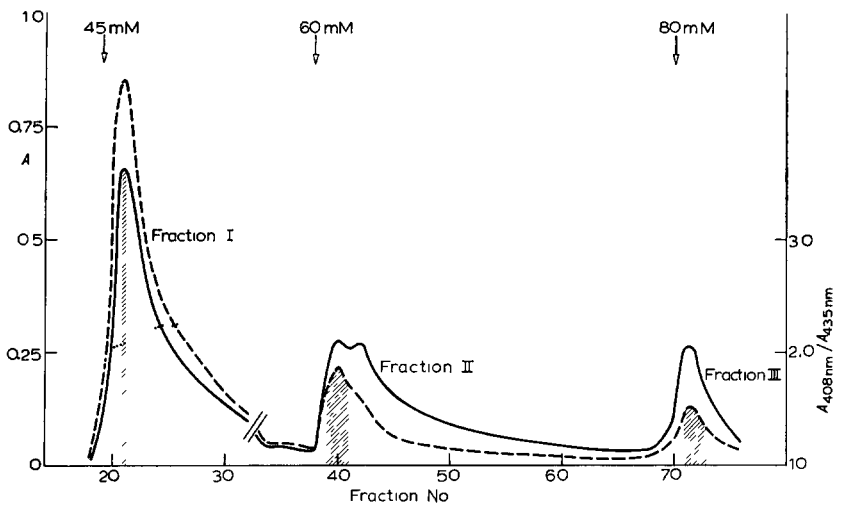

Fig 4 Elution profile of Micrococcus nitrite reductase from an hydroxylapatite column (1 5 cm × 22 cm) Equilibrated in 5 mM phosphate buffer (pH 6 0) and eluted batchwise with 45, 60 and 80 mM phosphate buffer (pH 6 0) The shaded areas represent the peak fractions which were combined to give the Fractions I, II and III described in the text Where the curves are broken, about 200 ml of 45 mM eluate were collected and not recorded ———, 280-nm absorption due to protein, — — —, 408-nm absorption of Soret band maximum, , ratio of $A_{408\ nm}/A_{435\ nm}$

Using gel filtration techniques (see MATERIALS AND METHODS) a molecular weight of 120 000 was obtained for the Micrococcus enzyme (Fig 5) Its isoelectric point determined by pH-focussing (see MATERIALS AND METHODS), was 3 85 (Fig 6), using ampholine buffers of pH range both 3–5 and 4–6

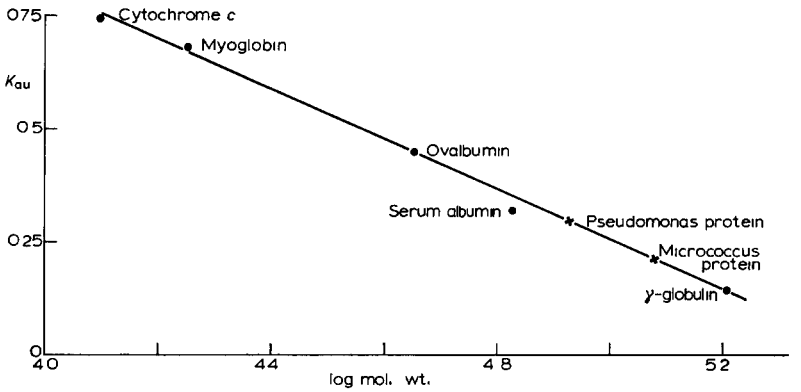

Fig 5 Determination of molecular weights of Micrococcus and Pseudomonas nitrite reductases on a calibrated Sephadex G-150 column (5 cm × 78 cm) For both calibration and experimental runs the phosphate buffer concentration was 0 1 M (pH 6 0)

Spectral properties

The unusual pH-dependent spectrum of the reduced enzyme, its CO spectrum, and some of the similarities to the Pseudomonas enzyme have already been described[2]. Further spectrophotometric studies are reported below, unless otherwise stated, a sample of purified enzyme of $A_{280\ nm}/A_{408\ nm}$ of approx 1 0, and estimated to contain not less than 95% of Component I, was used Table II summarises the spectral properties of the enzyme at different pH's, and of its derivatives formed with CO, pyridine, CN^-, imidazole, N_3^- and NO_2^- The bands in the 400–420-nm and 520–560-nm region are due to the *c*-type haem, and those in the 430–460-nm and 600–700-nm region are due to the green haem

Reaction with pyridine In alkaline pyridine *plus* dithionite, the *c*-type haem has

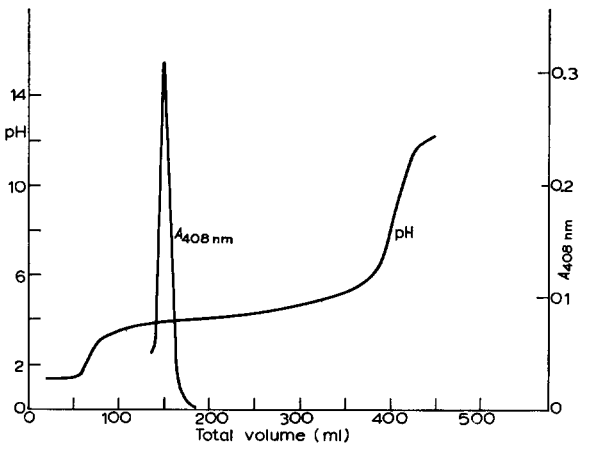

Fig 6 Determination of the isoelectric point of Micrococcus nitrite reductase by pH-focussing Ampholine buffer of pH range 3–5 was used, and the pH-focussing column was jacketed and cooled to 4 ± 0 2°

TABLE II

SPECTRAL PROPERTIES OF MICROCOCCUS NITRITE REDUCTASE

Reagent concentrations were CO, 1 mM, imidazole, 0 5 M, pyridine, 25% (v/v) in 0 1 M NaOH, CN^-, 0 05 M, N_3^-, 0 125 M, NO_2^-, 0 1 M The concentration of the enzyme was 5–10 μM, calculated from the 618-nm absorption band of the pyridine haemochrome, as described in MATERIALS AND METHODS Numbers in parentheses indicate minor absorption bands or shoulders on a major absorption band

Derivative	*Absorption maxima (nm)*			
Oxidised (pH 6 0)	408 (435)	525 (560)	640	702
Reduced (pH 6 0)	418 (460)	521 547 553	625 (655)	
Oxidized (pH 8 2)	408 (435)	522 (560)	640	700
Reduced (pH 8 2)	418 (460)	520 547 553	(620) 655	
Oxidized (pH 3 9)	408 (435)	525 (560)	640	700
Reduced (pH 3 9)	418 (460)	521 548 553	620	
Oxidized 0 2 M NaOH	411	530 (570)	640–670	
Reduced 0 2 M NaOH	418 (460)	520 (547) 553	625	
Reduced + CO (pH 6 0)	415	520 547 553	630–660	
Oxidized + imidazole (pH 7 8)	408 (435)	520 (560)	640	700
Reduced + imidazole (pH 7 8)	418 (460)	520 547 553	625	
Pyridine haemochrome, no dithionite	410 (460)	530–550 (570)	618	
Pyridine haemochrome + dithionite	412 (460)	520 549	618	
Pyridine haemochrome + ferricyanide	410 (435)	500–530 (570)	610–650 (700)	
Oxidized + CN^- (pH 7 5)	408 (435)	525 (560)	640	700
Reduced + CN^- (pH 7 5)	418 (445) (475)	520 547 553	625	
Oxidized + N_3^- (pH 5 5)	410 (440)	525 (560)	630–640	700
Reduced + N_3^- (pH 5 5)	418 (460)	520 547 553	620–650	
Oxidized + NO_2^- (pH 6 0)	409	523 (560)	638	
Reduced + NO_2^- (pH 6 0)				
Initial spectrum (brown)	417	520 547 553	615–660	
Final spectrum (green)	411 (455)	(520–560)	630	

a typical *c*-type haemochrome spectrum with a sharp α-band at 549 nm and an A_α/A_β ratio of 1 66 This is in sharp contrast to its atypical spectrum in the native protein, where the α-band is double (547 and 553 nm) and the A_α/A_β ratio is approx 1 The green haem forms a pyridine ferrohaemochrome in the absence of added reducing agent, the broad double band in the 600–700-nm region of the reduced native protein is replaced by a single symmetrical band at 618 nm The autoreduction of the green haem in alkaline pyridine is a marked contrast to its very slow reduction by dithionite in the native protein

Reaction with CN^- In the oxidized enzyme there was only a very slight spectral shift obtained with CN^-, apparent in the ferric + CN^- *minus* ferric difference spectrum as a small peak at 418 nm and trough at 408 nm In the reduced enzyme there was a marked reaction of the green haem with CN^-, the derivative formed had a single symmetrical band at 625 nm and two distinct bands at 440 and 480 nm in the Soret region The bands due to the *c*-type haem were unaltered

Reaction with imidazole Imidazole only reacted with the reduced enzyme, and the derivative formed with the green haem was similar in its α-band position (625 nm) to the CN^- derivative, but like the pyridine, and unlike the CN^- derivative, it had only one Soret band (460 nm) The bands due to the *c*-type haem were unaltered

Reaction with N_3^- This ligand reacted with only the oxidised enzyme, and a spectroscopically recognisable derivative was formed at acid pH only, indicating that the reactive species was HN_3 The reaction was with the green haem, and not the *c*-type

Reaction with NO_2^-. A series of complex reactions occurred, a bright green derivative was formed with the oxidised enzyme, and disappearance of the 435-nm shoulder and 700-nm band was observed as well as an increased height of the 640-nm band On addition of dithionite the colour changed from green to brown, the haem *c* was reduced and two bands at 615 and 660 nm, similar to those of the reduced green haem, appeared although there was no visible Soret shoulder at 460 nm On standing for a few minutes the colour changed to green, the haem *c* became oxidised and strong 455- and 630-nm bands developed On addition of more dithionite this cycle could be repeated many times A series of reactions such as this may occur when the enzyme is functioning as a nitrite reductase, and reflects electron transfer between the two haem groups

Low temperature spectra Spectra of the reduced enzyme at −196° show enhanced splitting of the double α-band of the cytochrome *c* component, but do not show any new bands, the 700-nm band of the oxidised enzyme is much less apparent at −196° than at room temperature (see Fig 7)

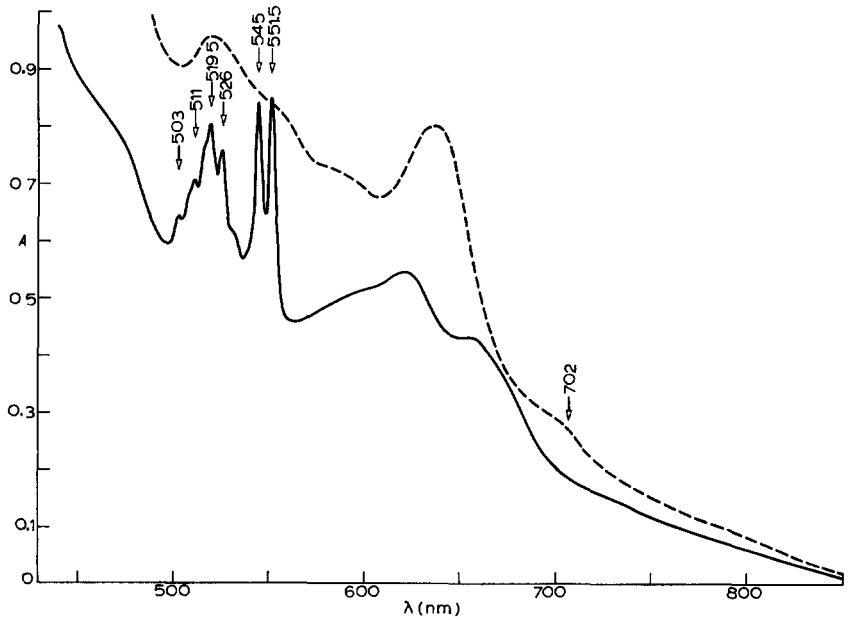

Fig 7 Cold temperature spectra (−196°) of Micrococcus nitrite reductase A concentrated solution of the enzyme (68 μM) was diluted 10 times with 0 1 M phosphate buffer (pH 6 0), containing 50% glycerol, and the spectra recorded in 2-mm cuvettes, as described in MATERIALS AND METHODS ———, reduced with dithionite, — — —, oxidized

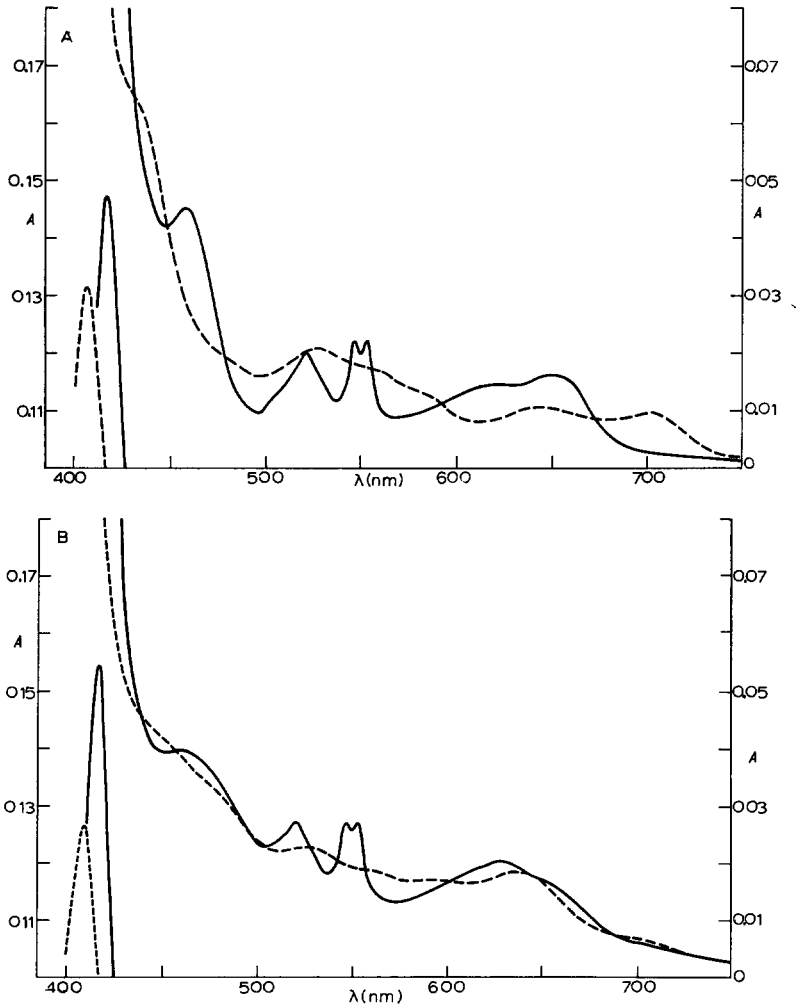

Fig 8 Spectra of Component I (A) and of Components II + III (B) of Micrococcus nitrite reductase as separated on hydroxylapatite The samples were diluted to approximately the same concentrations (approx 0 9 μM) in 0 05 M phosphate buffer (pH 6 0) and the spectra recorded using the 0–0 1, 0 1–0 2 A slidewire of a Cary 14R spectrophotometer ———, reduced with dithionite, — — —, oxidized

Different components. The major Component I and a mixture of the minor Components II and III which were inseparable, were examined, and some slight, but distinct, spectral differences were noted (Fig 8 and Table III) Most notably, the 700-nm band and the 435-nm shoulder of the 408-nm Soret band, so apparent in the oxidised spectrum of Component I, are much less obvious in Components II + III, they are absent in the Pseudomonas enzyme In the spectra of the reduced enzyme (30–40 min allowed for complete reduction of the green haem with dithionite) the 625-nm band predominates over the 655-nm band in Component I whereas the reverse is true for Components II + III and for the Pseudomonas enzyme Combination of

TABLE III

SPECTROSCOPIC RATIOS OF MICROCOCCUS AND PSEUDOMONAS PROTEINS

Reagent concentrations were CO, 1 mM, pyridine, 25% (v/v) in 0 1 M NaOH The absorbances for the ratios are measured at the band maxima, *e g*, 408, 409 and 410 nm for the Soret bands of oxidized Micrococcus Component I, Components II + III and Pseudomonas enzyme, respectively The slight variations in some of the band positions between different components are discussed later

Ratio	*Micrococcus*		*Pseudomonas*
	Component I	*Components II + III*	
Oxidized (pH 6 0)			
408/435	2 1	2 6	2 9
408/523	6 4	6 9	7 3
408/640	12 5	6 8	7 0
408/700	13 8	22 5	44 0
Reduced (pH 6 0)			
418/460	3 3	4 0	3 8
418/548	6 7	6 0	6 7
418/625	10 1	7 4	8 9
418/655	8 9	10 4	11 7
Reduced + CO (pH 6 0)			
415/455	5 6	4 3	5 8
415/650	11 6	9 5	10 9
Pyridine haemochrome			
408/618 (no dithionite)	6 1	6 1	6 8
550/618 (+ dithionite)	1 2	1 3	1 3

the green haem of the reduced enzyme with CO, measured by collapse of the 460-nm band, was only partial in Components II + III, whereas it was complete in Component I and the Pseudomonas enzyme The ratio of the two major bands of the pyridine haemochrome (550 nm for haem *c* and 618 nm for the green haem) did not differ, but the 618-nm band was shifted to 620 nm in Components II + III

Removal of the green haem The green haem was removed from the protein by titrating a sample of the enzyme at 0° to pH 4 5 with dilute HCl and repeatedly extracting with ethyl methyl ketone The precipitated pink haem *c* protein, could be redissolved with difficulty at pH 8–9 and the reduced spectrum still had the double α-band and low α/β ratio but was now much more strongly CO-reactive There was no trace of the characteristic absorption bands of the green haem at 435, 640, 700 nm in the oxidised, and 460, 620, 650 nm in the reduced spectra.

Comparison of the Pseudomonas and Micrococcus enzymes

The Pseudomonas enzyme is present in higher concentrations than the Micrococcus (Table I) and it was also found to be more amenable to purification It was less anionic and not adsorbed onto DEAE-cellulose but was readily purified on columns of CM-cellulose Its molecular weight, determined on Sephadex, was 85 000 (Fig 5) and its isoelectric point, determined by pH focussing, was 7 1 A preparation with $A_{280\ nm}/A_{410\ nm}$ ratio of 0 80 was obtained (*cf* ratio of 0 87 of YAMANAKA) and it behaved

electrophoretically as a single haemoprotein Spectroscopically it was similar to the Micrococcus enzyme, and the slight differences have already been noted (Table III)

DISCUSSION

The two-haem protein described above from *M denitrificans* appears to be concerned with dissimilatory (FEWSON AND NICHOLAS[14]) reduction of NO_2^- by the organism, it has nitrite reductase activity, it complexes in a highly specific manner with NO_2^- and it occurs only in cells grown anaerobically with NO_3^- as electron acceptor NO_2^- may also induce the enzyme, but because of the inhibitory effect of NO_2^-, experiments with a continuous anaerobic culture using low levels of this electron acceptor would be necessary to establish this Unlike the Pseudomonas enzyme[3], the enzyme from Micrococcus does not appear under aerobic growth in the presence of NO_3^- so the requirement for its production is the presence of NO_3^- and the absence of O_2 This difference may be explained if the particulate cytochrome a–a_3 system of aerobically grown organisms[1] supplies sufficient energy without supplementation by the cytochrome oxidase activity of the soluble two-haem enzyme or by reduction of NO_3^- and NO_2^- (ref 15) The particulate cytochrome oxidase of aerobically-grown *P aeruginosa* appears to contain cytochrome a_1 (ref 16) and may not be as effective as the a–a_3 cytochrome of *M denitrificans*

The cytochrome oxidase activity of the Micrococcus enzyme (Fig 1) is weak when compared with that of the particulate cytochrome a–a_3 of both aerobically and anaerobically grown cells[1], this fact and the absence of the green haemoprotein in aerobically-grown organisms point to it having no role in the O_2 respiration of this Micrococcus It is possible that the oxidase function is vestigial and may have an evolutionary significance It should be noted that the purified Micrococcus cytochrome c-550 used as substrate in both the nitrite reductase and cytochrome oxidase assays may not be the true physiological substrate another cytochrome c with a double α-band and low α/β band ratio, similar to that described by HORI[17] from a halotolerant Micrococcus, was observed in small quantities during the enzyme preparations, and this cytochrome may be the true substrate since the halotolerant Micrococcus sp cytochrome c-548, 554 is far more effectively oxidised by Pseudomonas cytochrome oxidase than are any of the cytochrome c species with the normal single α-band[18] YAMANAKA[19] observed that the ratio of nitrite reductase to cytochrome oxidase activities varied considerably with the different species of cytochromes c tested, and he has speculated on the evolutionary significance of these differences

Nitrite reductase enzymes from different organisms have widely differing properties and presumably different reaction mechanisms The enzymes from *P denitrificans*[20], Neurospora[21,22], soybean leaves[21] and *Azotobacter agile*[23] are believed to be metalloflavoproteins, but those from *Achromobacter fischeri*[24], *M denitrificans* and *P aeruginosa* are haemoproteins The enzymes from the latter two organisms are very similar and they contain two different haem groups (c and d-like) whereas that from *A fischeri* contains only haem c[24] Of the haemoprotein nitrite reductases only the two-haem type can also function as cytochrome oxidase On removal of the green haem (d-like) the enzyme loses practically all its cytochrome oxidase and nitrite reductase activities, but they are largely regained on reconstitution[25]

Although they have similar properties the enzymes from Pseudomonas and

Micrococcus have some distinct differences shown in this paper The Micrococcus protein has a considerably higher molecular weight (120 000) than the Pseudomonas (85 000), and the difference in their amino acid compositions is reflected in their isoelectric points (Micrococcus, 3.85 and Pseudomonas, 7 1) This value of 7 1, determined by pH-focussing, differs significantly from that of 5.8 determined by electrophoresis at different pH's and extrapolation to zero mobility[3], the higher value is consistent with its behaviour on ion-exchange resins The higher molecular weight of the Micrococcus protein is not reflected in the $A_{280\ nm}/A$ Soret band ratio which for the purified protein is almost identical with that of the Pseudomonas enzyme

The unusual band of the Micrococcus enzyme at 700 nm seems to be related to the 435-nm shoulder of the Soret band In Component I where the 700-nm band is prominent, so is the shoulder, the opposite is true in Components II + III (see Table III) For this reason, and because it disappears on the addition of pyridine and alkali and is no longer present in the spectrum of the haem *c* protein remaining after removal of the green haem, the 700-nm band appears to be associated with the presence of the green haem in its oxidized form Unlike the 695-nm band of oxidized cytochrome *c* it does not disappear following CN^- addition[26], nor does it disappear on dialysis for 20 h against 1 M glycine buffer (pH 10 0) as does the near infrared absorption band of cytochrome *c* oxidase at 830 nm, which is attributed to copper[27] As the 640-nm band is diminished when the 700-nm band is present (Table III), the green haem may exist in an environment where it has one intense band at 640 nm or where it has two less intense bands at 640 and 700 nm Something similar may happen in the Soret region, but here the changes are masked by the high Soret absorption bands of the *c*-type haem YAMANAKA[3] stated, and the present studies confirm, the variable absorption of the green haem, which is dependent on the purity of the enzyme preparation and on pH Gel electrophoresis and the elution profile from hydroxylapatite (Fig 4) show that Component I is much purer than Components II + III, and this may explain some of the observed spectral differences

The slow reduction of the green haem part of the enzyme by dithionite is a property shared by the haem *a* of cytochrome *a*–a_3 (ref 28) and cytochrome a_1 (J BARRETT, personal communication) The rapid autoreduction in aqueous alkaline pyridine is unusual, and was also observed for the green haem derived from the Pseudomonas enzyme[3] Chlorin-type haems of high potential are reduced under these conditions, (J BARRETT, personal communication), and some formyl-substituted haemins combine with non-aqueous pyridine to form ferrohaemochromes[29,30]. A slow ferrohaemochrome formation takes place with myeloperoxidase in aqueous pyridine in the absence of added reducing agents[31]

The pyridine haemochrome band at 618 nm is too far to the red for any haem with porphyrin conjugation the substitution of electrophilic groups at the β-pyrrole positions of a haem with such conjugation is unlikely to cause a shift beyond the haem *a* haemochrome band at 587 nm or the myeloperoxidase haemochrome band at 590 nm (ref 31) A shift to 618 nm may arise from a stable alteration or interruption to the conjugation pathway around the porphyrin nucleus, either by loss of a pyrrole $\beta\beta$ double bond to form a chlorin, or by substitution at an α-pyrrole position. Ferrosulphhaemoglobin (α-band 622 nm) contains a green ferrochlorin-type chromophore, but nevertheless has a pyridine haemochrome band at 558 nm because the addition to the pyrrole $\beta\beta$ double bond is unstable to alkaline pyridine[32], resulting in the reformation of protohaem

YAMANAKA[3] concluded that the green haem split off from the Pseudomonas nitrite reductase was the same as the haem *d* of *A aerogenes* which has been postulated to be an iron–chlorin having an hydroxyethyl side chain[5] Both the Micrococcus and Pseudomonas green haems do resemble haem *d* superficially, but there are several points of difference the α-band maxima of the pyridine haemochromes differ by 5 nm, and both the Micrococcus and Pseudomonas green haems are considerably more water-soluble than haem *d* Preliminary chemical studies indicate that the nitrite reductase haems are the iron complex of a new type of tetrapyrrole[33]

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