The purification and some equilibrium properties of the nitrite reductase of the bacterium *Wolinella succinogenes*

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The bacterium *Wolinella succinogenes* produces a nitrite reductase enzyme that can be purified to homogeneity in high yield by a combination of detergent extraction, hydroxyapatite chromatography and M_r fractionation. Nitrite reductase activity is found to be present in both a high- and a low- M_r fraction. The high- M_r fraction has been shown to consist of the low- M_r nitrite reductase enzyme associated with a hydrophobic 'binding protein'. The amino acid composition for both proteins is reported. The nitrite reductase enzyme shows spectral characteristics indicative of the presence of *c*-type haem groups. Measurements at 610 nm indicate the presence of some high-spin haem groups at neutral pH. This haem subgroup undergoes a pH-linked high-spin – low-spin transition at alkaline pH. Approximately two of the six haem groups present within the enzyme bind CO with low affinity ($K_D = 0.4$ mM). The enzyme also shows a range of redox activities with various inorganic reagents. The enzyme has been shown to exhibit dithionite reductase, oxygen reductase and CO₂ reductase activities.

INTRODUCTION

The bacterium *Wolinella succinogenes* was first isolated from the rumen of the cow by Wolin *et al.* (1961). Early observations suggested that the bacterium contained a nitrite reductase enzyme responsible for the six-electron reduction of nitrite to ammonia. The enzyme has subsequently been isolated by Liu *et al.* (1983) and more recently by Schroder *et al.* (1985). The initial reports on the isolation of the molecule raised the possibility that the enzyme might exist in two forms, a cytosolic soluble form and a membrane-bound form, and that an equilibrium between these forms might occur (Liu *et al.* 1983). Further characterization of the enzyme indicated the presence of six *c*-type haem groups associated with a single protein chain of approx. M_r 63 000 (Liu *et al.*, 1983). Apart from some steady-state measurements, very little work has been reported on the resolution of the apparent dichotomy of form of the enzyme or its characterization at the molecular level. In the present paper we report a new, simpler method of purification of the enzyme that produces higher yields together with the results of studies aimed at clarifying the molecular character of two forms of the enzyme previously identified. We have also further characterized the nature of the haem group present and have performed preliminary experiments on a range of redox activities associated with this enzyme.

MATERIALS AND METHODS

Protein concentration was determined by Peterson's (1977) modification of the Lowry method, bovine serum albumin being used as a protein standard.

The purification of the nitrite reductase enzyme was monitored by using reduced-versus-oxidized difference spectroscopy in the Soret region by the method of Chance

(1954). As isolated, all the cytochromes were fully oxidized. Reduction of the cytochromes was achieved by adding a few grains of sodium dithionite. The difference spectra were recorded with an Aminco DW2a spectrophotometer (American Instrument Co., Silver Spring, MD, U.S.A.).

The presence of nitrite reductase activity was monitored by measuring the nitrite-induced oxidation of dithionite-reduced samples. Nitrite reductase was quantified by using the published molar absorption coefficient of 548 mm⁻¹·cm⁻¹ at 410 nm for the oxidized enzyme (Liu *et al.*, 1983).

Electrophoresis was performed by the method of Laemmli (1970) in either 10% or 7.5%-(w/v)-polyacrylamide gels as appropriate, in both the presence and absence of dithiothreitol. All gels were scanned at 610 nm with a Helena Quickscan densitometer (Helena Laboratories, Beaumont, TX, U.S.A.). Preparative electrophoresis was performed in tube gels (11 cm × 0.7 cm). For the purpose of amino acid analysis, protein bands were electrophoretically eluted from slices of the gel by the method of Hunkapillar *et al.* (1983) and derivatives were produced by the method of Henrikson & Meredith (1984). The constituent amino acids were separated and quantified by comparison with a standard amino acid mixture by using h.p.l.c. on a C₁₈ reversed-phase column.

Standard dithionite solutions were prepared by the anaerobic addition of a weighed amount of sodium dithionite to thoroughly deoxygenated buffer. The active concentration of the resulting dithionite solution was determined by anaerobic titration against cytochrome *c*.

CO oxidation and CO₂ reduction reactions were performed by using the methods of Bickar *et al.* (1984). CO production was monitored by anaerobic addition of aliquots of the reaction mixture into a solution of reduced myoglobin and measuring the increase in A_{420} . The molar absorption coefficient given by Antonini & Brunori (1971) for the CO–myoglobin complex was used.

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Cell-culture methods

Wolinella succinogenes was grown in batch culture at 37 °C in stoppered 15-litre flasks under an atmosphere of O₂-free N₂. The growth medium used was as described by Bokranz *et al.* (1983) in which sodium formate and NaNO₃ act as electron donor and acceptor respectively.

Growth of *W. succinogenes* was initiated in batch culture by seeding 15 litres of sterile medium with a 1 litre pre-culture. When growth had ceased, the cells were harvested by using a Sorvall RC5 centrifuge at 4 °C. Cell separation was achieved using a Sorvall KSB continuous-flow attachment run at 24000 *g* at a flow rate of 60 ml/min. The cell pellet was re-suspended in 100 ml of 30 mM-potassium phosphate buffer, pH 7.6, and re-centrifuged at 17500 *g* for 30 min. The resulting cell pellet was re-suspended in 30 ml of 30 mM-phosphate buffer and stored at 80 °C until required.

RESULTS

Enzyme purification

(a) Cell lysis. Lysis of whole bacteria was achieved by using a French pressure cell (American Instrument Co.). The bacterial suspension was thawed and diluted 1:2 with 30 mM-potassium phosphate buffer at pH 7.6 containing 1 M-mannitol and 100 mM-MgCl₂. The resulting suspension was passed through the pressure cell at 21 MPa (3000 lbf/in²) at a rate of 10 drops/min at 4 °C. After this procedure, di-isopropyl fluorophosphate (final concn. 0.04%) was added to the lysed suspension in order to inhibit proteinase activity. A few crystals of deoxyribonuclease was also added at this stage in order to reduce the viscosity of the suspension.

(b) Solubilization of membrane nitrite reductase. Membrane fragments obtained by cell lysis were centrifuged at 17500 *g* for 30 min and the supernatant discarded. The

Fig. 1. Elution profile of proteins from the hydroxyapatite column

The detergent-solubilized protein fraction was applied to a column (10 cm × 2.5 cm) of hydroxyapatite and developed as described in the text. Fractions (9 ml each) were collected and the protein concentration (●) and A₄₂₅ of each fraction (▲) was determined. Peaks a, b and c correspond to the peaks investigated by difference spectroscopy as in Fig. 2.

Fig. 2. Difference spectra of the haem-associated bands obtained by hydroxyapatite chromatography

The dithionite-reduced – oxidized spectra of the three haem-associated bands determined by hydroxyapatite chromatography described in Fig. 1 are shown. The spectra (—, ---, — · —) correspond to the order in which the proteins were eluted from the hydroxyapatite column.

pellet was suspended in 40 ml of 60 mM-potassium phosphate buffer, pH 7.6, containing 1% Tween 80. The suspension was then centrifuged and the pellet retained. The pellet was re-extracted with buffer containing 1% Triton X-100, centrifuged and the supernatant retained. Polyethylenimine (0.04%) was added to the Triton X-100 extract and precipitated impurities were removed by centrifugation. All subsequent steps were performed at 4 °C using buffers containing 0.05% Triton X-100.

(c) Hydroxyapatite chromatography. The detergent-solubilized material was pumped on to a column (10 cm × 2.5 cm) of hydroxyapatite equilibrated with 60 mM-potassium phosphate buffer, pH 7.6. The cytochromes were absorbed as a tight red band on the top of the column, which was thoroughly washed with the equilibrating buffer. The column was then developed with a constant-pH buffer by using a linear potassium phosphate buffer gradient produced from 500 ml of 60 mM-buffer and 500 ml of 500 mM-buffer at pH 7.6. Development of the column yielded a number of protein bands, three of which were associated with haem (Fig. 1). Soret-region oxidized – reduced difference spectra of these bands identified the first haem band to be eluted as containing cytochrome *c*, the second band with cytochrome *b*, whereas the third band contained the nitrite reductase and was the only band sensitive to nitrite-induced re-oxidation (Fig. 2).

Fig. 3. Elution profile of nitrite reductase from the Ultrogel AcA 34 column

Fraction *c* obtained from the hydroxyapatite column was applied to a 100 cm × 2.5 cm column of Ultrogel AcA 34 and eluted with 60 mM-phosphate buffer, pH 7.6. Protein concentration (○) was determined in each fraction as described in the text and the presence of nitrite reductase was determined from A_{425} (△). Fractions (7 ml each) were collected.

Table 1. Summary of the purification of the nitrite reductase enzyme

Procedure	Recovery (%)	Purity (%)	Purification factor
Cells	100	2	—
Membrane fragments	50	6	3
Polyethylenimine	40	20†	10
Hydroxyapatite	35	40†	20
M_r fractionation	14*	>95†	50

* Values for the low- M_r form.

† Estimated from sodium dodecyl sulphate/polyacrylamide-gel electrophoresis after staining with Coomassie Brilliant Blue.

(d) M_r fractionation. Chromatography of the nitrite reductase-containing fraction, obtained after hydroxyapatite chromatography, on a column (100 cm × 2.5 cm) of Ultrogel AcA34 resolved three protein bands, two of which were associated with haem (Fig. 3). From the elution volumes of the nitrite reductase-associated fractions it was possible, by comparison with M_r standards, to obtain M_r values for the three fractions of 63000, 12000 and 360000. At this stage, nitrite reductase activity was present in the 63000- and 360000- M_r fractions. The 63000 M_r fraction is homogeneous and > 95% pure, as judged by polyacrylamide-gel electrophoresis, at this stage. The preparation of the enzyme is summarized in Table 1.

Characterization of the nitrite reductase structure

By using the Laurent & Killander (1964) formula, a Stokes radius of 3.3 nm was obtained for the 63000- M_r band. To further investigate the relationship of the three species separated by Ultrogel AcA34 chromatography, both the low- (63000) and high- (360000) M_r fractions were subjected to polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate, in both the

presence and absence of dithiothreitol (Fig. 4). The low- M_r protein migrated on the gel as a protein of 63000 M_r , both in the presence and in the absence of dithiothreitol. The large molecular weight protein was found to dissociate into two bands on the gel, both in the presence and absence of dithiothreitol, yielding a band with an M_r of 63000 associated with haem and a band of M_r 120000 not associated with haem. The haem content of the 63000- M_r form was estimated by a comparison of the optical absorbance of the pyridine haemochrome of a sample with the protein concentration of the same sample. This procedure yielded a value of more than five haem groups per molecule of protein.

To investigate the possibility that the protein of 120000 M_r might be related to the haem protein of 63000 M_r , an amino acid analysis of both proteins was carried out. The resulting amino acid composition of both proteins is shown in Table 2.

Spectra

In the Soret region the enzyme shows a broad absorption band with a peak at 404.5 nm. The dithionite-reduced enzyme has a maximum at 421 nm, with a narrower absorption band than that seen for the oxidized form. Addition of CO to the dithionite-reduced material produces an asymmetric Soret band with a maximum at 418 nm. The addition of an excess of nitrite to the CO complex leads to the rapid formation of a spectrum with a maximum at 415 nm, followed by the very slow conversion of this material into the fully oxidized species (Fig. 5).

In the $\alpha\beta$ region the enzyme shows spectra typical of

Fig. 4. A densitometer scan of the bands obtained by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of nitrite reductase fractions obtained by M_r fractionation

Both high- (—) and low- (---) M_r haem-containing fractions obtained by Ultrogel AcA 34 chromatography were subjected to electrophoresis on 10% (w/v) acrylamide gels in the presence of sodium dodecyl sulphate. Gels were stained with Coomassie Brilliant Blue before densitometry at 610 nm. For ease of comparison, the traces from two gels are shown displaced in the vertical axis.

Table 2. Amino acid analysis of nitrite reductase and its binding protein

Serine and threonine were estimated by extrapolation of hydrolysis data back to zero time. Tryptophan and cysteine were not determined. Values under '(a)' are for proteins obtained by electrophoresis and under '(b)' for proteins obtained by gel chromatography.

	Composition (mol/100 mol)			
	Nitrite reductase		Binding protein	
	(a)	(b)	(a)	(b)
Asx	8.1	7.0	5.4	4.9
Glx	10.2	10.5	4.0	7.6
Ser	4.7	5.8	6.0	4.8
Gly	9.7	12.1	14.3	14.5
His	2.7	1.9	3.0	1.5
Arg	4.3	3.9	6.9	5.3
Thr	5.9	6.4	7.4	6.4
Ala	10.6	10.5	4.6	7.5
Pro	4.7	4.7	5.7	5.7
Tyr	3.4	3.1	2.5	3.5
Val	6.7	6.2	8.0	6.8
Met	1.3	1.8	3.6	1.7
Ile	5.8	6.4	6.8	5.4
Leu	8.5	8.3	17.0	18.8
Phe	3.7	3.1	1.5	2.7
Lys	9.9	8.5	2.3	2.5

c-type haem groups for the reduced protein with maxima at 524 and 553 nm. The alkaline pyridine ferrochrome showed an α -peak maximum at 550 nm, identical with that of cytochrome *c*, showing all haem groups to be of the *c*-type. The oxidized spectrum shows a small peak at 610 nm. In the Soret region the spectra of both the oxidized and reduced forms were found to be pH-dependent. An increase in pH from 7.6 to 11 induced a 5% increase in the absorption of the oxidized Soret band and a shift to 408.5 nm. In the reduced form the Soret band showed a 30% increase in absorption and a shift to 418 nm. In the $\alpha\beta$ region the same pH change produced a loss of the 610 nm band in the oxidized form. A titration of the Soret band of the ferric nitrite reductase gave a titration curve centred at approx. pH 10.5 (Fig. 6).

Ligand binding

A titration of reduced nitrite reductase with CO showed that, even at 100 kPa (1 atm) pressure of CO, the titration had not reached an end point. The data were thus fitted to a binding isotherm by using non-linear regression analysis (Fig. 7). The data fitted well a hyperbolic binding curve with a K_D of 0.415 mM.

Electron-transfer activity

The anaerobic addition of 24 mM-ascorbate, pH 7.0, to 0.5 μ M-nitrite reductase was found to produce negligible reduction of the enzyme. The addition of 50 mM-*NNN'*-tetramethylbenzene-1, 4-diamine did, however, lead to partial reduction (20%), and this level of reduction was increased substantially (35%) if the reduction was performed under 100 kPa of CO, although the resulting species presented a very asymmetric spectrum with a broad maximum at 421 nm.

Fig. 5. Soret-region spectra of nitrite reductase and some of its derivatives

The spectra of oxidized (----), reduced (-----) and CO-bound complex (—) are shown together with the spectrum obtained immediately after the CO complex was treated with an excess of nitrite (— · —). All spectra were obtained by using 1 μ M-enzyme in 60 mM-phosphate buffer, pH 7.6.

The addition of a stoichiometric amount of dithionite to the nitrite reductase produced rapid reduction of the enzyme, which could be rapidly reversed by the addition of excess nitrite. The stoichiometrically reduced enzyme was, however, unstable and reverted to the oxidized form over a period of approx. 30 min. This reoxidation process was not associated with stable isosbestic points. Furthermore, the enzyme could be cycled many times through this reduction-oxidation cycle, and oxidation would occur even if a large excess of dithionite was added.

The enzyme showed a marked oxidase activity, as indicated by the complete oxidation of the enzyme on passage of a dithionite-reduced sample down a small Sephadex column that had been prewashed with N_2 -purged buffer.

Incubation of oxidized nitrite reductase with 100 kPa of CO for 24 h did not result in any measurable reduction of the enzyme. However, incubation of 0.5 μ M-nitrite reductase in the presence of $NaHCO_3$ and excess dithionite for 24 h was found to lead to the production of CO at a concentration of 2 μ M. No CO was produced in the same system in the absence of the nitrite reductase enzyme.

DISCUSSION

The preparation outlined above produces pure enzyme in significantly higher yields than those methods previously reported (Liu *et al.*, 1983; Schroder *et al.*, 1985). In terms of the purification and our understanding of the nature of the enzyme, the final M_r -fractionation

Fig. 6. A pH titration of the Soret band of oxidized nitrite reductase

Nitrite reductase ($1 \mu\text{M}$) was titrated by the addition of small volumes of 3 M-NaOH or 3 M-HCl and changes in the absorbance at 420 nm recorded as a function of the solution pH.

Fig. 7. CO titration of reduced nitrite reductase

Dithionite-reduced nitrite reductase ($1 \mu\text{M}$) in 60 mM-phosphate buffer, pH 7.6, was titrated anaerobically by adding small volumes of CO-equilibrated buffer. The experimentally observed changes in absorbance at 418 nm ($\square$) are compared with those of the best-fit hyperbola (—).

step is of particular significance. Gel filtration of the nitrite reductase-containing protein fraction yielded three protein bands, two of which showed identical spectra. The electrophoretic results together with the amino acid analyses shown in Table 2 show clearly that the high- M_r haem protein is in fact a complex of the intermediate- M_r protein together with the smaller haem protein. The absence of any effect of dithiothreitol on the dissociation of the complex suggests that the aggregation does not

involve the formation of disulphide links. The relatively high hydrophobicity of the intermediate- M_r fraction indicated by its amino acid composition might well indicate that this protein not only acts as an aggregation centre, but may also be a binding protein through which aggregates of the 63000- M_r haem protein bind to the membrane of the bacterium, particularly in light of the fact that electron-microscopic studies of the bacterium have shown the presence of particles of the appropriate size associated with the inside face of the inner membrane. These findings thus appear to resolve the conflict in the literature concerning the possible existence of two distinct nitrite reductase enzymes within this bacterium (Liu *et al.*, 1983). The previously reported findings now appear merely to reflect the existence of monomeric and aggregated forms, with the binding protein being unidentified in previous studies.

The broad Soret spectrum of the enzyme in the oxidized state suggests that this band may well arise from unresolved bands originating from the presence of different haem types within the protein. The presence of a 610 nm band may well indicate the presence of high-spin haem groups (Brittain & Greenwood, 1975). The absorption associated with this band is lower than would be expected if all the haem groups were high-spin and is equivalent to only approximately two haem groups per molecule of protein (Antonini & Brunori, 1971). The effect of pH on the spectrum of the enzyme is consistent with a pH-induced high-spin-to-low-spin transition (Brittain & Greenwood, 1975). The titration of this process is centred at approx. pH 10.5, and may well represent the ligation of a deprotonated lysine residue to the vacant haem iron site, inducing a transition to the low-spin six-co-ordinate form, in a manner similar to that suggested for cytochrome *c*. (Lambeth *et al.*, 1973). The titration is complicated, however, by irreversible processes at the highest pH values investigated. In light of the CO-binding data, the marked asymmetry of the Soret band of the CO-bound and nitrite-oxidized CO complex appears to arise from incomplete reaction of the enzyme with CO. This finding also resolves the apparent paradox of rapid nitrite-induced oxidation of the CO complex. If some of the haem groups are five-co-ordinate CO-binding sites, then these same sites would also be expected to be the nitrite-binding sites. The CO-binding data suggest that the addition of excess nitrite to the CO complex in fact results in the oxidation of the unligated fraction of the enzyme that is present even under 100 kPa of CO, whereas the complex form is totally unreactive. The subsequent slow oxidation process is due to the reaction of nitrite with the CO-bound haem groups, rate-limited by the CO dissociation rate. The spectral studies on the enzyme can thus be rationalized in terms of the existence of the two haem subgroups within the protein, namely one group of 'normal' *c*-type haem groups that do not react with CO, and another group of 'abnormal' *c*-type haem groups that react with CO, but only weakly. The spectral characteristics of all the haem groups, together with the finding that none of them can be extracted with acid acetone does, however, still imply that all the haem groups present in this enzyme are at least *c*-type.

The CO affinity of this enzyme is very low, being only 0.415 mM, and is comparable with that seen for *Rhodospirillum rubrum* cytochrome *c'* which has a K_D of 0.79 mM (Cusanovich & Gibson, 1973). Experimental data indicates the enzyme attains only 71% saturation

under 100 kPa pressure of CO, and so the end point for CO binding must be estimated. The calculated final absorbance for CO binding is consistent with the binding of approximately two molecules of CO per molecule of protein and the quality of the fit of the binding data to a simple hyperbola shows that the two sites of CO binding are equivalent.

No satisfactory measurements have been made of the redox potentials of the haem groups within the protein, but, when in excess, ascorbate does appear to reduce at least part of the enzyme preferentially. The effect of CO on the reduction presumably arises from its stabilizing of ferrous haem groups thus:

where c and c^* refer to the CO non-binding and binding haem groups respectively. The redox properties of this protein are also interesting in that it shows both dithionite reductase activity, similar to that seen in the nitrite reductase of *Desulfibrio desulfuricans* (Steenkamp & Peck, 1980), as well as considerable oxidase activity, such as is seen in the nitrite reductase of *Pseudomonas aeruginosa* (Horio *et al.*, 1958). The dithionite reductase reaction, which by analogy might be expected to produce sulphide in solution, is extremely slow. The oxidase reaction, on the other hand, is very rapid. It is not yet clear whether the differences in character between these two activities is thermodynamic or kinetic in origin. The oxidation of CO to CO₂ is known to occur in a range of metalloproteins, but the mechanism of the reaction is not clear (Young & Caughey, 1980; Bickar *et al.*, 1984). It has been proposed that reaction occurs via an equivalent of the water-gas shift reaction:

In the case of studies on haemoglobin and cytochrome c oxidase, the reduced product would bind, with high affinity, some of the large excess of CO present and hence become stabilized. The low affinity of the nitrite reductase for CO would preclude this in the present case. When nitrite reductase is incubated with a large excess of bicarbonate (HCO₃⁻) and reducing agent, the enzyme does produce CO, presumably by a reversal of the above reaction. The present findings are thus in agreement with the water-gas shift proposal made by Bickar *et al.* (1984).

In conclusion, the nitrite reductase from *Wolinella*

succinogenes has been purified and shown to exist in two forms, a monomeric 63000- M_r species and a membrane-bound aggregate form produced from the binding of monomeric subunits to a 120000- M_r hydrophobic binding protein that may be responsible for its attachment to the bacterial membrane. The six-haem-containing protein has some anomalous spectral features that may be accounted for by the presence of two subclasses of c -type haem groups. One set of haem groups appears to be high-spin at neutral pH and is responsible for the binding of CO to the protein. In addition the enzyme shows a wide range of redox activities, being able both to donate and accept electrons from a wide range of redox partners.

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