

## ***ESCHERICHIA COLI* CYTOCHROME c NITRITE REDUCTASE NrfA**

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### **Abstract**

The periplasmic cytochrome *c* nitrite reductase (Nrf) system of *Escherichia coli* utilizes nitrite as a respiratory electron acceptor by reducing it to ammonium. Nitric oxide (NO) is a proposed intermediate in this six-electron reduction and NrfA can use exogenous NO as a substrate. This chapter describes the method used to assay Nrf-catalyzed NO reduction in whole cells of *E. coli* and the procedures for preparing highly purified NrfA suitable for use in kinetic, spectroscopic, voltammetric, and crystallization studies.

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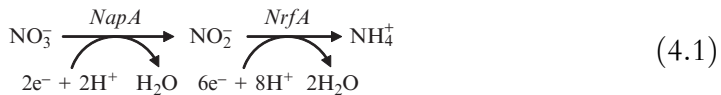
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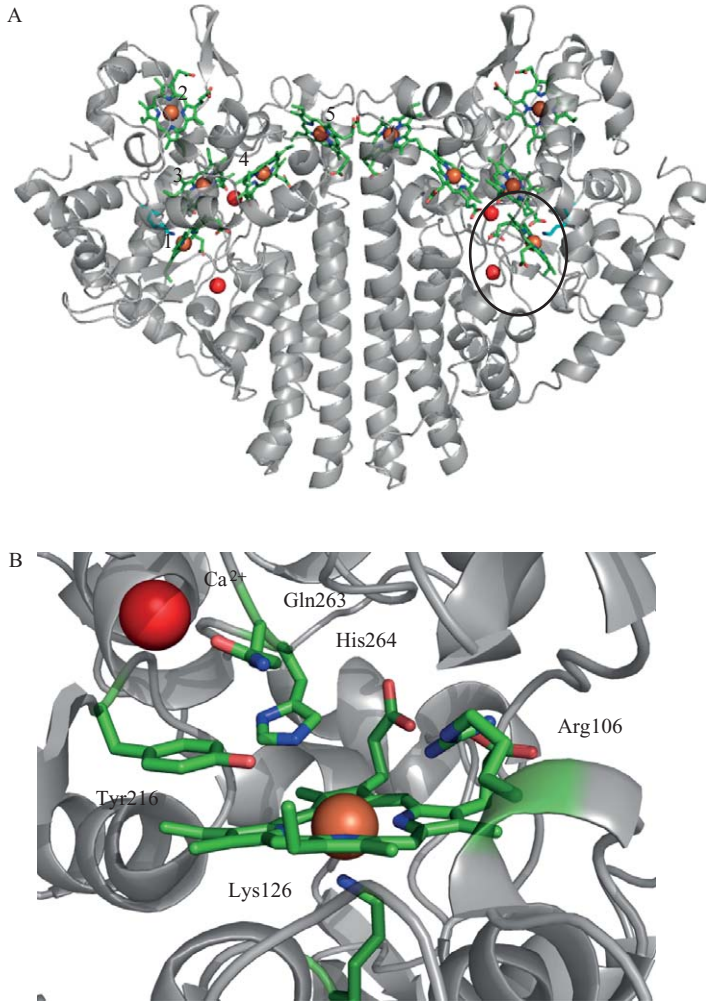
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## 1. INTRODUCTION

Enteric bacteria such as *Escherichia coli* and *Salmonella typhimurium* have evolved to survive in electron acceptor-limited anaerobic conditions. Under anoxic and microoxic conditions in the presence of low levels of nitrate, the periplasmic nitrate reductase (Nap) system and the periplasmic nitrite reductase (Nrf) system are expressed (Potter *et al.*, 2001); these conditions are similar to those found in the mammalian host environment, such as the gut and bloodstream. The NapA enzyme is responsible for the two electron reduction of nitrate to nitrite, while the NrfA enzyme reduces nitrite to ammonium through a six-electron reduction (Eq. [4.1]) proposed to involve bound intermediates of nitric oxide (NO) and  $\text{NH}_2\text{OH}$  (Einsle *et al.*, 2002). In *E. coli*, the reduction of nitrate to ammonia can be coupled to energy-conserving electron transport pathways with formate as an electron donor (Potter *et al.*, 2001).



The NrfA enzyme is a deca-heme homodimeric molecule with eight *bis*-His-coordinated hemes (hemes 2 to 5) and two active site hemes (heme 1) coordinated by lysine residues on the proximal side and by a water (hydroxide) molecule or substrate on the distal side (Figs. 4.1A and 4.1B). The proposed electron input site into NrfA is through heme 2 (see Fig. 4.1A), and there are two potential routes for electrons to move to an active site: electrons can either move directly from heme 2 to the nearest active site via heme 3 or across the NrfA dimer interface via heme 5 to the remote active site (Fig. 4.1A). The reduction of nitrite to ammonia requires six electrons, indicating that an additional electron is required by the NrfA monomers, either through a second electron-donating step or from the other NrfA monomer in the dimer. Crystal structures of NrfA are currently available from bacteria *Wolinella succinogenes* (Einsle *et al.*, 1999), *Sulfurospirillum deleyianum* (Einsle *et al.*, 2000), *E. coli* (Bamford *et al.*, 2002), *Desulfovibrio desulfuricans* (Cunha *et al.*, 2003), and a NrfAH complex from *Desulfovibrio vulgaris* (Rodrigues *et al.*, 2006). The active site in all NrfA structures contains five highly conserved residues providing a positive environment around the active site heme and acting as potential proton donors (see Fig. 4.1B). The ability of purified *E. coli* NrfA to act as an NO reductase was first identified in 1990 (Costa *et al.*, 1990), and its ability to catalyze this reaction in whole cells and confer resistance to NO was demonstrated in 2002 through comparative studies on wild-type and *nrf* mutant strains of *E. coli* (Poock *et al.*, 2002).



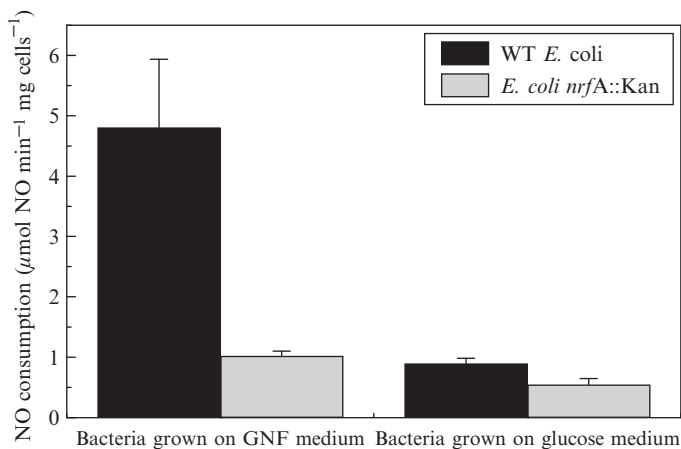
**Figure 4.1** Molecular structure of cytochrome *c* nitrite reductase NrfA. (A) The dimeric structure showing the 10 bound hemes. Hemes of one of the NrfA monomers are numbered to correlate with the description in the text. The active site heme of the other NrfA monomer is circled. (B) Detail of the active site showing the lysine coordinated to the heme. Figures were prepared using Pymol (Delano Scientific).

## 2. MEASUREMENT OF CYTOCHROME C NITRITE REDUCTASE-DEPENDENT CONSUMPTION OF NITRIC OXIDE IN WHOLE CELLS

For a rapid assessment of NrfA NO reductase activity in intact cells, 100 ml of *E. coli* is grown anaerobically in minimal media containing glycerol as the carbon and electron source and nitrate and fumarate as electron acceptors. The cultures are grown in a completely filled bottle until they enter the late exponential phase of growth. The bacterial cells are then centrifuged at 6000g for 10 min, and the cell pellet is resuspended in the growth medium in which they were grown, but without any electron acceptors present. An oxygen- and NO-sensitive amperometric Clarke electrode (Hansatech Instruments) is polarized to detect NO (Field *et al.*, 2007), and 3 ml of concentrated cell culture is loaded into the electrode chamber. The cells are incubated at room temperature until the oxygen present in the chamber is consumed, and saturated NO solution is then added to the chamber from a stock solution (Field *et al.*, 2007) to give a final NO concentration of 100  $\mu\text{M}$ . The rate of NO consumption is then measured from the change in signal over time. NO consumption is measured in nmol NO consumed  $\text{min}^{-1} \text{mg cells}^{-1}$  and the specific activity is highly dependent on the anaerobic growth procedures. For example, it is some 10-fold lower following anaerobic growth in minimal media containing glucose compared to anaerobic growth in the presence of glycerol with nitrate and fumarate (GNF) present as electron acceptors (Fig. 4.2). This pattern mirrors the pattern of *nrfA* expression in these different growth media (Potter and Cole, 1999) and reflects how the choice of growth medium is critical in assessing the contribution that NrfA makes to NO reduction. Because *E. coli* has other NO-consuming systems, such as cytoplasmic flavorubredoxin (NorV) and flavohemoglobin (HmpA), it is important to estimate the proportion of activity measured that arises from NrfA. This can be done by conducting identical experiments on a *nrfA* mutant. Data in Fig. 4.2 are taken from such an experiment in which the *nrfA* mutant carried a deletion of the *nrfA* gene.

## 3. GROWTH OF *E. COLI* OPTIMIZED FOR CYTOCHROME C NITRITE REDUCTASE PRODUCTION FOR USE IN ENZYME PURIFICATION

Production of NrfA in large quantities suitable for crystallographic and spectropotentiometric analysis is complicated by the requirement for multiple cofactors to be inserted into the apoprotein to form the active enzyme.



**Figure 4.2** Nitric oxide consumption by WT and *nrfA::kan* mutant strains of *E. coli*. Bacteria were grown under anaerobic respiratory conditions using glycerol as the electron donor and carbon source with nitrate and fumarate present as electron acceptors (GNF medium) or under anaerobic fermentative conditions with glucose as the carbon and energy source.

Four of the NrfA hemes are *bis*-His-coordinated *c*-type hemes and are incorporated through the standard *E. coli* cytochrome *c* biosynthesis pathway, which uses the CXXCH amino acid sequence motif for covalent heme incorporation. However, the active site *c*-type heme is bound by a unique CXXCK motif, which requires heme lyases encoded by the *nrfEFG* genes, for heme attachment. For production of active NrfA, the heme lyases from two separate cytochrome systems must therefore be encoded. To date, the most effective method of preparing NrfA protein is from *E. coli* strain LCB2048. This kanamycin-resistant strain contains lesions in the structural genes for both nitrate reductase A and Z (*narA* and *narZ*) and results in optimized expression of the periplasmic nitrate and nitrite reductase system genes of the *nap* and *nrf* operons (Potter and Cole, 1999). For optimal production, cells are grown anaerobically at 37° in minimal salts media containing 5% LB, 40 mM fumarate, 0.4% glycerol, and 20 mM nitrate (Potter and Cole, 1999). Initially a 5-ml culture is inoculated with a colony of *E. coli* strain LCB2048 and grown overnight at 37° anaerobically. This is then transferred to a 200-ml culture and grown overnight at 37°. This 200-ml culture is then used to inoculate a 1.5-liter culture, which in turn is used to inoculate a 100-liter New Brunswick 5000 fermenter, operated for ~16 h with an agitation of 200 rpm. Cells are harvested using a CEPA continuous flow centrifuge and stored at -80° until required. Typical cell yields are approximately 150 g.

NrfA can also be produced as a recombinant protein by a procedure that has been optimized with *E. coli* strain JCB4083a ( $\Delta narZ::\omega \Delta narL::Tn10$

$\Delta napGH \Delta nrfAB \Delta nirBDC::Kan^R$ ). The JCB4083a strain carries two plasmids: plasmid pEC86 expresses the cytochrome *c* maturation system (chloramphenicol resistant) and pJG1.9a contains the entire *nrf* operon (ampicillin resistant). Briefly, *E. coli* strain JCB4083a is freshly transformed with pEC86, colony purified on chloramphenicol plates, and subjected to a second transformation to introduce plasmid pJG1.9a followed by growth on plates containing ampicillin and chloramphenicol. Colonies of the resulting JCB4083a (pEC86, pJG1.9a) strain are used to inoculate 5 ml Terrific Broth containing ampicillin ( $100 \mu\text{g ml}^{-1}$ ) and chloramphenicol ( $30 \mu\text{g ml}^{-1}$ ) and are grown aerobically at  $37^\circ$  for 8 h. The 5-ml culture is used to inoculate 1 liter of Terrific Broth containing  $100 \mu\text{g ml}^{-1}$  ampicillin and  $30 \mu\text{g ml}^{-1}$  chloramphenicol and is grown aerobically at  $37^\circ\text{C}$  overnight. The 1-liter culture is then used to inoculate 90 liters of Terrific Broth in a 100-liter New Brunswick 5000 fermenter, with a controlled oxygen level of 60%. Cells are harvested using a CEPA continuous flow centrifuge and stored at  $-80^\circ$  until required. The typical weight of cells harvested using this method is approximately 2 kg.

#### 4. PURIFICATION OF CYTOCHROME C NITRITE REDUCTASE

The first stage of NrfA purification is to resuspend cells in spheroplasting buffer (0.5 M sucrose, 100 mM Tris-HCl, pH 8.0, 1 mM EDTA) with  $100 \text{ mg liter}^{-1}$  DNase 1 and  $1 \text{ g liter}^{-1}$  lysozyme to disrupt the cell wall and release the periplasmic contents. Cells should be stirred for 30 min at  $30^\circ$  to ensure complete cell lysis; the periplasm is then separated from the suspension by centrifugation at  $9000g$  for 1 h, and the periplasm-containing supernatant is retained. Ammonium sulfate is slowly added to the supernatant to give a final concentration of 65% (w/v) and left stirring for 3 h at  $4^\circ\text{C}$ . The supernatant is then centrifuged at  $30,000g$  for 1 h to pellet the precipitated protein. The supernatant is discarded and the pellet is resuspended in 50 mM Tris-HCl, pH 8.0, and incubated for 1 h with stirring at  $4^\circ$ . The solubilized NrfA-containing fraction is centrifuged at  $30,000g$  for 1 h at  $4^\circ$  to remove precipitated protein and is then dialyzed extensively with 50 mM Tris-HCl, pH 8.0, at  $4^\circ$  until the conductivity is less than 4 mS.

The dialyzed, solubilized, NrfA-containing fraction is loaded onto a  $25 \times 2$ -cm Q-Sepharose column (Amersham) equilibrated with 50 mM Tris, pH 8.0, and washed with 200 ml of the same buffer until the baseline absorbance has stabilized. Proteins are then eluted using a gradient of 0–150 mM NaCl over 200 ml, followed by 150–1000 mM NaCl over 150 ml. At this stage it is also possible to separate NrfB, the electron donor to NrfA (Clarke *et al.*, 2004, 2007), and the periplasmic nitrate reductase

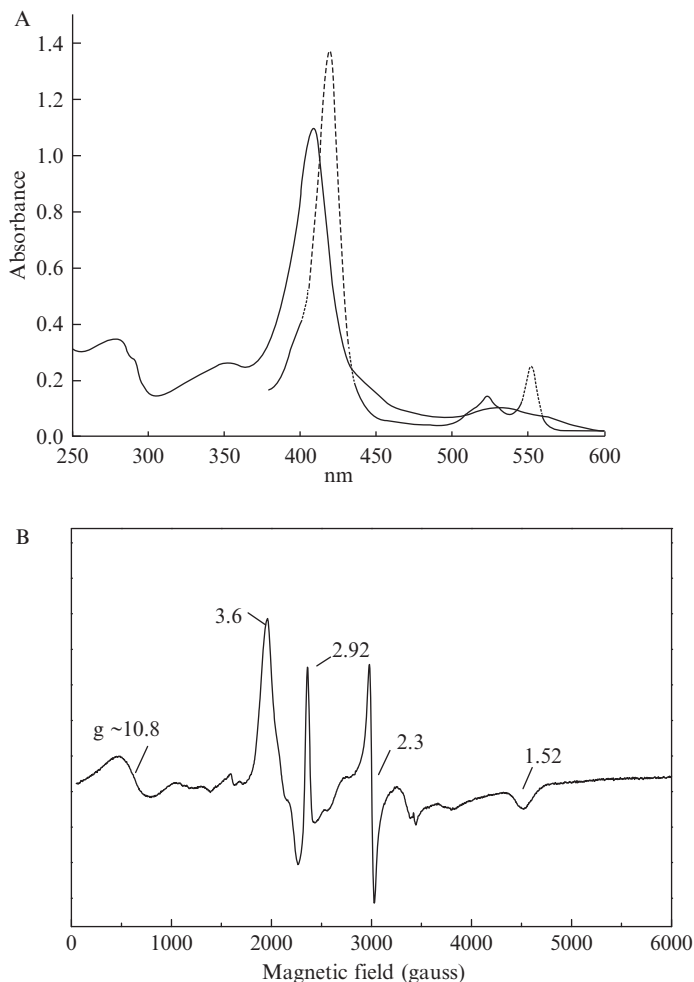
proteins NapA and NapB for further purification (Jepson *et al.*, 2007). Fractions containing NrfA can be identified using heme-stained gels. Briefly, fractions are loaded onto a 12 % SDS-PAGE gel, and the gel is run as normal. The gel is washed in water and transferred to 70 ml 50 mM Na-acetate buffer at pH 5.0 and left for 15 min in the dark. Methanol (30 ml) containing 5-tetramethylbenzidine ( $1 \text{ mg ml}^{-1}$ ) is added, and the gel is incubated in the dark for a further 15 min. A 30% (v/v)  $\text{H}_2\text{O}_2$  solution (300  $\mu\text{l}$ ) is added to initiate the reaction, and the gel is incubated in the dark until bands appear. Fractions containing NrfA can be identified by a band at 53 kDa.

Fractions containing NrfA are pooled and concentrated using a 30-kDa  $M_w$  cutoff Amicon filter (Millipore). The concentrated NrfA protein is then passed through a Superdex 200 26/60 column (Amersham) equilibrated with 50 mM Tris-HCl, pH 7.0, 50 mM NaCl and fractions containing NrfA (identified using conventional Coomassie-stained SDS-PAGE gels). As a final purification step, NrfA fractions are pooled, dialyzed into 50 mM Tris-HCl, pH 7.0, and the dialyzed NrfA is loaded onto a Dionex anion-exchange column equilibrated with 50 mM Tris, pH 7.0. The column is washed with 30 ml buffer, and NrfA is eluted from the column using a 50-ml gradient of 0–200 mM NaCl, 50 mM Tris-HCl, pH 7.0. NrfA fractions that appear as a single band on a Coomassie-stained SDS-PAGE gel are considered to be pure. These fractions are pooled and concentrated before dialysis into 50 mM HEPES, pH 7.0.

The purified protein is characterized, in the oxidized state, by an  $A_{410/280}$  ratio of 3.1 (Fig. 4.3A) and has an extinction coefficient of  $497 \text{ mM}^{-1} \text{ cm}^{-1}$  at 410 nm (Bamford *et al.*, 2002). The X-band EPR spectrum (see Fig. 4.3B) in the oxidized state has characteristic features at  $g = 10.5$  and  $3.6$  that arise from the magnetically coupled active site  $S = 5/2$  heme 1 and the nearby  $S = 1/2$  heme 3 and a rhombic trio at  $g_{z,y,x} = 2.92, 2.3, 1.52$  that arises from the  $S = 1/2$  heme 2 (see Fig. 4.3B). The  $g = 3.6$  feature has a shoulder at high field ( $g \sim 3.5$ ) that is a “Large  $g_{\text{max}}$ ” feature that arises from either (or both) heme 4 or heme 5 (Bamford *et al.*, 2002). Evidence for the integrity of the protein comes from assessing the high-spin heme signal at  $\sim g = 6$ . This reflects damaged enzyme and so the intensity of the signal should be low (see Fig. 4.3B). NrfA can be stored in 50 mM HEPES, pH 7.0, in 100- $\mu\text{l}$  aliquots at  $-80^\circ$  without a noticeable loss of activity.

## 5. ASSAYING THE CYTOCHROME *c* NITRITE REDUCTASE

The conventional method used to measure reduction of the nitrite substrate is to determine the rate of methyl viologen oxidation. Methyl viologen has a redox midpoint potential of  $-446 \text{ mV}$  at  $25^\circ$  (Mayhew, 1978), and the reduced form has a characteristic blue color that has an

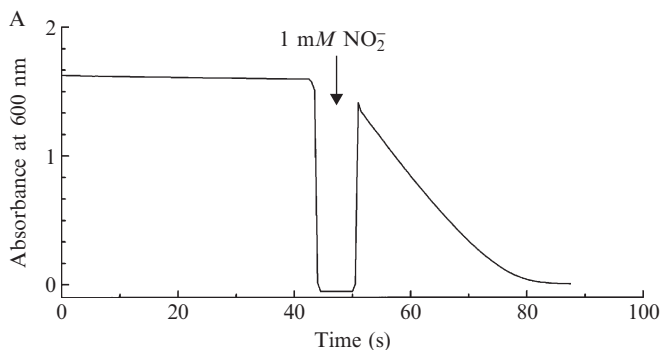


**Figure 4.3** UV-visible and X-band EPR spectroscopic properties of purified cytochrome *c* nitrite reductase. (A) UV-visible spectrum of a 2.2  $\mu\text{M}$  NrfA solution (solid line, oxidized; dashed line, reduced). (B) X-band EPR spectrum of 50  $\mu\text{M}$  NrfA collected at 10 K, 2 mW power, and 10 mT modulation amplitude.

$A_{\text{max}}$  at 600 nm with an extinction coefficient of  $13,700 \text{ M}^{-1}\text{cm}^{-1}$ . Methyl viologen assays are performed in 1-cm glass cuvettes containing 2 ml of 50 mM HEPES, pH 7.0, 2 mM  $\text{CaCl}_2$ , 1–2 nM NrfA protein, and 0.8 mM methyl viologen. The cuvettes are stoppered using rubber septa and sparged with air-free nitrogen gas for 10 min. Absorbance measurements are performed using a Varian 3100 dual-beam absorbance spectrophotometer at 600 nm. After sparging, the absorbance of the cuvette at 600 nm is used as a baseline, and 1–3  $\mu\text{l}$  of a degassed 10 mM sodium dithionite solution is

injected through the septa to increase the absorbance of the solution in the cuvette to between 1 and 1.5 AU at 600 nm. The absorbance is recorded for approximately 30 s, and then an aliquot of a 1 M stock  $\text{NaNO}_2$  solution sparged with nitrogen is added to give the desired  $\text{NO}_2^-$  concentration. The rate of the decrease in absorbance is used to determine the rate of oxidation of methyl viologen by NrfA (Fig. 4.4). The activity of a highly purified and fully active preparation of NrfA toward nitrite measured with this assay should be approximately  $625 \text{ NO}_2^- \text{ s}^{-1}$  at  $25^\circ$  at saturating nitrite, and the  $K_m$  should be in the region of  $38 \mu\text{M}$ . These values are subject to slight variation due to the interference of dithionite and/or dithionite degradation products but are reproducible for a given stock of reagents.

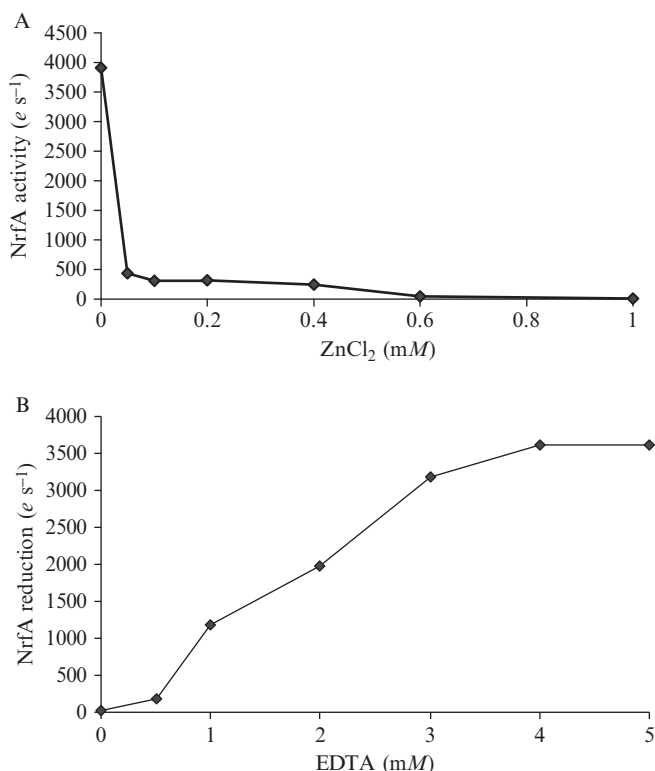
NrfA is also expressed by bacteria such as *Desulfovibrio desulfuricans* in the presence of sulfate, and the expressed NrfA enzymes are known to reduce sulfite (Clarke *et al.*, 2006); sulfite is a product of dithionite oxidation and so, to determine the maximal rate of NrfA-catalyzed nitrite reduction, it is necessary to use an alternative method to produce reduced methyl viologen such as zinc. Granules of zinc must be washed in  $\text{N}_2$ -sparged 1 M HCl for 30 s to remove the external layer of zinc oxide. The granules are then blotted dry before one is added to 100 mM methyl viologen in a glass vessel that is sealed and then made anaerobic by sparging with nitrogen. A color change is immediately apparent. After 5 min, the granule of zinc is removed, and the reduced methyl viologen is sparged with nitrogen to remove any oxygen. Ten microliters of a zinc-reduced methyl viologen solution is added to 2 ml of 50 mM HEPES, pH 7.0, 2 mM  $\text{CaCl}_2$ , 1–2 nM NrfA, and 5 mM EDTA in a  $\text{N}_2$ -sparged sealed cuvette. This produces an absorbance of 2 AU at 600 nm and, on addition of nitrite, a decrease in the absorbance at 600 nm is observed. Using this protocol, the  $V_{\text{max}}$  of NrfA for



**Figure 4.4** Methyl viologen-based assay for the cytochrome *c* nitrite reductase NrfA. Methyl viologen oxidation assay: change in absorbance at 600 nm of a 2-ml solution containing 50 mM HEPES, pH 7.0, 2 mM  $\text{CaCl}_2$ , 1.6 nM NrfA, and 0.8 mM  $\text{Na}_2\text{S}_2\text{O}_4$  reduced methyl viologen. At the indicated time, 1 mM  $\text{NO}_2^-$  is injected into the cuvette to initiate NrfA-catalyzed oxidation of methyl viologen.

nitrite was determined as  $769 \text{ NO}_2^- \text{ s}^{-1}$  and the  $K_m$  was  $22 \mu\text{M}$ . EDTA is present in this assay because, in control experiments,  $\text{Zn}^{2+}$  is found to be an inhibitor of NrfA activity (Fig. 4.5A) but the enzyme is protected by the chelator EDTA (see Fig. 4.5B). Because the EDTA protects NrfA from zinc inhibition, it suggests that, in the concentration range used, it is not disrupting the active site through chelation of the active site calcium ion (see Fig. 4.1B), although this possibility is something to be aware of when adding this chelator.

An alternative method of assessing NrfA activity utilizes protein film voltammetry (PFV) in which NrfA is immobilized onto an electrode surface

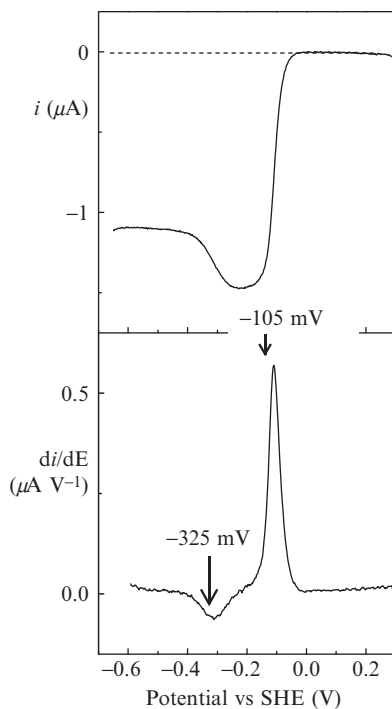


**Figure 4.5** Zinc ion inhibition of the cytochrome *c* nitrite reductase NrfA. (A) NrfA activity measured using dithionite-reduced methyl viologen assays in the presence of increasing concentrations of zinc chloride. Zinc chloride was added to cuvettes immediately prior to the addition of NrfA. The concentration of NrfA in these assays was  $1.6 \text{ nM}$ , and  $1 \text{ mM NO}_2^-$  was used to initiate the reaction. (B) Activity of  $1.6 \text{ nM}$  NrfA in the presence of  $1 \text{ mM}$  zinc chloride and  $0\text{--}5 \text{ mM}$  EDTA as indicated. Reagents were incubated for 5 min before addition of dithionite to reduce methyl viologen. Activity assays were initiated by the addition of  $1 \text{ mM NO}_2^-$ . In all experiments,  $0.8 \text{ mM}$  methyl viologen was used in  $50 \text{ mM}$  HEPES, pH 7.0,  $2 \text{ mM}$   $\text{CaCl}_2$ .

that can then serve to donate electrons to the enzyme. This method removes the need for chemical reductants that might inhibit the enzyme or react directly with a substrate such as nitric oxide. PFV is performed in a three-electrode cell configuration with the sample chamber equilibrated at the required temperature (Angove *et al.*, 2002). The cell is placed inside a Faraday cage housed in a N<sub>2</sub>-filled chamber with atmospheric O<sub>2</sub> <2 ppm. Pyrolytic graphite edge working electrodes of 3 mm diameter must be polished immediately prior to use with an aqueous slurry of 0.3 μm Al<sub>2</sub>O<sub>3</sub>, sonicated, rinsed, and dried with a tissue. The freshly polished electrodes are then taken into the anaerobic chamber, together with an aliquot of frozen NrfA (routinely around 0.5 to 1 μM). Immediately after the NrfA sample has thawed, 3–5 μl is placed on the electrode surface and after approximately 15–20 s excess solution is removed from the electrode, taking care not to dry the electrode surface. The electrode is then placed in the electrochemical cell and voltammetry commences. Voltammetry is performed with an Autolab electrochemical analyzer under the control of GPES software, and electrode rotation is driven with an EG&G Model 636 electrode rotator. A typical catalytic current collected under substrate-limiting conditions with nitrite at pH 7 is shown in Fig. 4.6. The upper part of Fig. 4.6 shows the current measured as a function of potential. The Y axis can be replotted as a derivative of the current with respect to potential (bottom part of Fig. 4.6) to more clearly resolve features in the catalytic waveshape. The positive peak at –105 mV approximates to the midpoint potential of the active site heme (see Fig. 4.1B) measured from the dependence of the *g* = 3.5 and 10.6 EPR signals on electrochemical potential (Bamford *et al.*, 2002). The negative peak at –300 mV reflects attenuation in NrfA activity at a potential that approximates to the midpoint potential of the species giving rise to the *g* ~3.5 Large *g*<sub>max</sub> signal (see Fig. 4.3B). The magnitude of the catalytic current is dependent on substrate concentration and can be used to determine the *K*<sub>m</sub>. In the case of nitrite, this is close to that determined in solution state studies (Angove *et al.*, 2002). The catalytic waveshape is dependent on many factors, including substrate concentration, pH, electrode rotation rate, and scan rate, which must be carefully assessed in any study of the interaction between a substrate or inhibitor and cytochrome *c* nitrite reductase (Gwyer *et al.*, 2004, 2005, 2006).

## 6. CRYSTALLIZATION OF *E. COLI* CYTOCHROME *C* NITRITE REDUCTASE

For effective crystallization, NrfA must be concentrated to 10 mg ml<sup>-1</sup>. The crystal screens 1, 2, and 3 (described by Jancarik and Kim, 1991) can be used to identify suitable crystallization conditions for NrfA.



**Figure 4.6** Protein film voltammetry-based assay for the cytochrome *c* nitrite reductase NrfA. The assay is performed in the presence of 1.7 mM nitrite, 2 mM CaCl<sub>2</sub>, and 50 mM HEPES, pH 7.0, with data collected at 20° at a scan rate of 20 mV s<sup>-1</sup> and an electrode rotation rate of 3000 rpm. (Top) Current measured as a function of potential; (bottom) Y axis is replotted as a derivative of the current with respect to potential.

Hanging drop vapor diffusion methods have yielded red crystals (for an example, see Fig. 4.7) in five conditions that resulted in several different crystal packing motifs (Table 4.1). The red color of the crystal arises from the five hemes bound by each NrfA molecule. The original structure of NrfA was obtained at 2.5 Å through crystal condition type I (Bamford *et al.*, 2002). However, higher-resolution structures to 1.7 Å have been obtained using crystal condition type II.

## 7. CONCLUDING REMARKS

The cytochrome *c* nitrite reductase or NrfA from *E. coli* is a fascinating enzyme because of the complex six-electron reaction it can catalyze and the number of substrates, including nitric oxide, that it can use. The physiological relevance of the NO reduction reaction and the detailed mechanism of



**Figure 4.7** Cytochrome *c* nitrite reductase crystals. Crystals of NrfA were formed using the vapor diffusion method. NrfA ( $1 \mu\text{l}$ ) at a concentration of  $10 \text{ mg ml}^{-1}$  was mixed with  $1 \mu\text{l}$  mother liquor containing  $100 \text{ mM}$  HEPES, pH 7.5, 20% PEG 10K and incubated in hanging drop trays with a 1-ml well solution at  $4^\circ$ . Crystals typically developed in 5–14 days.

**Table 4.1** Crystallographic parameters of NrfA crystals

Type	Shape	Space group	Cell ( $\text{\AA}$ )	Conditions
I	Rectangular plates	$P2_12_12_1$	$81 \times 90 \times 294$	10% isopropanol, 20% PEG 4K, 100 mM HEPES, pH 7.5
II	Square plates	$P2_1$	$89 \times 80 \times 142$	20% PEG 10K, 100 mM HEPES, pH 7.5
III	Trapezoid	$C222$	$83 \times 91 \times 362$	100 mM MES, pH 6.5, 25% PEG 8K, 0.8 M Na,K tartrate
IV	Cubes	$P2_13$	$188 \times 188 \times 188$	2 M $(\text{NH}_4)_2\text{SO}_4$ , 100 mM $\text{NH}_4\text{Ac}$ , pH 4.0
V	Rectangular plates	$I222$	$158 \times 178 \times 264$	50% K phosphate, pH 7.0

the enzyme as an NO reductase remain to be determined. However, the assay, purification, and crystallization procedures presented here provide a strong foundation from which to explore studies on the NO reductase activity of native and engineered enzymes in whole cells and as purified enzymes.

## ACKNOWLEDGMENTS

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