NrfA is a pentahaem cytochrome present in a wide-range of γ-, δ- and ε-proteobacteria. Its nitrite and nitric oxide reductase activities have been studied extensively and contribute to respiratory nitrite ammonification and nitric oxide detoxification respectively. Sulfite is a third substrate for NrfA that may be encountered in the micro-oxic environments where nrfA is expressed. Consequently, we have performed quantitative kinetic and thermodynamic studies of the interactions between sulfite and *Escherichia coli* NrfA to provide a biochemical framework from which to consider their possible cellular consequences. A combination of voltammetric, spectroscopic and crystallographic analyses define dissociation constants for sulfite binding to NrfA in oxidized (∼54 μM), semi-reduced (∼145 μM) and reduced (∼180 μM) states that are comparable with each other, and the $K_m$ (∼70 μM) for sulfite reduction at pH 7. Under comparable conditions $K_m$ values of ∼22 and ∼300 μM describe nitrite and nitric oxide reduction respectively, whereas the affinities of nitrate and thiocyanate for NrfA fall more than 50-fold on enzyme reduction. These results are discussed in terms of the nature of sulfite co-ordination within the active site of NrfA and their implications for the cellular activity of NrfA.

**Key words:** cytochrome, electron paramagnetic resonance, magnetic circular dichroism, nitrite reductase, NrfA, protein film voltammetry.

**INTRODUCTION**

The pentahaem cytochrome NrfA was first identified in the enteric pathogen *Escherichia coli*, where its role in anaerobic nitrate respiration is now well-established. At low nitrate concentrations expression of *napABC* and *nrfABCD* allows quinol oxidation to be coupled to the periplasmic reduction of nitrate to nitrite and nitrite to ammonium respectively. In themselves these reactions are not proton motive. However, they contribute to maintaining a transmembrane proton gradient by regenerating the quinone required for the proton-motive oxidation of formate by formate dehydrogenase-A. NrfA contributes to this process by catalysing the six-electron reduction of nitrite to ammonium, and homologues have been identified in a wide range of γ-, δ- and ε-proteobacteria that have environmental, medical and biotechnological importance [1].

Enzymes of the NrfA family have a high degree of sequence similarity and this is reflected in their highly conserved three-dimensional structures [2–5]. Four *bis*-histidine co-ordinated c-haem groups form two branches that converge on the active site from distinct areas on the surface of the enzyme. The active site pocket is defined by the side chains of conserved histidine, tyrosine and arginine residues, together with the distal face of a c-haem with a lysine residue as its proximal ligand. A crystal structure of *Wolinella succinogenes* NrfA (PDB code 3BNF) shows nitrite co-ordinated to the distal face of the active-site haem through its nitrogen atom and with its oxygen atoms hydrogen-bonded to histidine and arginine residues [6]. These residues together with tyrosine may facilitate catalysis by delivering the protons needed for the reductive transformation of nitrate to ammonium: $\text{NO}_2^-+6\text{e}^-+8\text{H}^+\rightarrow\text{NH}_4^++2\text{H}_2\text{O}$.

NrfA also catalyses the reduction of nitric oxide to ammonium [7–9]. Nitric oxide is cytotoxic and the presence of NrfA has been shown to contribute to defence against exogenous nitric oxide in *Salmonella enterica* serovar Typhimurium and *E. coli* [10,11]. Thus NrfA has the capacity to contribute to both respiration and detoxification as necessitated by the prevailing conditions, and some insight into the likely activity of NrfA in the presence of both nitric oxide and nitrite is provided by the steady-state kinetic parameters for these reductions. For *E. coli* NrfA a combination of PFV (protein film voltammetry) and spectrophotometric analyses has defined values of $k_{\text{cat}}$ and $K_m$ for nitric oxide reduction of ∼840 s$^{-1}$ and 300 μM respectively at pH 7 [8]. Nitrite reduction occurs with a $k_{\text{cat}}$ of ∼770 s$^{-1}$ and $K_m$ of ∼30 μM under comparable conditions [8,12]. It is likely that both nitrite and nitric oxide bind as distal ligands to the active-site haem prior to their reduction. Certainly similar electrochemical potentials are required to observe appreciable activity towards both substrates, which is consistent with their reduction at a common site. Thus, at neutral pH, if the levels of NrfA in the cell are such that the enzyme can be saturated with the nitrogenous substrates, high levels of nitric oxide relative to nitrite will be required to favour nitric oxide detoxification over nitrate respiration.

A third substrate of NrfA is sulfite [3,7,13,14] (for simplicity we use the term ‘sulfite’ to encompass the species $\text{SO}_3^{2-}$, $\text{HSO}_3^-$ and $\text{SO}_2^-$ that are formed in a pH-dependent equilibrium when $\text{Na}_2\text{SO}_3$ dissolves in aqueous solution). This is an essential nutrient and metabolite, which may be present at high levels in micro-aerobic aquatic niches and be cytotoxic. It is produced by neutrophils as part of the host defence against microbial invasion and added to certain food and beverages as an antimicrobial [15,16]. Consequently, NrfA may well encounter sulfite in a cellular...
context. Sulfite reduction by NrfA generates sulfide in a six-electron process that appears to parallel nitrite ammonification, although the reaction pathway, and indeed the physiological role of this reaction, are presently unclear [13]. Steady-state parameters describing NrfA sulfite reduction that may inform on the possible cellular consequences of interactions between sulfite and NrfA have not been reported to date. However, where rates of sulfite reduction are documented they are at least as high as those of dedicated sulfite reductases, although several orders of magnitude less than those for nitrite reduction under comparable conditions. It may also be significant that \( \text{SO}_4^{2-} (\text{HSO}_3^-) \) can bind as the distal ligand to the active-site haem of \( \text{W. succinogenes} \) NrfA [13]. This suggests that sulfite will compete with nitrite and nitric oxide for binding to NrfA and, since it is reduced considerably more slowly than those substrates, its presence may have a significant impact on the rates of reduction of the nitrogenous substrates. In this context we have performed quantitative kinetic and thermodynamic studies to provide greater insight into the interactions between sulfite and \( \text{E. coli} \) NrfA in multiple oxidation states. Our results provide a detailed biochemical framework describing the interactions between a redox enzyme and its substrate and one from which the possible cellular consequences of these interactions can be considered.

**EXPERIMENTAL**

**Materials**

NrfA was purified from \( \text{E. coli} \) strain LCB2048 as described previously and concentrations were determined using \( \varepsilon_{410\mu \text{m}} = 497650 \, \text{M}^{-1} \cdot \text{cm}^{-1} \) for the oxidized enzyme [4,17]. Unless stated otherwise, experiments were performed in 50 mM Hepes, pH 7.0, containing 2 mM CaCl\(_2\) at 20°C [prepared with water of resistivity of \( >18 \) M\( \Omega \) cm (Purelab Maxima; Elga) or Trace Select Ultra Water (\( \leq 1 \mu \text{g} \) of nitrite \( \cdot \text{kg}^{-1} \); Fluka)]. Fresh NaNO\(_2\) and Na\(_2\)SO\(_3\) stock solutions (pH 7.0) were prepared daily as required.

**PFV**

All potentials are quoted against the SHE (standard hydrogen electrode) after adding 0.197 V to the value measured with a Ag/AgCl (saturated KCl) reference electrode. The experimental arrangement, in addition to data collection and analysis, were essentially as described previously [12,18]. Briefly, pyrolytic graphite ‘edge’ working electrodes were coated with NrfA and rapidly rotated during experiments performed in nitrogen-filled chambers. Sulfite concentrations above 1 mM were avoided because currents arising from electrode sulfite reduction interfered with accurate assessment of the currents arising from NrfA dependent processes.

The Michaelis constant (\( K_m \)) and maximum catalytic current (\( i_{\text{max}} \)) describing NrfA nitrite reduction in a solution of defined sulfite concentration were determined from the catalytic currents measured by chronoamperometry in response to titrating nitrite into the sample. Before fitting these currents to the Michaelis–Menten equation, they were corrected for first-order loss of magnitude over time and Koutecky–Levich analysis was used as appropriate to determine the response at infinitely high rotation rates, i.e. in the absence of mass-transport limitations. Where it was necessary to compare results from different films this was done after normalizing the response of each film to that recorded during cyclic voltammetry at 30 mV \( \cdot \) s\(^{-1} \) and 3000 rev./min in 5 \( \mu \text{M} \) nitrate.

For competitive inhibition, the dissociation constant, \( K_d \), describing binding of the inhibitor I to the enzyme was determined from the equation:

\[
K_d = K_d^0 (1 + [I]/K_d),
\]

where \( K_d^0 \) is the Michaelis constant in the absence of inhibitor. For mixed inhibition, values for the dissociation constants \( K_d^0 \) and \( K_d^{R-nitrite} \) describing inhibitor binding to the enzyme and enzyme–nitrite complex respectively were determined from the equation:

\[
1/i_{\text{max}} = 1/i_{\text{max}}^0 \left[ \left( 1 + [I]/K_d^0 \right) / \left( 1 + [I]/K_d^{R-nitrite} \right) \right]
\]

where \( i_{\text{max}}^0 \) is the maximum catalytic current in the absence of the inhibitor [19]. We note that the major conclusions drawn from the data are not changed by linear analysis of both the plots of \( 1/i_{\text{max}} \) against [I] that yields \( K_d^0 \) of \( \sim 87 \mu \text{M} \) and \( K_d^{R-nitrite} \) of \( \sim 1270 \mu \text{M} \).

**Spectroscopic assessment of sulfite binding to NrfA**

Sulfite binding to NrfA was monitored by X-band EPR, MCD (magnetic CD) and electronic absorption spectroscopies [4]. Values for the dissociation constant \( K_d^0 \) describing sulfite binding to oxidized NrfA were determined from the variation of signal intensity with sulfite concentration fitted to the equation describing reversible binding of a single molecule of sulfite to a single molecule of NrfA:

\[
[NrfA – sulfite]/[NrfA]_{\text{total}} \cdot [\text{sulfite}]_{\text{total}} = \frac{C - \sqrt{C^2 - 4[NrfA]_{\text{total}}[\text{sulfite}]_{\text{total}}}}{2}
\]

where \( [\text{NrfA-sulfite}] \) is the equilibrium concentration of the complex formed between NrfA and sulfite, \( [\text{NrfA}]_{\text{total}} \) and \( [\text{sulfite}]_{\text{total}} \) are the total concentrations of NrfA and sulfite respectively, and \( C = [\text{NrfA}]_{\text{total}} + [\text{sulfite}]_{\text{total}} + K_d^0 \). The concentration of NrfA–sulfite in the MCD sample was calculated using \( \varepsilon_{532\mu \text{m}} = 53210 \, \text{M}^{-1} \cdot \text{cm}^{-1} \) as electronic absorption spectroscopy showed that both oxidized NrfA and its complex with sulfite had the same absorbance at this wavelength (Supplementary Figure S1 at http://www.BiochemJ.org/bj/431/bj4310073add.htm).

**Crystallization, X-ray data collection, structure solution and refinement**

NrfA was crystallized under the conditions reported previously [17]. A single crystal was soaked in the crystallization reservoir solution plus 50 mM Na\(_2\)SO\(_3\) for 10 min at 277 K prior to transfer to a similar solution supplemented with both 50 mM Na\(_2\)SO\(_3\) and 20 % (v/v) ethylene glycol as cryoprotectant. X-ray diffraction from a single crystal maintained at 100 K was measured using an ASDC Q4 detector on beamline ID14.2 at the ESRF (European Synchrotron Radiation Facility, Grenoble, France). The crystal was of space group \( P \), cell dimensions \( a = 90.5 \, \text{Å} \) (1 Å = 0.1 nm), \( b = 82.2 \, \text{Å} \), \( c = 142.2 \, \text{Å} \) and \( \beta = 101.1^\circ \). Diffraction data were obtained to a resolution of 2.3 Å using X-rays at a wavelength of 0.933 Å.

Microspectrophotometry established that exposure to X-rays was accompanied by photo-reduction of the NrfA haem groups (Supplementary Figure S2 at http://www.BiochemJ.org/bj/431/bj4310073add.htm). This reduction appeared complete within 100 s of X-ray exposure. Consequently, to avoid combining data from both oxidized and reduced states of NrfA, only those images collected after 100 s of cumulative X-ray exposure, i.e. those arising from reduced NrfA, were processed using MOSFLM.
and integrated using SCALA as part of the CCP4 crystallography package [20–22]. A summary of data collection statistics is provided in Supplementary Table S1 at http://www.BiochemJ.org/bj/431/bj4310073add.htm.

The structure of sulfite-bound NrfA was determined by molecular replacement with MOLREP [22] employing a single monomer from the 1.7 Å resolution NrfA structure (PDB code 2RDZ) as a search model [23]. This revealed four monomers of NrfA in the crystallographic asymmetric unit. This model was refined using several rounds of manual building using COOT [24] and automatic refinement using REFMAC [25]. Addition of 957 water molecules using ARP [26] and COOT, as well as eight sulfite molecules and 12 ethylene glycol molecules gave a final structure with a final R
tot (Rfree) of 17.7 (23.8) for data in the range 50.4–2.3 Å. When analysed using MOLPROBITY [27], 1756 residues in the unit cell were in the allowed regions, with four residues in unfavoured regions being the histidine residues of the active sites.

Assays of sulfite reduction

Steady-state rates of sulfite reduction were measured in a spectrophotometric assay through the NrfA-dependent oxidation of zinc-reduced methyl viologen (0.3 μM NrfA with 1 mM zinc-reduced methyl viologen) in 50 mM HEPES, pH 7.0, containing 2 mM CaCl2 and 5 mM EDTA, and the desired concentration of sulfite, under anaerobic growth conditions [17]. To verify sulfide as the product of sulfite reduction, NrfA and dithionite were incubated in nitrogen-sparged sealed cuvettes. Dithionite oxidation was quantified using ε315 = 8 mM⁻¹·cm⁻¹ for S2O4²⁻ [28] and sulfide determined colorimetrically by the Cline method [29].

Construction of an E. coli nrfA-deletion strain and assessment of the impact of sulfite on anaerobic growth

The phage λ Red recombinase PCR mutagenesis system was used to delete nrfA from E. coli strain DH10B [30,31]. Primers were designed to generate a PCR product consisting of the kanamycin-resistance gene from pKPD13 flanked by regions homologous with nucleotides adjacent to nrfA. This PCR product was used to electrottransform E. coli DH10B, expressing the λ Red recombinase via the arabinose-inducible plasmid pKD46. A kanamycin-resistant transformant was then selected and the chromosomal mutation verified by colony PCR. To confirm the expected phenotype periplasmic extracts were prepared from cultures of the nrfA-deletion strain and DH10B after overnight anaerobic growth in glycerol/nitrate/fumarate medium for optimal nrfA expression [17]. The extract from DH10B, but not that from the nrfA-deletion strain, displayed nitrite reductase activity in a spectrophotometric assay using dithionite-reduced methyl viologen as the electron donor and a haem-staining band of ∼50 kDa, corresponding to NrfA [31], was resolved by SDS/PAGE.

To assess the impact of sulfite on anaerobic growth of these strains, 10 ml cultures were initially grown overnight in aerobic LB (Luria–Bertani) medium (10 g/l of tryptone, 10 g/l of NaCl and 5 g/l of yeast extract, pH 7.0). These cultures were used to inoculate sealed Hungate tubes containing 14 ml of minimal salts medium supplemented with 1 % LB, 10 μM selenate and 10 μM molybdate with the complete medium brought to pH 6.4 with phosphate [32]. In this medium the main carbon source was 43 mM glycerol and the terminal electron acceptors were 2 mM sodium nitrate and 40 mM sodium fumarate. After 6 h of growth the cultures were used to inoculate 14 ml of nitrogen-flushed minimal salts medium in sealed Hungate tubes. These anaerobic cultures were grown to an absorbance of ∼0.15 at 600 nm and 140 μl of either 250 mM aqueous Na2SO3 (pH 6.4) or water was added as desired. Bacteria were grown at 37°C, and kanamycin (25 μg·ml⁻¹) was added where appropriate.

RESULTS

Sulfite inhibition of NrfA nitrite reduction

The low rates reported for NrfA sulfite reduction when compared with those for nitrite reduction, taken together with crystallographic resolution of these molecules as distal ligands to the active-site haem of W. succinogenes NrfA, suggested that sulfite would inhibit NrfA nitrite reduction [7,13,14,33]. Previously, PFV was used to resolve inhibition of NrfA nitrite reduction by ions, such as cyanide, azide, sulfate and nitrate, across the electrochemical potential domain, i.e. as a function of the enzyme oxidation state [34]. Consequently, PFV was used to assess the impact of sulfite on steady-state nitrite reduction by NrfA (Figure 1A). A ‘film’ of electrocatalytically active NrfA was adsorbed on an electrode surface and placed in 5 μM nitrite. Cyclic voltammetry revealed a negatively signed catalytic current

Figure 1 PFV of E. coli NrfA in the presence of sulfite and/or nitrite

(A) Baseline-subtracted steady-state voltammetry from a NrfA film in 5 μM nitrite with 0, 20, 100, 200, 500 or 1000 μM sulfite. (B) Baseline-subtracted voltammetry from a NrfA film in 1 mM sulfite (heavy solid line) and after transfer to 10 μM nitrite (light solid line). The catalytic response from a freshly polished electrode placed in 1 mM sulfite (broken line). The buffer electrolyte was 50 mM HEPES, pH 7.0, containing 2 mM CaCl2; measurements were taken at 20°C with a scan rate of 30 mV·s⁻¹ and an electrode rotation at 3000 rev./min.

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below \sim -0.1 \text{ V} that quantified the rate at which electrons moved from the electrode to nitrite via NrfA as a function of the potential applied to the electrode. The magnitude of the catalytic wave decreased on addition of sulfite, but the original magnitude was restored when the film was returned to a solution containing 5 \mu M nitrite with no sulfite. Thus sulfite acted as a reversible inhibitor of NrfA nitrite reduction.

In an attempt to identify a catalytic response describing sulfite reduction PFV was also performed in 1 mM sulfite (Figure 1B). Although catalysis was clearly displayed when the film was subsequently transferred into 10 \mu M nitrite, there was no indication of a catalytic response arising from sulfite reduction by NrfA, as the currents below \sim -0.4 \text{ V} could be attributed to direct reduction of sulfite by the electrode. This result was consistent with sulfite reduction occurring at a rate significantly less than that for nitrite reduction, see below. Although this precluded analysis of sulfite reduction by PFV it had the benefit of allowing sulfite to be considered simply as an inhibitor of nitrite reduction, such that its interactions with NrfA could be readily quantified as described below.

Sulfite inhibition of NrfA catalysis in 5 \mu M nitrite was described by IC_{50} values of \sim 150 and \sim 350 \mu M at -0.25 and -0.60 V respectively (Figure 1A). Thus the potency of sulfite as an inhibitor of NrfA was dependent on applied potential and hence the effective oxidation state of the enzyme. To gain greater insight into this behaviour, values for K_m and the maximum catalytic current i_{max} (equivalent to V_{max}) were determined as a function of sulfite concentration at these two potentials. For simplicity we relate the behaviours displayed by NrfA poised at -0.25 V and -0.60 V to SR (semi-reduced) and R (reduced) states of NrfA respectively, given that the catalytic current magnitude is independent of potential below \sim -0.50 \text{ V} and thus presumably arises from fully reduced enzyme. When NrfA was poised at -0.25 V, sulfite had negligible effect on i_{max}, but increased K_m in a manner consistent with competitive inhibition with a dissociation constant for sulfite binding to semi-reduced NrfA (K_{SR}) of \sim 145 \pm 50 \mu M (Figures 2A and 2B, circles).

For NrfA poised at -0.60 V the presence of sulfite decreased i_{max} and increased K_m indicating a form of mixed-inhibition (Figures 2A and 2B). Plots of 1/i_{max} and K_m/i_{max} against sulfite concentration showed hyperbolic- and linear-behaviour respectively (Figures 2C and 2D). This is consistent with formation of a dead-end complex when sulfite binds to reduced NrfA, but an active complex when sulfite binds to nitrite-bound reduced NrfA. From the plot of 1/i_{max} against sulfite concentration the dissociation constants describing inhibitor binding to reduced NrfA (K_{SR} and its nitrite complex (K_{R-nitrite}), were found to be 178 \pm 50 \mu M and 389 \pm 50 \mu M respectively [17]. These results are summarized in Figure 3.

**Spectroscopic studies of sulfite binding to oxidized NrfA**

Spectroscopically, the various haem groups of oxidized, i.e. all-ferric NrfA, are most readily distinguished by X-band EPR spectroscopy in the perpendicular mode [4]. The four bis-histidine residue co-ordinated haem groups of NrfA are low-spin (S = \frac{1}{2}). The lysine residue co-ordinated haem group of the active site is high-spin (S = \frac{3}{2}) where it exists as a mixed population with distal ligation from water and hydroxide. Weak spin-coupling between the high-spin ferric haem and its neighbouring bis-histidine residue co-ordinated ferric haem results in two prominent features in the perpendicular mode X-band EPR spectrum of oxidized NrfA at 10 K (Figure 4A). These features, a high-field peak with a g-value of \sim 10.8 and a mid-field derivative having a peak at a

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**Figure 2** Summary of kinetic parameters for E. coli NrfA nitrite reduction in the presence of sulfite

Results from PFV with the NrfA film poised at -0.25 V (○) or -0.6 V (■) and experimental conditions as in Figure 1. The lines represent best fits to the data as described in the text: in (B) K_{SR} = 145 \mu M and in (C) K_{SR} = 178 \mu M and K_{R-nitrite} = 389 \mu M. In (D) the line shows the best linear fit to the data.

**Figure 3** Summary of thermodynamic parameters describing sulfite inhibition to E. coli NrfA

The square represents NrfA in different oxidation states: oxidized, open square; semi-reduced, half-filled square; and reduced, filled square. Dissociation constants for reversible binding of a single sulfite molecule to NrfA were determined for oxidized NrfA (K_{SR}) from spectroscopic studies, and for semi-reduced (K_{SR}) and reduced (K_{R-nitrite}) NrfA from PFV defining sulfite inhibition of NrfA nitrite reduction at -0.25 and -0.6 V respectively. See the text for details.
There were no changes in the EPR or MCD spectra on sulfite binding to oxidized NrfA that would indicate changes of haem oxidation or spin-state. In the MCD spectrum the region below 600 nm, which is dominated by contributions from low-spin ferric haem, was essentially unchanged by the presence of sulfite. There was no indication of sharp and relatively intense features in the α/β-region (500 to 550 nm) that would indicate the formation of low-spin ferrous haem or a trough at 600 nm indicative of high-spin ferrous haem. In the EPR spectra, the sharp features at g-values of ~3.1 and 2.7 that arise from low-spin ferric haem groups, either as large g_{max} or spin-coupled features, are not changed by the presence of sulfite. Similarly, sulfite had no impact on the rhombic trio of features at g-values of ~2.9, 2.3 and 1.5 which arise from a single bis-histidine residue co-ordinated low-spin ferric haem. Consequently, the changes seen during the EPR-monitored titration of sulfite with oxidized NrfA can be attributed simply to sulfite binding to the oxidized enzyme and perturbing the environment of the active-site haem. The variation of the g = 3.53 signal intensity with sulfite concentration is well-described by reversible one-to-one binding of sulfite to oxidized NrfA with a dissociation constant (K_{d}) of 53 ± 20 μM (Figure 4A).

The electronic absorbance spectrum of oxidized NrfA is dominated by contributions from the four low-spin ferric haem groups, which makes it less sensitive than EPR and MCD to changes at the high-spin ferric haem. Nevertheless there were changes in the electronic absorbance, consistent with the results from EPR and MCD spectroscopy, when sulfite was titrated with oxidized NrfA (Supplementary Figure S1). Changes in the Soret region, highlighted in the sulfite-bound minus oxidized NrfA difference spectrum that had a trough at 408 nm, an isosbestic point at 423 nm and peak at 430.5 nm, were consistent with an altered environment of the high-spin ferric haem. In addition, the absorbance at 430.5 nm varied with sulfite concentration in the manner expected for reversible binding of a single molecule of sulfite to a single NrfA subunit with K_{d} = 25 ± 20 μM and in acceptable agreement with the EPR results given the distinct features and errors associated with each type of experiment. The presence of sulfite did not induce changes in the α/β-region, ruling out the formation of low-spin ferrous, or ferric, haem.

Crystallographic resolution of sulfite binding to reduced NrfA

Molecular resolution of the site of sulfite binding to reduced E. coli NrfA was afforded by crystals of NrfA that had been soaked with 50 mM Na_{2}S{O}_{3} prior to harvesting, which diffracted X-rays to 2.3 Å. Microspectrophotometry established that exposure to X-rays was accompanied by relatively rapid photoreduction of the NrfA haem groups (Supplementary Figure S2). This reduction appeared complete within 100 s of X-ray exposure. Consequently, to avoid combining data from both the oxidized and reduced states of NrfA, only those diffraction images collected after 100 s of cumulative X-ray exposure, i.e. those arising from reduced NrfA, were processed. Refinement using these data showed that the majority of electron density overlaid that resolved from native crystals, i.e. those prepared in the absence of inhibitors and substrates. However, significant additional electron density was observed in the active site of each of the four NrfA monomers within the unit cell. This electron density could be attributed to SO_{4}^{2−} (HSO_{3}^{−}) positioned with the sulfur bound to the iron of the lysine residue-co-ordinated haem at a distance of 2.35 ± 0.1 Å, with one oxygen hydrogen-bonded to Arg^{108}, another oxygen hydrogen-bonded to both His^{104} and Tyr^{126} and the third oxygen co-ordinated by a conserved water molecule that was in turn hydrogen-bonded to the propionate groups of the
Lysine residue-co-ordinated haem (Supplementary Figure S3 at http://www.BiochemJ.org/bj/431/bj4310073add.htm). This was the only orientation of $\text{SO}_3^{2-}$ that could provide a satisfactory fit to the electron density, and it was similar to that reported for $\text{SO}_3^{2-}$ ($\text{HSO}_3^{-}$) bound to W. succinogenes NrfA [13]. The average temperature factors (B-factor) of the active-site sulfite molecules were similar to the average B-factors of the other ligands (Supplementary Table S1), indicating that the electron density at the active site could be adequately attributed to the presence of $\text{SO}_3^{2-}$ ($\text{HSO}_3^{-}$). Additional electron density was also identified on the surface of one NrfA subunit and this could be attributed to a fifth sulfite ion in the unit cell co-ordinated via the side-chains of Lys322 and the backbone amide groups of Phe310 and Ala311. However, the significance of this finding is unclear since it may arise from adventitious association of $\text{SO}_3^{2-}$ ($\text{HSO}_3^{-}$) with the surface of NrfA under the conditions of co-crystal formation. When the positions of the main chain atoms from the 1.7 Å resolution structure of the native E. coli NrfA monomer were superposed with atoms in the corresponding 2.3 Å resolution structure of the $\text{SO}_3^{2-}$ ($\text{HSO}_3^{-}$)-bound NrfA monomer, the overall RMSD (root mean square deviation) was 0.483 Å. This indicates that $\text{SO}_3^{2-}$ ($\text{HSO}_3^{-}$) binds to NrfA without causing significant conformational changes.

**Sulfite reductase activity of NrfA**

Steady-state analysis of NrfA sulfite reduction was performed using a spectrophotometric assay with zinc-reduced methyl viologen as the electron donor. The results were well described by the Michaelis–Menten equation with $K_m = 70 \pm 15 \mu \text{M}$ and $k_{cat}/K_m = 0.03 \pm 0.005$ molecules of sulfite reduced s$^{-1}$ giving a specificity constant ($k_{cat}/K_m$) of the order of $4.3 \times 10^7$ M$^{-1}$ s$^{-1}$ (Figure 5). In the same assay, nitrite reduction by NrfA was described by $K_m = 22 \pm 7 \mu \text{M}$, $k_{cat} = 769 \pm 20$ s$^{-1}$ and $k_{cat}/K_m$ of the order of $3.5 \times 10^7$ M$^{-1}$ s$^{-1}$. To verify the product of sulfite reduction, NrfA ($3 \mu \text{M}$) was incubated anaerobically with dithionite ($10 \mu \text{M}$) and sulfite and electrons ($\text{SO}_3^{2-}+2\text{H}_2\text{O} \rightarrow 2\text{HSO}_3^{-}+2\text{H}^++2\text{e}^{-}$). Dithionite oxidation occurred at a rate of $0.8 \pm 0.05$ electrons s$^{-1}$ and sulfide was formed at a rate of $0.14 \pm 0.1$ molecules s$^{-1}$. The ratio of these rates is $5.7 \pm 2$, consistent with the six-electron reduction of sulfite to sulfide.

**Figure 5** Sulfite reductase activity of E. coli NrfA

Rates were measured using zinc-reduced methyl viologen as the electron donor with 0.3 $\mu \text{M}$ NrfA in 50 mM Hepes, pH 7.0, containing 2 mM CaCl$_2$ and 5 mM EDTA, at room temperature. The line shows best fit to the Michaelis–Menten equation with $K_m = 70 \mu \text{M}$ and $V_{max} = 0.029$ s$^{-1}$. The error bars represent the standard deviation of three assays at each sulfite concentration.

**Impact of nrfA deletion on anaerobic growth in the presence of sulfite**

Anaerobic growth of the E. coli nrfA-deletion strain and the parent strain DH10B were compared in the presence and absence of sulfite to assess whether NrfA conferred resistance to sulfite (Figure 6). A pulse of 35 $\mu$mol of sulfite was added to both cultures during early exponential growth with nitrate and fumarate as the terminal electron acceptors and glycerol as the non-fermentable carbon source. Growth of both strains was arrested by the addition of sulfite (Figure 6A). However, whereas the parent strain resumed growth after $\sim 12$ h, the growth of the nrfA-deletion strain was arrested for at least 20 h. The ability of NrfA to confer resistance to sulfite was confirmed in control experiments where water, of equal volume to that containing 35 $\mu$mol of sulfite, was added to the cultures (Figure 6B). Both strains reached stationary phase within $\sim 12$ h with the nrfA-deletion strain having a slightly lower final attenuation than the parent strain.

**DISCUSSION**

The ability of NrfA to reduce sulfite to sulfide has been known for some time [3,7,13,14]. However, the possible cellular consequences of this activity have received little attention. For E. coli NrfA, a periplasmic enzyme in an enteric pathogen, exogenous sulfite may be encountered in various contexts during colonization of a human host. Sulfite can be encountered in the gastrointestinal tract as it is added to certain foods and beverages as an antimicrobial [15]. E. coli that enters the blood stream as a prelude to sepsis or meningitis may also encounter sulfite as it is produced by neutrophils as part of host defence against microbial invasion [16]. Consequently, the dissociation constants and kinetic parameters determined in the present study provide not only a quantitative description of interactions between a redox enzyme and its substrate, but a biochemical framework from which to consider the cellular consequences of the interactions between sulfite and the wide-spread multi-haem cytochrome NrfA.

Resolution of dissociation constants describing the affinity of a substrate for multiple oxidation states of an oxidoreductase...
is relatively rare. Substrate binding to a catalytically competent oxidation state is typically followed by relatively rapid events leading to product formation and that preclude isolation of the binding event. In the present study the interactions of sulfite with catalytically competent semi-reduced and fully reduced NrfA were resolved by taking advantage of the relatively low rate of sulfite reduction, as compared with that of nitrite. This allowed well-established models of enzyme inhibition to provide dissociation constants for various redox states of NrfA as defined by the potential applied to the electrode in the PFV experiments. For both fully and semi-reduced NrfA there was competition between sulfite and nitrite for binding to the active site. This is a finding that is consistent with both SO$_3^{2−}$ (HSO$_3^−$) and nitrite being substrates for NrfA and the resolution of both of these molecules as distal ligands to the active site haem in crystals of oxidized W. succinogenes NrfA [6,13]. Crystals of reduced E. coli NrfA co-ordinate SO$_3^{2−}$ (HSO$_3^−$) in a manner indistinguishable from that in the W. succinogenes enzyme, as expected from the extensive homology between these enzymes. That crystals of E. coli NrfA contain reduced catalytically competent enzyme may be explained by a turnover number that, at the cryogenic temperatures of the X-ray diffraction, must be significantly lower than that of 0.03 sulfite molecules reduced every second at room temperature. However, other interpretations cannot be excluded. It may be that SO$_2^−$, present in equilibrium with the anionic forms SO$_3^{2−}$ (HSO$_3^−$) in aqueous solution, is the true substrate of NrfA and that the anionic forms bind preferentially under the conditions of crystallization. Equally it may be that the crystallized forms do not lie on the catalytic pathway, as has been observed for other enzymes with relatively small anionic substrates [36].

In the present study information on sulfite binding to solutions of E. coli NrfA was provided by optical spectroscopies and complemented by information on frozen solutions afforded by EPR spectroscopy. Each of these spectroscopies indicated that sulfite bound to oxidized NrfA at, or in close proximity to, the high-spin lysine residue co-ordinated ferric haem of the active site. It is less certain that binding reflects sulfur ligation of the ferric haem as found in the co-crystals. Both EPR and optical spectroscopies showed that the active-site haem remains in a high-spin ferric state when sulfite is bound to NrfA. The MCD between 600 and 700 nm of as-prepared and sulfite-bound NrfA are very similar. This region of the spectrum is diagnostic of the chemical nature of the ligands to high-spin ferric haem and the troughs that are observed from as-isolated NrfA are consistent with a mixed population of haem with lysine–hydroxide and lysine–water ligation, in line with the crystal structure [4]. We have not been able to find literature precedent for spectroscopic characterization of a high-spin ferric c- or b- haem with axial ligation from nitrogen and sulfur. However, a discernable change in the MCD spectrum between 600 and 700 nm is expected if sulfur replaces oxygen as a haem ligand to give axial co-ordination as resolved by the crystal structure. Instead, the spectra are more readily explained by retention of nitrogen and oxygen ligation. This may arise from a small displacement of the overlapping derivatives from lysine–hydroxide and lysine–water co-ordinated haem groups if sulfite binds in the vicinity of the active-site-leaving water/hydroxide as axial ligand(s). Alternatively sulfite may bind the active-site haem through an oxygen atom. In either case, reduction of NrfA must be accompanied by reorganization within the active site if sulfur ligation of the active-site haem is a requirement for sulfite reduction.

The affinity of the active site for sulfite decreases on reduction of NrfA, as summarized in Figure 3. However, the decrease is minor when compared with that for other anions [34]. Thiocyanate, nitrate and azide bind oxidized NrfA with dissociation constants in the order of 42, 73 and 38 μM respectively, which is comparable with that of 54 μM for sulfite. On reduction of NrfA the affinities for thiocyanate, nitrate and azide fall 50-, 140- and 380-fold, whereas that for sulfite falls only 3-fold. Thus it appears that NrfA has a mechanism to retain sulfite within its active site when it accumulates the reducing equivalents needed for reduction of this molecule. This mechanism may be reflected in the changes of co-ordination within the active site described above and that presumably contrast with the modes of binding to thiocyanate, nitrate and azide. The $K_m$ for sulfite reduction of $\sim 70 \mu$M is comparable with the dissociation constants describing sulfite binding to the active site of NrfA in its oxidized, semi-reduced and reduced states. Consequently, the $K_m$ is effectively a measure of the affinity of sulfite for the active site and the same is likely to be true for the other NrfA substrates, nitrite and nitric oxide, for which the $K_m$ values are 30 and 300 μM respectively under comparable conditions [8]. Thus sulfite may well compete effectively with nitric oxide and also nitrite for binding to E. coli NrfA in vivo, for example in blood plasma where the concentration of sulfite may rise to 30 μM and exceed that of nitrite at $\sim 1 \mu$M [37,38]. Assessing the impact of exogenous sulfite on rates of nitrite respiration and nitric oxide detoxification was beyond the scope of the present study. However, the finding that NrfA confers resistance to exogenous sulfite during anaerobic growth of E. coli demonstrates that interactions between NrfA and sulfite are possible in the cellular context. It is likely that the sulfite reductase activity of NrfA, rather than a capability to sequester sulfite, underpins this finding as growth of E. coli is restored after several hours of incubation with sulfite, which may be detoxified via its removal from the culture by precipitation as an inorganic sulfide or conversion into the volatile gas hydrogen sulfide.

Finally, we note that bioinformatic and structural studies have shown the NrfA enzymes to be highly homologous [1]. Many of the amino acid residues defining not only the active sites, but also the channels linking these sites to the protein surfaces, are identical. Thus the quantitative descriptions of the interactions between sulfite and the active site of E. coli NrfA should prove to be applicable to many, if not all, members of the NrfA family. It is difficult to predict whether the second site for sulfite binding to E. coli NrfA, as revealed by mixed-inhibition of nitrite reduction at low potentials, will be found in other members of the enzyme family. We were unable to provide unambiguous resolution of this secondary sulfite-binding site, although crystallographic resolution of sulfite bound to the surface of reduced NrfA near a low potential histidine–histidine co-ordinated haem is one possibility. It is hoped that in the future PFV of the NrfA enzymes from other organisms and/or site-specific engineering of E. coli NrfA will resolve this. For the moment it is worth noting that the catalytic wave describing reduction of 5 μM nitrite changes from a peaked response to one containing two sigmoidal increases of activity as sulfite is titrated into the experiment. The same change of wave shape is seen when nitrite is titrated into such an experiment, although it is accompanied by an increase rather than a decrease in the magnitude of the response (Supplementary Figure S4 at http://www.BiochemJ.org/bj431/bj4310073add.htm) [12]. These similarities may arise because nitrite, in addition to sulfite, can bind to the site on NrfA that is revealed on full reduction of the enzyme.

**AUTHOR CONTRIBUTION**

Gemma Kemp, Thomas Clarke, Sophie Marritt, Colin Lockwood, Susannah Poock and Andrew Hemmings performed experiments and participated in data analysis. Julea Butt,
Myles Cheesman and David Richardson provided scientific guidance and financial support. Julea Butt planned and prepared the manuscript.

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SUPPLEMENTARY ONLINE DATA

Kinetic and thermodynamic resolution of the interactions between sulfite and the pentahaem cytochrome NrfA from Escherichia coli

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Figure S1 Analysis of sulfite binding to NrfA by electronic absorbance spectroscopy

Top panel: electronic absorbance spectra of NrfA (5.3 μM) equilibrated with 0 (thin solid line), 25, 100, 250 and 2500 (thick solid line) μM sulfite. The buffer was 50 mM Hepes, pH 7.0, containing 2 mM CaCl2 and spectra are corrected for dilution on addition of sulfite. Middle panel: difference spectrum from that of the sulfite–NrfA complex (2500 μM sulfite to 5.3 μM NrfA) minus that of NrfA. Bottom panel: intensity change at 410 nm as function of sulfite concentration (○) with the line showing the behaviour predicted for reversible binding of a single sulfite molecule to a single NrfA with a dissociation constant of 23 μM.

Figure S2 Photoreduction of NrfA crystals on beamline ID-14 at the ESRF

Visible spectrum of mounted NrfA crystal at 100 K as isolated (black line) and after 70 s of unattenuated X-rays from the ID-14 beamline at the ESRF (grey line).

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The refined co-ordinates for the E. coli NrfA–sulfite complex have been deposited in the PDB under code 3L1T.
Figure S3  Active site of reduced E. coli NrfA with sulfite bound at 2.3 Å resolution

(A) The |Fo–Fc| omit map contoured at 5σ is shown in green (calculated using the experimentally determined structure factor amplitudes with phases calculated for the final structure minus the active-site sulfites). The structure shown is the final experimentally determined structure with the active-site haem, sulfite and active site residues shown in stick representation. The broken lines indicate hydrogen bonds with distances less than 3.1 Å.

Figure S4  PFV of NrfA nitrite reduction in 1 mM nitrite (solid lines) and 5 μM nitrite with 1 mM sulfite (broken lines)

Experimental conditions: scan rate of 30 mV·s⁻¹; rotation rate of 3000 rev/min; temperature of 20°C; and buffer consisting of 50 mM Hepes, pH 7.0, containing 2 mM CaCl₂.
### Table S1  X-ray data collection and refinement statistics

Values in parentheses indicate the highest resolution shell. $R = |F_o - F_c|/ F_o$. $R_{	ext{crys}}$ is calculated with the 95% of data used during refinement. $R_{\text{free}}$ is calculated with a 5% subset of data not used during refinement. The protein atoms number includes atoms in residues with dual conformations observable at 2.3 Å resolution. RMSD, root mean square deviation.

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