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Prosthetic groups of the NADH-dependent nitrite reductase from *Escherichia coli* K12

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A substantially improved purification of *Escherichia coli* NADH-dependent nitrite reductase was obtained by purifying it in the presence of 1 mM-NO_2^- and 10 μM-FAD. The enzyme was obtained in 20% yield with a maximum specific activity of 1.04 kat·kg^{-1}: more than 95% of this sample subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis migrated as a single band of protein. This highly active enzyme contained one non-covalently bound FAD molecule, and, probably, 5 Fe atoms and 4 acid-labile S atoms per subunit. No FMN, covalently bound flavin or Mo was detected. The spectrum of the enzyme shows absorption maxima at 386, 455, 530 and about 575 nm with a shoulder at 480–490 nm. The Soret-band/α-band absorbance ratio is about 4 : 1. These spectral features are characteristic of sirohaem, apart from the maximum at 455 nm, which is attributed to flavin. The enzyme also catalyses the NADH-dependent reduction of horse heart cytochrome *c*, 2,6-dichlorophenol-indophenol and $K_3Fe(CN)_6$. The presence of sirohaem in *E. coli* nitrite reductase explains the apparent identity of the *cysG* and *nirB* genes of *E. coli* and inability of *hemA* mutants to reduce nitrite.

The NADH-dependent nitrite reductase (NADH–nitrite oxidoreductase, EC 1.6.6.4) of *Escherichia coli* K12 catalyses the six-electron reduction of NO_2^- to NH_4^+, 3 NADH molecules being oxidized for each NO_2^- ion reduced (Coleman *et al.*, 1978*a*). The enzyme also catalyses the two-electron reduction of hydroxylamine to NH_4^+ with the oxidation of one NADH molecule. Full activity of nitrite reductase requires the presence of NAD^+, a product of the reaction (Coleman *et al.*, 1978*b*).

The NADH-dependent nitrite reductase has been purified in this laboratory to better than 95% homogeneity from a chlorate-resistant mutant of *E. coli* K12, OR75Chl5 (Coleman *et al.*, 1978*a*). Yields and specific activities of the purified enzyme were low, however. The enzyme was apparently a dimer of two identical or similar-sized subunits with a subunit M_r 88000 as estimated by SDS/polyacrylamide-gel electrophoresis. Further studies have now enabled us to stabilize the enzyme *in vitro* and to obtain pure preparations with far higher yields and specific activities. As a result, we have now obtained clear evidence for the presence of flavin, haem and iron–sulphur components as prosthetic groups of the enzyme.

Abbreviation used: SDS, sodium dodecyl sulphate.

Experimental

Buffers

All buffers were based on TE buffer (50 mM-Tris/HCl/5 mM-EDTA, pH 8.0). This was supplemented with 5 mM-ascorbate (TEA buffer), 5 mM-ascorbate and 1 mM-NO_2^- (TEA/NO_2^- buffer), 5 mM-ascorbate, 1 mM-NO_2^- and 10 μM-FAD (TEA/NO_2^-/FAD buffer), 1 mM-NO_2^- (TE/NO_2^- buffer) or 10 μM-FAD (TE/FAD buffer). Buffers were prepared at room temperature (18–20°C) and degassed before use.

Bacteria

Escherichia coli strain OR75Ch15 was grown essentially as described in Coleman *et al.* (1978*a*). Bacterial extracts were prepared as described there, except that the bacteria were suspended in TEA/NO_2^- buffer and the preliminary centrifugation was omitted. The pellet after centrifugation at 75000 ***g*** was resuspended in TEA/NO_2^- buffer and passed through the French pressure cell a second time as if it were a fresh bacterial pellet.

In later experiments, cultures were supplemented with 1 μM-$(NH_4)_6Mo_7O_{24}$, 1 μM-Na_2SeO_3 and 1 ml of a sulphur-free trace salts solution (Cole *et al.*, 1974)/l, because these additions caused the yield of

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nitrite reductase to be doubled (to 33 μkat per 100g of cells).

Preparation of enzyme for chemical analyses and spectra

Enzyme from the DEAE-Sephadex pool was precipitated by 50% saturation with $(NH_4)_2SO_4$ at pH8.0 and 4°C. It was redissolved in TEA/NO_2^- buffer (or TE/NO_2^- buffer in the case of flavin analyses) and desalted on a Sephadex G-25 column (17cm × 1.2cm diam.) equilibrated with the same buffer. The enzyme was prepared in the same way for measurement of the NADH-dependent 'diaphorase' activity except that the buffer was TE/FAD buffer containing 50% (w/v) glycerol and the enzyme was stored at −20°C.

Measurement of the rate of NADH-dependent reduction of NO_2^- and hydroxylamine

Nitrite reductase and hydroxylamine reductase activities were assayed by the method of Coleman *et al.* (1978*a*), except that the concentration of hydroxylamine was 100mM rather than 10mM, so that the enzyme was nearly saturated by hydroxylamine.

When NO_2^- or hydroxylamine was present as a stabilizing agent in the enzyme stock solution, the rate of NADH oxidation in the absence of oxidized substrate was determined from the approximately linear rate obtained after complete reduction of NO_2^- or hydroxylamine. In the absence of added NO_2^- or hydroxylamine the rate of NADH oxidation was determined as previously (Coleman *et al.*, 1978*a*).

One katal of nitrite reductase activity is defined as the amount of enzyme that catalyses the oxidation of 3mol of NADH in 1s. This unit is equivalent to 1.8×10^{11} of the units defined by Coleman *et al.* (1978*a*).

Measurement of NADH-dependent 'diaphorase' activities

Open sample and reference cuvettes (1.5ml) contained 1.0ml of 0.2mM-NADH, 1mM-NAD^+ and 0.1mM electron acceptor in TE buffer at 30°C. The reaction was initiated by addition of enzyme to the sample cuvettes. Diaphorase activities were calculated from the rate of change of absorbance at 340, 550 or 600nm when $K_3Fe(CN)_6$, horse heart cytochrome *c* or 2,6-dichlorophenol-indophenol respectively was the electron acceptor. Rates were corrected for change in absorbance in the absence of electron acceptor.

Determination of protein and cofactors etc.

Protein concentrations were determined by the micro tannin turbidometric procedure of Mejbaum-Katzenellenbogen & Dobryszycka (1959), with bovine serum albumin (10–100 $\mu g \cdot ml^{-1}$) as standard.

Non-covalently bound FAD and FMN in purified extracts were determined as described by Faeder & Siegel (1973). Covalently bound flavin was assayed by the method of Singer *et al.* (1971).

Iron was assayed by the bathophenanthroline method of van de Bogart & Beinert (1967), with the use of water that had been distilled twice in all-glass apparatus.

The acid-labile sulphur content of purified extracts was determined by the method of King & Morris (1967), except that the enzyme sample (0.7ml) was preincubated for 1h with 0.1ml of 1.5M-NaOH and 0.5ml of 0.2M-zinc acetate before the addition of the *NN'*-dimethyl-*p*-phenylenediamine reagent.

Molybdenum in purified extracts was assayed by the method of Johnson & Arkley (1954).

Spectra were recorded with a Perkin–Elmer 356 double-beam spectrophotometer.

Polyacrylamide-gel electrophoresis and assessment of purity of purified samples

Proteins were separated by electrophoresis in the presence of SDS. The discontinuous buffer system of Laemmli (1970) was used, with a 5.5% (w/v) stacking gel and a 9% (w/v) resolving gel. Electrophoresis was performed as described by Newman *et al.* (1980), but the concentration step was omitted.

Gells were stained with Coomassie Brilliant Blue R, scanned with a Zeineh laser scanning densitometer (T. and J. Crump, Rayleigh, Essex, U.K.) and the quantity of each polypeptide was estimated from the area under the peak on the scan. A minimum of two tracks was scanned in duplicate for each purified sample.

Proteins were also separated by electrophoresis in the presence of 8M-urea by the procedure of Perrie & Perry (1970), with an 8%-acrylamide (w/v) polyacrylamide gel.

Results

Stability studies

Various chemicals were tested for their ability to stabilize nitrite reductase so that the low specific activity and poor yield reported by Coleman *et al.* (1978*a*) might be improved. The oxidized substrates 1mM-NO_2^- and 50mM-hydroxylamine were equally effective in decreasing the loss of activity after 24h at 4°C from 40% to 10%. Contrary to the previous report by Kemp & Atkinson (1966), no increase in catalytic activity was detected when enzyme was preincubated for 15min with either of these substrates, even when NAD^+ was omitted from the assay mixture. Nitrite and hydroxylamine therefore increase the stability of the enzyme rather than activate it. A further increase in stability was

observed when the TEA/NO_2^- buffer was supplemented with 10μM-FAD, but the addition of FAD to TEA buffer was insufficient for full stability to be maintained.

Purification of nitrite reductase from cell-free extracts

Nitrite reductase was purified by a modification of the method described by Coleman *et al.* (1978*a*), in which TEA buffer was supplemented with 1mM-NO_2^- and 10μM-FAD (Table 1). The DEAE-cellulose (DE-52; Whatman Biochemicals, Maidstone, Kent, U.K.) column (28cm × 4.8cm diam.) was washed with 200ml of 0.12M-KCl in TEA/NO_2^-/FAD buffer and eluted with a 1680ml linear gradient of 0.13–0.35M-KCl in TEA/NO_2^-/FAD buffer. The DEAE-Sephadex A-50 column (24cm × 4.8cm diam.) was washed with 200ml of 0.2M-KCl in TEA/NO_2^-/FAD buffer and eluted with a 1200ml linear gradient of 0.25–0.65M-KCl. The final yield and specific activity were about 20% and 0.5–1.05 kat·kg^{-1}, compared with 0.4% and 0.028 kat·kg^{-1} obtained by Coleman *et al.* (1978*a*). More than 95% of the sample (DEAE-Sephadex pool; Table 1) subjected to SDS/polyacrylamide-gel electrophoresis migrated as a single band of protein. The molar catalytic activity is therefore 195 mol of NO_2^- reduced·(mol of dimer)$^{-1}$·s^{-1}. Throughout the paper statements about purity refer to estimates from the behaviour in SDS/polyacrylamide-gel electrophoresis.

Subunit structure of nitrite reductase

Only one type of subunit was detected by Coleman *et al.* (1978*a*) after SDS/polyacrylamide-gel electrophoresis. In some samples, however, we have since noted two bands each with M_r about 88000, differing in M_r by 700–1000. The ratio of intensities of the upper to the lower band in one sample that had been dissociated in SDS/mercaptoethanol/Tris/HCl buffer, pH6.8, for 16h at 37°C was 1:1. This ratio for the same sample that had been dissociated at 100°C for 10min was 1:1.9. This behaviour is consistent with the occurrence of nitrite reductase both with and without a prosthetic group. Both FAD and sirohaem have M_r in the range 750–1000. Furthermore other preparations have given only a single band after SDS/polyacrylamide-gel and urea/polyacrylamide-gel electrophoresis.

Flavin

Okuda *et al.* (1979) reported that the substrates of D-amino acid oxidase and β-glucose oxidase inhibit the dissociation of FAD from these enzymes. Nitrite reductase that had been purified in TEA/NO_2^- buffer contained 0.45–0.75 mol of FAD per mol of subunit; in contrast, samples purified by Coleman *et al.* (1978*a*) in the absence of NO_2^- contained only 0.2 mol of FAD per mol of subunit. Two samples of nitrite reductase that had been separately purified in TEA/NO_2^-/FAD buffer contained 0.92 and 0.95 mol of FAD per mol of subunit. Neither FAD nor FMN was detected in fractions eluted on both sides of the nitrite reductase peak from the Sephadex G-25 column: the FAD that was eluted with nitrite reductase is therefore bound specifically. These samples were 85% and 92% pure respectively, but no contaminating polypeptide was present at a concentration greater than 2.5% of nitrite reductase. Similar results were obtained with two slightly less pure preparations.

In the most highly purified samples tested 0.02 mol of FMN was present per mol of subunit. Thus FMN is unlikely to be associated with nitrite reductase, and these low concentrations observed may be due to inaccuracies in the method of assay. Less than 0.05 mol of covalently bound FAD and less than 0.05 mol of covalently bound FMN were present per mol of subunit. Thus nitrite reductase contains one non-covalently bound FAD molecule per subunit, but no covalently bound flavin.

Haem component

A haem signal has been detected spectroscopically in five separate purified extracts of nitrite reductase (Fig. 1). The Soret band at 386nm [$\varepsilon = 63000\,cm^{-1}\cdot$(M-subunit)$^{-1}$], the α-band at 573–578nm [$\varepsilon = 16000\,cm^{-1}$. (M-subunit)$^{-1}$] and the Soret-band/$\alpha$-band absorbance ratio of about 4:1

Table 1. *Purification of nitrite reductase activity from E. coli strain K12 OR75Chl5*

Nitrite reductase activities were determined at 30°C with 1mM-NAD^+ and 0.25mM-NADH. Experimental details are summarized in the text.

Step	Volume (ml)	Total protein (mg)	Total activity (μkat)	Yield (%)	Specific activity (kat·kg^{-1})	Purification (fold)
Crude extract	298	3220	42.0	100	0.0087	1
$(NH_4)_2SO_4$ fractionation	54	1250	42.8	102	0.0357	4.1
DEAE-cellulose	373	112	23.5	56	0.214	24.7
$(NH_4)_2SO_4$ fractionation	22.6	32	19.4	46	0.617	71.0
DEAE-Sephadex	128	8.3	8.6	22	1.04	121.0

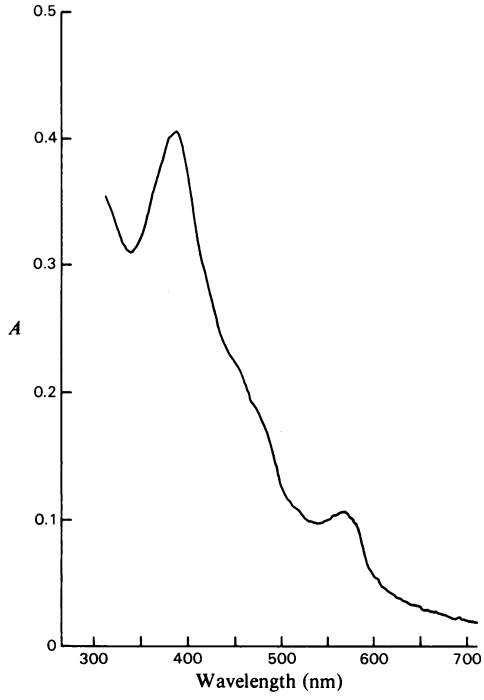


Fig. 1. *Absorbance spectrum of nitrite reductase*
Enzyme (92% pure) from the DEAE-Sephadex pool was precipitated by 50% saturation with $(NH_4)_2SO_4$, redissolved in TEA/NO_2^- buffer and desalted on a Sephadex G-25 column (17 cm × 1.2 cm diam.). The protein concentration was 0.6 mg · ml^{-1} and the concentration of nitrite reductase monomer was 6.3 μM. No contaminant subunit was present at a concentration greater than 0.1 μM. The reference cuvette contained TEA/NO_2^- buffer.

are consistent with the spectral properties of other sirohaem-containing enzymes (Siegel *et al.*, 1973; Vega *et al.*, 1975; Hucklesby *et al.*, 1976; Vega & Kamin, 1977). A peak at 455 nm and a shoulder at 480–490 nm are due to FAD associated with the enzyme. The absorption maxima of the enzyme in TE buffer and of the enzyme in TEA/NO_2^- buffer (Fig. 1) are at the same wavelengths. Assuming that the molar absorbance coefficient at 386 nm of enzyme-bound sirohaem is 7.6×10^4 cm^{-1} · (M-haem) (as for spinach nitrite reductase; Lancaster *et al.*, 1979) and of FAD at 386 nm is 3.6×10^3 $cm^{-1} \cdot M^{-1}$, these preparations contain 0.52–0.78 mol of sirohaem per mol of subunit.

No haem signal was detected in preliminary studies with enzyme that had frozen and thawed (Jackson *et al.*, 1979). However, after freezing and thawing, nitrite reductase loses its original orange–red colour and the Soret-band absorbance decreases by 90%. The orange–red colour was also rapidly lost at 4°C in the absence of NO_2^-. A rapid loss in absorbance was noted for sirohaem extracted from the NADPH-dependent sulphite reductase of *E. coli* (Siegel *et al.*, 1973), especially in aqueous neutral or alkaline solution.

Metal determination

Samples of enzyme were prepared for iron analysis by exhaustive dialysis against 50 mM-Tris/HCl buffer, pH 8.0, containing 0.1 mM-EDTA and 1 mM-NO_2^-, the last dialysis being against buffer prepared in double-distilled water to remove adventitious iron. Enzyme that had been frozen before dialysis contained 3.5 and 3.9 Fe atoms per subunit (corrected for degree of purity) for 85%-pure and 86%-pure samples respectively. In contrast, 4.5, 4.7 and 4.8 Fe atoms per subunit (corrected) were found in 92%-pure, 86%-pure and 72%-pure samples respectively that had not been frozen. These results are consistent with the hypothesis that nitrite reductase contains 5 Fe atoms per subunit and that haem iron dissociates on freezing and thawing.

A sample of nitrite reductase (1.5 ml containing 10.5 nmol of monomer) was analysed for Mo; none was detected.

Acid-labile sulphur

Two separately purified 85%-pure samples of nitrite reductase contained 3.5 and 3.7 labile S atoms per subunit. No contaminant polypeptide was present at a concentration greater than 3.5% of nitrite reductase.

NADH-dependent 'diaphorase' activities

Nitrite reductase (90% pure) catalysed the NADH-dependent reduction of the one-electron acceptors horse heart cytochrome *c*, 2,6-dichlorophenol-indophenol and $K_3Fe(CN)_6$. Various inhibitors were preincubated with nitrite reductase for 5 min before assay and were also added to assay mixtures. The reduction of cytochrome *c*, $K_3Fe(CN)_6$ and the two-electron acceptor hydroxylamine was completely inhibited (more than 99%) by 20 μM-*p*-chloromercuribenzoate, but, although 1 mM-KCN completely inhibits hydroxylamine reduction, it does not inhibit the reduction of $K_3Fe(CN)_6$ and only decreased the rate of cytochrome *c* reduction by 12%. Hence the NADH-dependent reduction of cytochrome *c* and $K_3Fe(CN)_6$ is not haem-dependent but requires at least one SH group/subunit.

Discussion

E. coli NADH-dependent nitrite reductase contains one non-covalently bound FAD and probably 5 Fe atoms and 4 acid-labile S atoms per subunit (M_r 88000) and also a haem component with the spectral characteristics of sirohaem. If 1 Fe atom per

subunit is associated with haem, it is likely that the remaining 4 non-haem Fe atoms and 4 acid-labile S atoms are associated in either two binuclear or one tetranuclear iron–sulphur centre(s). It is as yet unclear whether there are one or two polypeptide chains per sirohaem prosthetic group.

Sirohaem is a highly polar haem, an octacarboxyl isobacteriochlorin (Murphy *et al.*, 1973) whose structure was fully elucidated by Scott *et al.* (1978). Sirohaem is derived from uroporphyrinogen III, the first tetrapyrrole in the biosynthetic pathway of haem, by methylation of two adjacent rings, oxidation and iron insertion (Scott *et al.*, 1978). It has been identified as a prosthetic group of nitrite reductase and sulphite reductase, both of which catalyse six-electron transfer reactions (Table 2). The sirohaem-containing nitrite reductases of plants and fungi are assimilatory enzymes. However, the synthesis of the NADH-dependent nitrite reductase in *E. coli* is induced by nitrite and apparently repressed by oxygen but not by ammonia (Cole *et al.*, 1974). The role of the enzyme therefore is probably to reoxidize reduced nicotinamide nucleotides that limit the rate of fermentative growth. *Achromobacter fischeri* nitrite reductase is the only other bacterial enzyme that catalyses the reduction of NO_2^- to NH_4^+ to be purified and characterized: it contains haem *c* (Prakash & Sadana, 1972). Enzymes involved in the dissimilation of nitrite to gaseous products (NO, N_2O, N_2) do not contain the sirohaem prosthetic group. Sirohaem has been identified as the binding site for nitrite in the nitrite reductase of *Neurospora crassa* (Vega *et al.*, 1975) and spinach (*Spinacia oleracea*) (Lancaster *et al.*, 1979). Studies with vegetable-marrow (*Cucurbita pepo*) nitrite reductase suggest that, during the catalytic cycle, nitrite will only bind to the reduced haem (Cammack *et al.*, 1978).

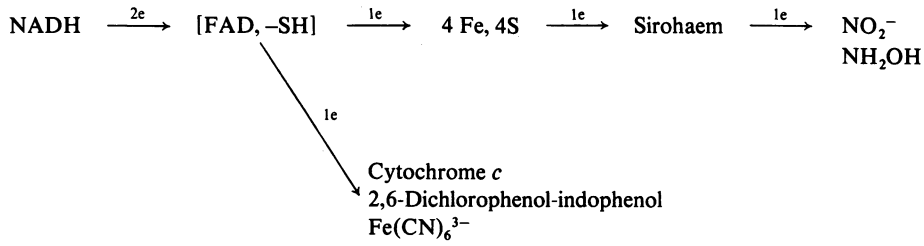
The presence of haem explains the complete inhibition of NADH-dependent nitrite reduction by 1mM-KCN and its partial inhibition by 1mM-sulphite (Coleman *et al.*, 1978*a*). Both NADH-dependent nitrite reduction and the NADH-dependent 'diaphorase' activities are completely inhibited by 20μM-*p*-chloromercuribenzoate. This inhibitor reacts with thiol groups and with acid-labile sulphide (Malkin, 1973). Hence an iron–sulphur centre or a thiol group (or groups) or both are essential for the NADH-dependent reduction of nitrite and 'diaphorase' acceptors. The 'diaphorase' activities, however, are only slightly inhibited by 1mM-KCN, showing that they are independent of the haem. The slight inhibition of NADH-dependent reduction of cytochrome *c* may be due to shifts of the redox potentials of prosthetic groups on the binding of CN^- to the haem. The minimal pathway of electron flow shown in Scheme 1 can therefore be proposed. We have no evidence for the position of the iron–sulphur centre(s) in the sequence of electron flow, and it is moreover possible that it is the final electron donor for the diaphorase activity.

The *Neurospora crassa* NADPH-dependent nitrite reductase shows striking similarities to the *E. coli* NADH-dependent enzyme, as it has exactly the same complement of prosthetic groups (Table 2) as well as a thiol group essential for NADPH-depen-

Table 2. *Properties of enzymes containing sirohaem*

Organism	Electron donor	Electron acceptor	Other prosthetic groups	References
Plants				
Spinacia oleracea	Ferredoxin*	NO_2^-*	Fe_4S_4*	Aparicio *et al.* (1975)
		NH_2OH		Vega & Kamin (1977)
				Lancaster *et al.* (1979)
Cucurbita pepo	Ferredoxin*	NO_2^-*	Fe_4S_4*	Hucklesby *et al.* (1976)
		NH_2OH		Hucklesby *et al.* (1978)
Chlorella fusca	Ferredoxin*	NO_2^-*		Zumft (1972)
Fungi				
Neurospora crassa	NADPH*	NO_2^-*	FAD	Lafferty & Garrett (1974)
	NADH	NH_2OH	5Fe, 4S	Vega *et al.* (1975)
Saccharomyces cerevisiae	NADPH*	SO_3^{2-}*	FAD, FMN	
		NH_2OH	Iron–sulphur centre(s)	Yoshimoto & Sato (1968)
Bacteria				
Escherichia coli	NADPH*	SO_3^{2-}*	α-Subunit Fe_4S_4*	Siegel *et al.* (1973)
		NO_2^-	β-Subunit $\frac{1}{2}$FAD, $\frac{1}{2}$FMN	Siegel (1978)
		NH_2OH		
Escherichia coli	NADH*	NO_2^-*	FAD, 5Fe, 4S	Present study
		NH_2OH		
Desulfovibrio gigas	Ferredoxin*	SO_3^{2-}*	Fe_4S_4*	Murphy & Siegel (1973)
		NO_2^-		Hall *et al.* (1979)

* Physiological donor or acceptor.



Scheme 1. *Possible pathway for electron flow in nitrite reductase*
The position of the non-haem iron–sulphur centre relative to the flavin and sirohaem is uncertain, as is the existence of an independent and labile thiol associated with the FAD.

dent 'diaphorase' activity (Garrett, 1978). It is also a homodimer, though its subunits (M_r 145000) are considerably larger than those of the *E. coli* enzyme. Product activation has not been reported for the *N. crassa* enzyme.

The presence of sirohaem in both nitrite reductase and sulphite reductase from *E. coli* provides an explanation for the apparent identity of the *cysG* and *nirB* genes (Cole *et al.*, 1980). Mutants defective in the *cysG* gene were isolated on the basis of their inability to reduce sulphite to sulphide (Jones-Mortimer, 1968), and *nirB* mutants on their ability to reduce NO_2^- to NH_4^+ (Newman & Cole, 1978). Results of experiments to map the *nirB* gene relative to the *cysG* gene suggested that the two genes were indistinguishable: the *cysG* and *nirB* mutations were 100% co-transducible and they reverted simultaneously. It is therefore probable that the *cysG*-gene product catalyses one of the reactions on the biosynthetic pathway from uroporphyrinogen III to sirohaem, possibly a methylation, as oxidation and iron insertion need not involve enzymes specific for sirohaem biosynthesis. The inability of *hemA* mutants to reduce nitrite is also explained, because 5-aminolaevulinate synthase would be required for sirohaem synthesis.

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