Siroheme: A Prosthetic Group of the Neurospora crassa Assimilatory Nitrite Reductase*

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The Neurospora crassa assimilatory nitrite reductase (EC 1.6.6.4) catalyzes the NADPH-dependent reduction of nitrite to ammonia, a 6-electron transfer reaction. Highly purified preparations of this enzyme exhibit absorption spectra which suggest the presence of a heme component (wavelength maxima for oxidized enzyme: 390 and 578 nm). There is a close correspondence between nitrite reductase activity and absorbance at 400 nm when partially purified nitrite reductase preparations are subjected to sucrose gradient centrifugation. In addition, a role for an iron component in the formation of active nitrite reductase is indicated by the fact that nitrate-induced production of nitrite reductase activity in Neurospora mycelia in vivo requires the presence of iron in the induction medium.

The heme chromophore present in Neurospora nitrite reductase preparations is reducible by NADPH. Complete reduction, however, requires the presence of added FAD. The NADPH-nitrite reductase activity of the enzyme is also dependent upon addition of FAD. A spectrally unique complex is formed between the heme chromophore and nitrite (or a reduction product thereof) when nitrite is added to NADPH-reduced enzyme.

Carbon monoxide forms a complex with the heme chromophore of nitrite reductase with an intense α-band maximum at 590 nm and a β-band of lower intensity at 550 nm. CO is an inhibitor of NADPH-nitrite reductase activity. Spectrophotometrically detectable CO complex formation and CO inhibition of enzyme activity share the following properties. (a) Both require the presence of a reducing agent (NADPH). (b) Both are incomplete unless FAD is added in addition to NADPH. (c) At 0.83 mM CO, both exhibit the same kinetics, with an apparent second order rate constant for enzyme·CO complex formation of 9 M⁻¹s⁻¹ when both NADPH and FAD are present. (d) Both can be reversed by passage of CO-treated enzyme through a column of Sephadex G-25. (e) Each can be both prevented and reversed by addition of nitrite to CO-treated enzyme. Agents which behave as competitive inhibitors (with respect to nitrite) of nitrite reductase activity, such as cyanide, sulfite, and arsenite, can partially protect the enzyme from inhibition by CO, a result which indicates that CO and these agents (and presumably nitrite) share a common enzymatic site.

The heme chromophore was extracted from Neurospora nitrite reductase with acetone/0.015 M HCl. The extracted heme was found to be identical with siroheme (extracted from Escherichia coli NADPH-sulfite reductase) by each of the following criteria: (a) spectrum of extracted heme in pyridine; (b) spectrum of CO complex of extracted heme (this spectrum is qualitatively similar to that of the CO complex of nitrite reductase itself); (c) chromatographic behavior of the extracted heme; and (d) fluorescence excitation and emission spectra of the porphyrin cation derived from the heme following removal of iron.

These results demonstrate that Neurospora NADPH-nitrite reductase contains siroheme as a prosthetic group, and that the siroheme component functions in the enzymatic catalysis of nitrite reduction. The results suggest that siroheme may actually provide the site of interaction between nitrite reductase and nitrite. The following scheme of electron flow is proposed for the Neurospora NADPH-nitrite reductase:

NADPH → FAD → Siroheme → Nitrite

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Assimilatory nitrite reductases catalyze the following 6-electron transfer reaction:

\[
\text{NO}_3^- + 8 \text{H}^+ + 6 e^- \rightarrow \text{NH}_4^+ + 2 \text{H}_2\text{O}
\]

The electron donor in plants and algae appears to be ferredoxin (1-5), while in fungi and some bacteria the donor is reduced pyridine nucleotide (6-11). Plant ferredoxin-nitrite reductases have been purified to near homogeneity (2, 3, 12-14). These enzymes have molecular weights of approximately 60,000 and exhibit absorption spectra which suggest that they are hemo-proteins. Murphy et al. (15) have recently identified the heme moiety of spinach ferredoxin nitrite reductase as "siroheme," an iron tetrahydroporphyrin of the isobacteriochlorin type with eight carboxylic acid-containing side chains. The structure of siroheme proposed by Murphy et al. (16) is shown in Fig. 1. No direct evidence was presented, however, which implicated the heme moiety in the catalytic mechanism of spinach nitrite reductase.

Siroheme was originally identified as a prosthetic group of *Escherichia coli* NADPH-sulfite reductase (17). This novel heme has subsequently been found in several other bacterial sulfite reductases (18, 19), all of which catalyze the following 6-electron transfer reaction:

\[
\text{SO}_4^{2-} + 8 \text{H}^+ + 6 e^- \rightarrow \text{H}_2\text{S} + 3 \text{H}_2\text{O}
\]

Siegel et al. (20) have shown that a number of agents which bind to the heme (as determined by spectral perturbations), such as CO, cyanide, and arsenite, are potent inhibitors of sulfite reduction catalyzed by the *E. coli* enzyme. Kinetic data of CO binding to and dissociation from the enzyme-bound siroheme have been directly correlated with the kinetics of inhibition and restoration of sulfite reductase activity. In addition, sulfite binds tightly to the *E. coli* enzyme under appropriate conditions, and this binding is associated with marked changes in the spectral properties of the heme (20). Thus, siroheme appears to be the site of interaction between sulfite reductase and sulfite.

*E. coli* sulfite reductase, like a number of other sulfite reductases from bacteria and fungi (20-25), can utilize nitrite as an alternate electron acceptor to sulfite and catalyze the reduction of nitrite to ammonia. Nitrite does not appear to be reduced by these enzymes in *vivo*, however, probably because the \( K_m \) values of the sulfite reductases for nitrite are too high to permit nitrite to serve as a substrate under physiological conditions (22). Organisms which can assimilate both sulfate and nitrate appear, then, to possess physically and (in *E. coli* and Neurospora crassa (22, 24) at least) genetically distinct sulfite and nitrite reductases. On the other hand, the presence of siroheme in sulfite reductases and in at least one nitrite reductase, taken together with the ability of a number of sulfite reductases to reduce nitrite in a 6-electron transfer reaction, suggests that there may be a common functional role for siroheme in the mechanism of sulfite and nitrite reduction.

Recently, Lafferty and Garrett (7) have reported the partial purification of a pyridine nucleotide-dependent nitrite reductase from *N. crassa*. The enzyme, molecular weight 290,000, requires added FAD to utilize either NADPH or NADH as electron donor, although the enzyme can use the artificial dye reduced benzyl viologen1 in the absence of FAD. Studies on the effects of mercurials and of heat on the NAD(P)H- and reduced benzyl viologen-nitrite reductase activities indicated that the sites of interaction of *Neurospora* nitrite reductase with nitrite and with pyridine nucleotide are not identical. Cyanide and sulfite served as competitive inhibitors of nitrite reduction. The absorption spectrum of the purified nitrite reductase was suggestive of a hemoprotein.

In the present investigation, we have attempted to examine the nature of the heme-like chromophore associated with *Neurospora* nitrite reductase and a possible role for the chromophore in catalysis. The results of this study are: (a) *Neurospora* nitrite reductase is a hemoprotein; (b) the heme prosthetic group is siroheme; and (c) the enzyme-bound siroheme functions in catalysis of nitrite reduction by the enzyme.

**EXPERIMENTAL PROCEDURE**

Materials—NADPH, NADH, and NADP+ were purchased from Calbiochem; FAD, protoporphrin, and glucose 6-phosphate from Sigma; benzyl viologen from Schwarz/Mann; Sephadex G-25 and G-200 from Pharmacia; and spectrophotometric grade acetone and pyridine from Mallinckrodt. All other chemicals were reagent grade and used as supplied.

**Growth of Mycelia—**A nitrate reductase-deficient strain of *Neurospora crassa*, nit-1 allele 34547 (Fungal Genetics Stock Center No. 54), was used in this study to eliminate the previously reported partial contamination of purified nitrite reductase with nitrate reductase, a heme-containing enzyme (25). The mycelia were cultured and grown in large amounts as described by Lafferty and Garrett (7), with the exception that the Fries medium used was modified in two ways. First, the sole sulfur source used for growth in the present study was 0.2 mM DL-methionine, a compound reported to repress production of sulfite reductase in *Neurospora* (21); and, second, sodium nitrite served as the only source of nitrogen (other than methionine) in the present study, the level of nitrite being maintained at 5 to 10 mM during the growth period by periodic addition of sodium nitrite.

**Purification of Enzymes**—NADPH-sulfite reductase was purified from *Escherichia coli* R by the procedure of Siegel et al. (17). The preparation used in these studies exhibited an \( A_{260}/A_{450} \) ratio of 3.54, a value reported to be characteristic of electrophoretically and ultracentrifugally homogeneous enzyme. To determine sulfite reductase concentration, the extinction coefficient of 3.1 x 10^4 M^-1 cm^-1 determined by Siegel et al. (17) was used.

NADP'H-nitrite reductase was purified from *N. crassa* mutant nit-1 through Step 6 of the procedure of Lafferty and Garrett (7) with the following modification: except where indicated, 10 mM FAD and 10 mM sodium dithionite were added to all buffers used in the purification, since addition of these agents markedly stabilized the enzyme during the purification process. In subsequent purification steps, the...
term "standard buffer" refers to 0.2 M potassium phosphate buffer (pH 7.5) containing 5 mM EDTA, 10 μM FAD, and 10 mM sodium dithionite. Two-milliliter aliquots of Step 6 enzyme dissolved in standard buffer containing 10% glycerol were applied to a column (60 x 3 cm) of Sephadex G-200 equilibrated with the same buffer, and gel filtration was performed at a flow rate of 10 to 15 ml per hour. Fractions of 2.5 ml each were collected. The fractions of highest specific activity were pooled and subsequently dialyzed for 3 hours versus standard buffer containing 561 g of (NH₄)₂SO₄ per l (80% saturation). The precipitated protein was collected by centrifugation and then dissolved in 1.0 to 1.5 ml of standard buffer from which dithionite and FAD had been omitted. Aliquots of this solution (0.0 ml) were carefully layered upon 11 ml of 15.5 to 30% linear gradients of sucrose in standard buffer from which dithionite and FAD had been omitted, and the gradient was then centrifuged, at 25,000 rpm for 23 hours at 4°C, in an SW 41 rotor of a Spinco L2-65B centrifuge. Fractions of approximately 0.2 ml each were collected from the bottom of each tube. The pool of fractions of highest specific activity was dialyzed versus standard buffer from which FAD and dithionite had been omitted in order to remove the dithionite. The dialysis period was kept to a minimum since the enzyme was quite unstable (NADPH-nitrite reductase activity) under these conditions. The final preparation of nitrite reductase contained 1 to 2% of the total activity of the crude extract and a specific activity of 3 to 4 units per mg of protein. Lafferty and Garrett (7) previously reported a specific activity of 2.2 units per mg of protein for Neurospora nitrite reductase. The marked instability of this enzyme makes a correlation between specific activity and degree of purity difficult.

**Protein Determination**—Protein concentrations were measured by the method of Lowry et al. (28).

**Enzyme Assays**—Benzyl viologen-nitrite reductase activity was assayed as described previously (7).

NADPH-nitrite reductase activity was measured as follows. The rate of nitrite-dependent NADPH oxidation was followed at 340 nm with a Cary model 14 spectrophotometer. The reaction mixture contained, in a total volume of 1.0 ml, 0.1 M potassium phosphate buffer (pH 7.5), 10 μM FAD, 1 mM sodium nitrate, 0.2 mM NADPH, and enzyme. Assays were performed at 25°C in cells of 1-cm path length.

Control mixtures contained buffer in place of the reactant. A unit of activity is defined as that amount of enzyme which catalyzes the oxidation of 1 μmol of NADPH per min under the given assay conditions. (Note that this is not the unit defined by Lafferty and Garrett (7); when comparisons are made between activities measured in this paper and those reported by Lafferty and Garrett (7), the activities are expressed as units defined in this paper.) Specific activity is defined as units per mg of protein.

NADPH-sulfite reductase activity was measured by the procedure used for NADPH-nitrite reductase activity, except that sulfite replaced nitrite in the reaction mixture.

NADH nitrite reductase was assayed by the same procedure as that used for NADPH-nitrite reductase, except that NADH replaced NADPH in the reaction mixture.

Glucose-6-phosphate dehydrogenase activity was measured by following the production of NADP at 340 nm in a Cary model 14 spectrophotometer. The reaction mixture contained, in a total volume of 1.0 ml, 0.07 M Tris-HCl buffer (pH 7.5), 7.5 mM MgCl₂, 20 mM glucose-6-phosphate, and 10 μM NADP⁺.

**Absorption Spectra**—Absorption spectra were measured at room temperature in silica cuvettes of 1-cm path length with a Cary model 14 spectrophotometer equipped with 0.1 nm 0.1 A and 0.1 nm 0.2 A slide wires. Reference cells contained appropriate solvent blanks in all cases.

**Concentration and Gel Filtration**—Ultrafiltration of enzyme solutions was performed with an Amicon concentrator equipped with a Diaflo PM-30 membrane. For removal of low molecular weight solutes from enzyme in ligand binding experiments, 1.0-ml samples were applied to a column (1.5 x 15 cm) of Sephadex G-25 equilibrated with 0.1 M potassium phosphate buffer (pH 7.5) and 2 ml fractions were collected.

**RESULTS**

**Formation of Nitrite Reductase Requires Iron**—The data presented in Fig. 2 indicate that the presence of iron in the growth medium is necessary for in vitro production of nitrite reductase activity in Neurospora. In this experiment, mycelia were grown on an iron-deficient medium in the presence of NH₄Cl, a nitrogen source which represses nitrite reductase activity (29). These mycelia, containing low levels of nitrite reductase activity, were then transferred to media containing NaNO₃ as sole nitrogen source and the indicated concentrations of FeCl₃. After 4 hours of incubation in the "induction medium," mycelia were harvested, washed, and crude extracts prepared as described previously (30). Each extract was assayed for NADPH-nitrite reductase activity ( ), NADH-nitrite reductase activity ( ), reduced benzyl viologen (BVH)-nitrite reductase activity ( ), and glucose-6-phosphate dehydrogenase activity ( ).

**Fe⁺⁺ ADDED (μM)**

**Fig. 2** Dependence of Neurospora nitrite reductase activity on the presence of iron in the culture medium. Mycelia were grown on iron-free Fries medium with 10 mM NH₄Cl as nitrogen source. After 48 hours, the mycelia were harvested, washed, and transferred to Fries medium containing 10 mM NaNO₃ as nitrogen source and the indicated concentrations of FeCl₃. After 4 hours of incubation in the "induction medium," mycelia were harvested, washed, and crude extracts prepared as described previously (30). Each extract was assayed for NADPH-nitrite reductase activity (○—○), NADH-nitrite reductase activity (———), reduced benzyl viologen (BVH)-nitrite reductase activity ( — — —), and glucose-6-phosphate dehydrogenase activity (Δ—Δ).
tase (7) has prevented us from achieving complete purification of this enzyme (three protein bands were observed upon electrophoresis of the purified enzyme preparation used in this paper on 5% acrylamide gels at pH 8.3), an analysis of partially purified enzyme by sucrose gradient centrifugation (Fig. 4) showed an exact correspondence between nitrite reductase activity and the absorption in the putative heme Soret region (400 nm) in individual fractions of the gradient.

Effect of Nitrite Reductase Substrates and FAD Cofactor on Spectral Properties of Heme-like Chromophore—Catalysis of NADPH-dependent nitrite reduction by purified Neurospora nitrite reductase requires addition of FAD to the assay mixture (7). This result suggests that the Neurospora enzyme is a flavoprotein which can lose its flavin prosthetic group upon purification and/or dilution of the enzyme. We have found that the absorption spectrum of nitrite reductase in most preparations is altered upon addition of NADPH. A quantitatively much more significant alteration of the spectrum is observed in all preparations, however, when both NADPH and FAD are added to the enzyme. As shown in Fig. 5 (Curve B), the "reduced" nitrite reductase species formed in the presence of NADPH + FAD exhibits visible wavelength maxima at 588 and 556 nm. The absorbance of NADPH prevents detection of alteration in the spectrum in the "Soret" region. When nitrite reductase is treated with sodium dithionite, a strong reducing agent for most hemoproteins, the resulting absorption spectrum is virtually identical with that produced upon addition of NADPH + FAD to the enzyme. These results indicate that the heme-like chromophore present in the nitrite reductase preparations can be completely reduced by NADPH + FAD; reduction is not complete, however, when NADPH serves as reducing agent in the absence of FAD.

We have as yet found no evidence for spectral perturbation in the heme-like chromophore upon addition of NaNO₂ to oxidized enzyme. When excess nitrite is added to NADPH + FAD reduced enzyme, however, a species is formed with an absorption spectrum different from that of either oxidized or reduced nitrite reductase (Fig. 5, Curve C). This species exhibits wavelength maxima at 585 and 560 nm and may represent a complex between enzyme and nitrite or a reduction product thereof. Although enzyme which exists in this "complexed" state with nitrite is clearly enzymatically active (vide infra), we have no evidence that this nitrite-"complexed" form of the enzyme is a participant in the normal catalytic cycle of the enzyme. Nonetheless, the demonstrated spectral perturbations induced in the heme-like chromophore upon addition of the presumed physiological substrates for nitrite reductase are in keeping with a possible functional role for the heme-like chromophore in nitrite reduction.

Effects of CO on Spectral Properties of Heme-like Chromophore—As shown in Fig. 5 (Curve D), carbon monoxide forms a complex with the heme-like chromophore of the nitrite reductase preparation. The CO complex exhibits an intense α-band maximum at 590 nm and a β-band maximum, of lower intensity, at 550 nm. Formation of the CO complex requires the addition of reducing agent (NADPH + FAD) to oxidized enzyme (compare Curves D and E in Fig. 5). Fig. 6 (Curves A and B) shows that, while CO complex formation can occur with NADPH as reducing agent in the absence of FAD, formation of this complex is incomplete unless FAD is added in addition to NADPH and CO. The data of Fig. 7 demonstrate that the effect of the FAD is not simply to enhance the rate of CO complex formation with nitrite reductase, but that FAD actually increases the total amount of CO complex formed when NADPH serves as reducing agent. This result supports the hypothesis that FAD is required for electron transfer between NADPH and the heme-like chromophore of nitrite reductase, and that a fraction of the enzyme molecules have lost their flavin during the course of enzyme preparation.

The kinetic data of complex formation between enzyme and

Fig. 3 (left). Absorption spectrum of Neurospora nitrite reductase. Enzyme was purified through the sucrose density gradient centrifugation step and dissolved in 0.1 M potassium phosphate buffer (pH 7.5). Curve A, 1.8 mg per ml; Curve B, 3.6 mg per ml.

Fig. 4 (center). Sucrose density gradient sedimentation of Neurospora nitrite reductase. Enzyme was purified through the Sephadex G-200 chromatography step of the purification procedure, concentrated by precipitation with 80% saturated (NH₄)₂SO₄, and subjected to sucrose density gradient centrifugation as described under "Experimental Procedure." Each fraction (approximately 0.2 ml) was diluted 1:5 with 0.05 M potassium phosphate (pH 7.5)/5 mM EDTA buffer and absorbancy at 280 nm (●—●) and 400 nm (O—O) determined in cells of 1-cm path length with a Cary model 14 spectrophotometer. An aliquot of each fraction was then assayed for NADPH-nitrite reductase activity (▲—▲). Fraction 1 corresponds to the bottom of the gradient.

Fig. 5 (right). Spectra of nitrite reductase in the presence of reducing agents, nitrite, and carbon monoxide. In all of the experiments, the sample cell contained nitrite reductase, 3.6 mg per ml, in 0.1 M potassium phosphate buffer (pH 7.5). Spectra were recorded versus a buffer blank 7.5 min after addition of the indicated components to the enzyme solution. Incubations were carried out at room temperature. Additions: A, none; B, 2 mM NADPH + 10 μM FAD; C, 2 mM NADPH + 10 μM FAD + 2 mM NaNO₂; D, 2 mM NADPH + 10 μM FAD + 0.9 mM CO; E, 0.9 mM CO.
Fig. 6 (left). Effects of FAD and nitrite on the formation of nitrite reductase complex with carbon monoxide. Conditions were the same as in Fig. 5. Additions to oxidized enzyme: A, 2 mM NADPH + 10 μM FAD + 0.9 mM CO; B, 2 mM NADPH + 0.9 mM CO; C, 2 mM NADPH + 10 μM FAD + 0.9 mM CO + 2 mM NaNO₂.

Fig. 7 (right). Time course of formation of nitrite reductase complex with carbon monoxide. The reaction was started by addition of enzyme to a CO-saturated solution of NADPH + FAD in 0.1 M potassium phosphate buffer (pH 7.5). The final concentrations were: nitrite reductase, 3.6 mg per ml; 2 mM NADPH; 0.83 mM CO; and, where indicated, 10 μM FAD. Successive absorption spectra were recorded in the region 500 to 650 nm versus a buffer blank. The amount of enzyme CO complex formed at any time (t) is measured as the ΔA₅₉₀₋₅₆₅ at that time (ΔAₜ) minus the ΔA₁₅₅₋₁₆₅ of enzyme incubated with all reagents except CO (ΔA₀). The wavelength pair 590 nm and 565 nm was chosen because the difference in absorbance between these two wavelengths is the same for oxidized enzyme and for enzyme reduced with NADPH + FAD. Thus, by using this wavelength pair, no correction need be made for incomplete reduction of enzyme when FAD is omitted. O—O, NADPH and FAD both present at zero time; O—O, NADPH present at zero time. FAD added after 12 min of incubation of enzyme with NADPH and CO. All incubations were carried out at room temperature.

Inhibition of Nitrite Reductase Activity by CO—The results presented in Fig. 8 demonstrate that CO is a potent inhibitor of NADPH-nitrite reductase activity. The inhibition was observed, however, only when CO was preincubated with enzyme in the presence of a reducing agent, NADPH (+ FAD), prior to assay by dilution of the preincubation mixture into the reagents of the standard NADPH-nitrite reductase assay mixture. If FAD was omitted from the NADPH + CO-containing preincubation mixture, only a partial inhibition of nitrite reductase activity could be achieved. Although the kinetics of CO inhibition are complicated by the previously reported (7, 30) slow inactivation of nitrite reductase by NADPH + FAD in the absence of CO, it is clear from Fig. 9 that the time course of CO inhibition of nitrite reductase activity is similar, if not identical, with the time course for formation of the spectrophotometrically detectable CO complex of the heme chromophore when both inhibition and complex formation are measured at the same concentrations of NADPH, FAD, and CO. Thus, CO inhibition of enzyme activity and formation of CO complex with the heme chromophore share the following characteristics: (a) both require the presence of a reducing agent; (b) both are incomplete unless FAD is added; (c) the two processes occur with the same kinetics.

Like formation of the spectrophotometrically detectable enzyme CO complex, CO inhibition of nitrite reductase activity is reversible. Thus, if enzyme is preincubated with
FIG. 8. Inhibition of NADPH-nitrite reductase activity by carbon monoxide. Solutions of nitrite reductase, 8 mg per ml, in 0.1 M potassium phosphate buffer (pH 7.5) were incubated at room temperature together with the indicated components. Concentrations of components other than enzyme were the same as in Fig. 7. In all cases, enzyme was the last component added to incubation mixtures. At the times indicated, aliquots of the incubation mixtures were diluted (1:50 or more) into solutions containing reagents for the standard assay of NADPH-nitrite reductase and the latter activity measured as described under "Experimental Procedure." Additions to enzyme in the incubation mixture: O-O, none, CO; ••, NADPH; Δ-Δ, NADPH + FAD; ••, NADPH + CO; □-□, NADPH + FAD + CO.

FIG. 9. Comparison of the time course of enzyme-CO complex formation with the time course of development of inhibition of NADPH-nitrite reductase activity by CO. Enzyme was incubated at room temperature with NADPH, FAD, and CO as indicated in Figs. 7 and 8. O—O, per cent NADPH-nitrite reductase activity remaining at time t; •—•, fraction of enzyme-CO complex formed at time t. ΔA and ΔA are defined in Fig. 7. The ΔAobserved after 20 min of incubation of enzyme with NADPH, FAD, and CO, under the conditions of Fig. 7 was taken to be ΔA.

NADPH, FAD, and CO under the conditions of Fig. 8 until only 10% of the activity remains, and the solution is then passed through a column of Sephadex G-25, the eluted enzyme is 90% as active as enzyme which had not been preincubated with CO. We have shown previously that formation of the enzyme-CO complex, determined spectrophotometrically, can be both prevented and reversed by addition of nitrite. The data of Fig. 10 show that addition of NaN3 to a reaction mixture containing enzyme, CO, NADPH, and FAD can both prevent and reverse the CO inhibition of nitrite reductase activity as well. (It should be noted that there is a lag period of approximately 2 to 3 min under the experimental conditions of Fig. 10 before activity begins to reappear following addition of nitrite to CO-inhibited enzyme. This result suggests that nitrite does not rapidly displace CO from its complex with reduced enzyme and also that the reappearance of enzymatic activity is not simply due to a progressive (first order) dissociation of CO from the reduced enzyme-CO complex, since in either case one would not expect to see a lag in the reappearance of enzymatic activity upon nitrite addition to CO-complexed enzyme. It seems probable that nitrite, by oxidizing the NADPH during turnover by the small amount of residual free enzyme activity always found in CO-treated enzyme (see Figs. 8 to 10) may simply be removing one of the factors required for maintenance of the reduced enzyme-CO complex. The reversal of CO inhibition by nitrite addition and by gel filtration may then operate by similar mechanisms.)

Lafferty and Garrett (7) and Vega et al. (30) have reported that cyanide, sulfite, and arsenite are competitive inhibitors (with respect to nitrite) of Neurospora NADPH-nitrite reductase activity. If these agents compete with CO for a common enzyme site on nitrite reductase, it might be expected that they could protect the enzyme from inhibition by CO. In an experiment not shown, nitrite reductase was incubated with CO, NADPH, and FAD in the presence of either cyanide (50 µM), sulfite (10 mM), or arsenite (10 mM). After 2.5, 5, 10, and 15 min, aliquots of each incubation mixture were diluted 50-fold into the standard reagents for assay of NADPH-nitrite reductase activity. The concentrations of cyanide, sulfite, arsenite, and nitrite were chosen so that inhibition by these competitive inhibitors would be expected to be negligible (less than 7% inhibition in all cases) in the final NADPH-nitrite reductase assay itself. In this experiment, the CO inhibition of nitrite reductase activity was reduced from 94% in the absence of any of the competitive inhibitors to a maximum of 40%, 52%, and 79%, respectively, when CN−, SO3−, or AsO3− was present...
general similarity in spectra (even though peak positions may position of the a-band to wavelengths as high as 610 nm. The components other than pyridine see Fig. 12) causes shifts in the spectral positions reported previously for the siroheme.CO complex; this shift is due to the presence of pyridine in the enzyme molecule. It is also apparent that CN-, SO₃⁻, and AsO₃ readily dissociate from the enzyme upon dilution, whereas the reduced enzyme-CO complex is relatively stable under these experimental conditions.

The studies of CO reactivity with nitrite reductase indicate, then, that the heme-like chromophore observed spectrophotometrically in the enzyme preparation represents the site of interaction of CO with the enzyme and that the CO-binding component is involved in catalysis of nitrite reduction by the enzyme. Thus, it may be concluded that this heme-like chromophore is both an integral component of nitrite reductase itself (and not merely an adventitious contaminant) and essential to its catalytic function.

Identification of Nitrite Reductase Heme-like Chromophore as Siroheme—Attempts to find either protoheme or heme c in our best nitrite reductase preparations by means of the pyridine hemochromogen test (31) were negative. The absorption spectrum of the Neurospora nitrite reductase complex with CO (Fig. 6) is quite similar to published spectra for the CO complexes of spinach nitrite reductase (15) and of several bacterial sulfite reductases (17, 19, 32), all of which enzymes have been shown to contain siroheme as prosthetic group. In order to compare their respective hemes, solutions of Neurospora nitrite reductase and the siroheme enzyme Escherichia coli sulfite reductase were treated in parallel as described below. The results indicate that Neurospora nitrite reductase contains siroheme as its prosthetic group. The preparation of nitrite reductase used in these experiments exhibited no detectable NADPH-sulfite reductase activity. Thus, the nitrite reductase preparation is not contaminated with Neurospora sulfite reductase (33).

Heme prosthetic groups were extracted from nitrite reductase and sulfite reductase by treatment of each enzyme with 9 volumes of acetone containing 0.015 N HCl at 0°C. The chromatophore extracted from the nitrite reductase was very unstable in acetone/0.015 N HCl; it could be stabilized, however, if the acetone-HCl solution was made 20% (v/v) in pyridine following extraction and centrifugation. When the extracted heme chromatophores were transferred to pyridine as solvent, they exhibited the absorption spectra shown in Fig. 11. Both the nitrite reductase and sulfite reductase extracted hemes exhibited the spectrum previously reported (17) to be characteristic of siroheme in pyridine solution; i.e. absorption maxima at 401 and 558 nm (absorptivity ratio 2.8 ± 0.1) and a shoulder at approximately 520 nm.

Spectra of the CO complexes of the extracted sulfite and nitrite reductase hemes (measured in the presence of alkaline dithionite and a small amount of pyridine) are shown in Fig. 12. The spectra are clearly similar, with both exhibiting maxima at 600 nm (a-band) and 558 nm (β-band). It should be noted that these maxima are shifted toward the red from the spectral positions reported previously for the siroheme-CO complex; this shift is due to the presence of pyridine in the enzyme solution. Addition of greater amounts of pyridine to solutions containing the extracted heme of E. coli sulfite reductase, CO, and alkaline dithionite (for concentrations of components other than pyridine see Fig. 12) causes shifts in the position of the a band to wavelengths as high as 610 nm. The general similarity in spectra (even though peak positions may be shifted) between the CO complex of intact nitrite reductase and its extracted heme strengthens the argument that CO does indeed bind to the heme component of nitrite reductase. In addition, the spectral similarity between extracted heme and the enzyme chromophore CO complexes argues against the possibility that the extracted heme represents only a minor heme component of the nitrite reductase preparation.

Siroheme is a highly polar compound which migrates quite differently from protohemin in a number of chromatographic systems (17). As shown in Fig. 13, the hemes extracted from nitrite and sulfite reductases migrate with nearly identical Rf values in a thin layer chromatographic system which readily separates these hemes from protohemin.

Since siroheme is the iron chelate of a reduced porphyrin, the spectral properties of its demetallized derivative (termed sirohydrochlorin) are markedly different from those exhibited by oxidized porphyrins (16, 18). Iron was removed from the heme prosthetic groups extracted from nitrite and sulfite

![Fig. 11 (left)](http://www.jbc.org/)

**Fig. 11 (left).** Absorption spectra of nitrite reductase and sulfite reductase extracted heme chromophores in pyridine. To 0.1-ml solutions of Neurospora nitrite reductase (A₂₅₀ = 2.4) and Escherichia coli NADPH-sulfite reductase (27 μM) in 0.05 M potassium phosphate buffer (pH 7.7) were added 0.9 ml of acetone/0.015 N HCl at 0°C. After 5 min, the mixtures were centrifuged, 0.5 ml of pyridine added to each supernatant, and the resulting mixture again centrifuged. Each of the supernatant fluids was then evaporated to near dryness in a stream of N₂ at room temperature, and pyridine added to a total volume of 1.0 ml for the nitrite reductase-extracted heme and 2.7 ml for the sulfite reductase-extracted heme, respectively. Absorption spectra were recorded versus a pyridine blank. --. Nitrite reductase heme; ----, sulfite reductase heme.

![Fig. 12 (right)](http://www.jbc.org/)

**Fig. 12 (right).** Absorption spectra of CO complexes of the extracted hemes from nitrite reductase and sulfite reductase. One milliliter of each of the heme solutions in pyridine prepared in Fig. 11 was evaporated to near dryness with a stream of N₂, and the heme dissolved in 20 μl of pyridine. A 10-μl aliquot of this heme solution was added to a cuvette containing 0.1 ml of 1 N KOH and 0.65 ml of CO-saturated 0.05 M potassium phosphate buffer (pH 7.7), and a few crystals of sodium dithionite were then added. The absorption spectrum of the resulting solution was recorded as quickly as possible versus a blank containing 10 μl of pyridine, 0.1 ml of 1 N KOH, and 0.65 ml of CO-saturated potassium phosphate buffer (pH 7.7). ----, Nitrite reductase heme; ----, sulfite reductase heme.
FIG. 13. Thin layer chromatography of the extracted heme chromophores from sulfite reductase and nitrite reductase. Concentrated solutions of sulfite and nitrite reductase-extracted heme chromophores were prepared as described in Figs. 11 and 12. A solution of protohemin was prepared by dissolving 2 mg of equine hemin in 10 ml of pyridine. A 5-μl aliquot of each heme solution was applied to a 5-cm wide plastic strip coated with Brinkman Instruments "Polyamide-6." After the pyridine had evaporated from the applied samples, the strip was developed for 26 min with acetone/HCl/formic acid (70/30/3). The position of the solvent front is indicated. Positions of the heme spots were detected by means of their color. Identical results were obtained when the hemes were detected by spraying the plate for peroxidatic activity with the benzidine/HCl/pyridine reagent of Connelly et al. (39). In a separate control experiment it was shown that the migration of the protohemin standard is not altered if the equine hemin is subjected to the same procedures (i.e. acetone-HCl, centrifugation, transfer to pyridine, drying with a stream of N₂) used for preparation of the nitrite and sulfite reductase-extracted heme solutions. N, nitrite reductase-extracted heme; S, sulfite reductase-extracted heme; P, protohemin.

reductases by a scaled down version of the procedure described previously for demetallization of siroheme (16). Fluorescence activation and emission spectra of the resulting porphyrin cations in 2 N HCl are shown in Fig. 14. The spectra of the porphyrin cations derived from the two enzymes are nearly identical. Excitation maxima were observed at (in order of decreasing intensity): 406, 385, 376, 612, 573, 529, and 499 nm for the sulfite reductase-demetallized heme, and at 405, 385, 375, 612, 573, 530, and 499 nm for the nitrite reductase-demetallized heme. A single emission maximum at 626 nm, with a shoulder in the region 665 to 675 nm, was observed for each of the demetallized hemes.

These results indicate that the heme prosthetic group of *Neurospora* nitrite reductase is a highly polar compound identical in its spectral properties (with metal or without metal) with the heme prosthetic group of *E. coli* sulfite reductase. We may conclude, then, that siroheme is a prosthetic group of *Neurospora* nitrite reductase.

Fig. 14. Fluorescence excitation and emission spectra of sulfite reductase and nitrite reductase-demetallized heme chromophores in 2 N HCl. Concentrated solutions of sulfite and nitrite reductase-extracted heme chromophores in pyridine were prepared as described in Figs. 11 and 12. Each heme solution was taken to near dryness with a stream of N₂ and 0.4 ml of glacial acetic acid added. The heme solution was then placed under a stream of N₂ at room temperature, and 10 μl of a freshly prepared solution of 1% FeSO₄·7H₂O in concentrated HCl added. The appearance of an orange-red fluorescence (indicating conversion of heme to porphyrin) was detected with a Mineralight UVS-25 long wavelength lamp almost immediately following addition of the FeSO₄·HCl solution to the heme. The solution of demetallized heme was evaporated to near dryness under a stream of N₂ and the porphyrin taken up in 2.2 ml of 2 N HCl. Fluorescence spectra were recorded at room temperature (at a speed of 0.3 nm per s) with a Turner Spectro 210 spectrophotofluorometer equipped with constant energy attachment. For excitation spectra, the emission wavelength was 640 nm. For emission spectra, the excitation wavelength was 405 nm. Bandwidths were 10 nm. -, nitrite reductase-demetallized heme; ---, sulfite reductase-demetallized heme.

**DISCUSSION**

The results presented in this paper demonstrate that *Neurospora* NADPH-nitrite reductase contains siroheme as a prosthetic group. That the siroheme component functions in the enzymatic process of nitrite reduction is indicated by the following data.

1. The heme component of the nitrite reductase preparation is reducible by the "natural" electron donor for the enzyme, NADPH, and a spectrally unique form of the enzyme is generated when nitrite, the "natural" electron acceptor for the enzyme, is added to the reduced enzyme, a result which
Whether the flavin-dependent and siroheme-containing pro-
teraction of the enzyme with electrons derived from NADPH.
spora nitrite reductase may be considered, then, as a func-
tional complex of a siroheme-containing protein portion, which
provides the site of interaction of nitrite with the enzyme, and
a flavin-binding protein portion, which provides the site of
interaction of the enzyme with electrons derived from NADPH.

Whether the flavin-dependent and siroheme-containing pro-
ten moieties are physically associated with separate polypep-
tide chains or a single polypeptide remains to be determined.
The previously reported finding that treatment of the enzyme
with mercurials or with heat can cause loss in ability to utilize
NADPH as electron donor, while nitrite reducing ability (with
the artificial electron donor reduced benzyl viologen) is re-
tained (7), supports the functional distinction presented here
between the sites of NADPH oxidation and nitrite reduction in the
Neurospora enzyme.

While siroheme has now been identified in two different
“assimilatory” nitrite reductases, it is not evident that all
enzymes capable of catalyzing nitrite reduction to ammonia
need contain this prosthetic group. Thus, Prakash and Sadana
(34) have reported that an enzyme which catalyzes reduction of
nitrite to ammonia presumably as part of the process of nitrate
respiration in Achromobacter fischeri contains heme c as its
sole iron-containing prosthetic group. Enzymes catalyzing the
reduction of nitrite to gaseous products (NO, NO, N2, etc.)
are frequently associated with nitrate “respiration” do not appear

3. Nitrite both prevents and reverses the complexation of
enzyme heme with CO and inhibition of nitrite reductase
activity. Sulfite, cyanide, and arsenite, which are competitive
inhibitors of nitrite reduction, reduce the ability of CO to
interact with nitrite reductase. These results, taken together
with the observed formation of a complex between nitrite (or a
reduction product of nitrite) and the enzyme-bound heme,
suggest that siroheme may represent the site of interaction
of nitrite (as well as inhibitors competitive with respect to nitrite)
with Neurospora nitrite reductase.

Neurospora nitrite reductase represents the second nitrite
reductase which has been found to contain siroheme as a
prosthetic group. Murphy et al. (15) reported previously that
spinach ferredoxin-nitrite reductase contains siroheme; how-
ever, no clear demonstration of a functional role for this heme
component in nitrite reduction by the spinach enzyme was
presented. The spinach nitrite reductase was shown to form a
complex with CO (15). The absorption spectra of the CO
complexes of the Neurospora and spinach nitrite reductases are
quite similar. Indeed, the similarity in absorption spectra of
the highly purified nitrite reductases from Chlorella (2) and
vegetable marrow (13) to that of the spinach enzyme suggests
that siroheme is a general component of nitrite reductases from
the plant kingdom. The Chlorella nitrite reductase was re-
ported to be inhibited by CO (2), although no spectro-
photometrically detected complex between enzyme and CO
was reported.

The plant ferredoxin-nitrite reductases are considerably
smaller in size than the pyridine nucleotide-linked nitrite
reductase of Neurospora (60,000 versus 290,000 (2-7, 12-14)).
The low molecular weight nitrite reductase hemoproteins do
not contain flavin and are reported to contain only about 2
atoms of iron per molecule of enzyme (2, 12-14). These
enzymes do not catalyze nitrite reduction with reduced pyri-
dine nucleotide as electron donor unless ferredoxin and the
exogenous flavoprotein ferredoxin-NADP+ oxidoreductase are
added along with NADPH. The high molecular weight Neuro-
spora nitrite reductase may be considered, then, as a func-
tional complex of a siroheme-containing protein portion, which
provides the site of interaction of nitrite with the enzyme, and
a flavin-binding protein portion, which provides the site of
interaction of the enzyme with electrons derived from NADPH.

and the kinetic data of CO inhibition of enzyme
activity are nearly identical with those for CO binding to the
enzyme heme when both types of measurement are performed
on solutions containing the same concentrations of NADPH,
FAD, and CO. Both spectrophotometrically determined CO
complex formation and CO inhibition of enzyme activity are
not complete unless FAD is present together with enzyme,
NADPH, and CO. The latter result suggests that the site for
interaction of enzyme with NADPH is located on the same
molecule as the CO binding heme chromophore, since if FAD
catalyzed intermolecular electron transfer between the
NADPH-oxidizing site and the CO binding site one would expect
the rate of NADPH-dependent CO binding to increase
in the presence of FAD, but not the extent of total CO binding.

Although in plants, bacteria, and fungi, the physiologically
important 6-electron reductions of sulfite to sulfide and of
nitrite to ammonia appear to be catalyzed by physically
distinct enzymes termed sulfite and nitrite reductases, respec-
tively, the finding that siroheme is a prosthetic group common
to both types of enzyme suggests that there may be a common
mechanism underlying the reduction of sulfite and nitrite. This
possibility is strengthened by the fact that a number of sulfite
reductases can catalyze both sulfite and nitrite reduction in
vitro, while sulfite can act as a competitive inhibitor for nitrite
reductases (7, 11). E. coli sulfite reductase forms spectro-
photometrically detectable complexes with either sulfite or
nitrite (or reduction products thereof) in the presence of
NADPH (17). The “complex” of E. coli sulfite reductase with
nitrite bears spectral similarity to that of the Neurospora
nitrite reductase “complex” with nitrite reported in this paper.
E. coli sulfite reductase and Neurospora nitrite reductase share
a common set of inhibitors, all of which have been shown to
promote spectral alterations in the heme chromophore of the
former enzyme: cyanide, CO, and arsenite.

Another property shared by Neurospora nitrite reductase
and bacterial sulfite reductases is the unusually slow rate of
the reaction between CO and the enzyme-bound siroheme pro-
thetic groups, when compared to the rates of reaction of CO
with hemoproteins, the normal function of which is to react
with O2. Thus, the apparent second order rate constants for the
reaction E + CO → E-CO (in the presence of reducing agent)
are as follows: E. coli sulfite reductase, 3 to 7 $10^9$ M$^{-1}$ s$^{-1}$ (depend-
ning on reducing agent used (17, 35); Desulfotomaculum nigri-
cans sulfite reductase, 15 $10^9$ M$^{-1}$ s$^{-1}$ (35); Neurospora nitrite
reductase, 9 $10^8$ s$^{-1}$; versus 8 to $10^4$ M$^{-1}$ s$^{-1}$ for cytochrome
oxidase (36); 5 $10^6$ M$^{-1}$ s$^{-1}$ for myoglobin (37); and 5 to $69$ $10^6$
M$^{-1}$ s$^{-1}$ for hemoglobin (37). Murphy et al. (35) have
demonstrated that the low rate of reaction of CO with siroheme
bound to E. coli sulfite reductase is due to the environment of
the enzyme-bound heme and not to its unusual chemical
structure, since CO reacts with free siroheme at $10^8$ times
the rate of its reaction with enzyme-bound siroheme (35).
The steric barriers to CO reaction with the siroheme prosthetic
group are present in both sulfite and nitrite reductases. It is
possible that this slow reactivity reflects the marked structural difference between CO and the anionic substrates which must interact with the catalytic sites of sulfite and nitrite reductases.

In conclusion, the data reported in this paper, together with that reported previously by Lafferty and Garrett (7), suggest the following sequence of electron flow in Neurospora NADPH-nitrite reductase:

\[ \text{NAD(P)H} \rightarrow \text{FAD} \rightarrow \text{Siroheme} \rightarrow NO_2^- \]

This scheme is similar to one proposed by Siegel et al. (20, 38) for electron flow in the NADPH-sulfite reductase of E. coli.

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