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

(Received for publication, April 16, 1975)

JOSE M. VEGA* AND REGINALD H. GARRETT

From the Department of Biology, University of Virginia, Charlottesville, Virginia 22901

LEWIS M. SIEGEL

From the Department of Biochemistry, Duke University Medical Center, and the Veterans Administration Hospital, Durham, North Carolina 27710

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⁻¹ s⁻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 N 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
Assimilatory nitrite reductases catalyze the following 6-electron transfer reaction:

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

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 heme-proteins. Murphy et al. (15) have recently identified the heme moiety of spinach ferredoxin nitrite reductase as "siroheme," an iron tetrahedrorporphyrin 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:

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

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, however, to possess an enzyme which can reduce nitrite to ammonia even under conditions which prevent sulfate reduction (23-26). The presence of an enzyme which can reduce nitrite to ammonia under conditions which prohibit sulfate reduction 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 60,000, requires added FAD to utilize either NADPH or NADH as electron donor, although the enzyme can use the artificial dye reduced benzyl viologen 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, protoporphin, 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 (26). 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 L-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 *Fusarium oxysporum* as described by Siegel et al. (17). The preparation used in these studies exhibited an A_260/430 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 × 10⁴ 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 μM 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 *Neurospora* enzyme in the absence of benzyl viologen, it is not clear to what extent in our assays dithionite and reduced benzyl viologen are each serving as the actual immediate reductant for nitrite reduction.
term "standard buffer" refers to 50 mM 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 × 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 56 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 (0.1 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 tubes were centrifuged, at 20, for 24 hours at 41,000 rpm in the 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 5 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 mM potassium phosphate buffer (pH 7.5), 10 mM FAD, 1 mM NaN₃, 0.2 mM NADPH, and enzyme. Assays were performed at 25°C in cells of 1-cm path length.

**Glucose-6-phosphate dehydrogenase activity was measured by** the production of 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** the production of NADPH at 340 nm with a Cary model 14 spectrophotometer. The reaction mixture contained, in a total volume of 1.0 ml, 0.07 mM Tris-HCl buffer (pH 7.5), 7.5 mM MgCl₂, 20 mM glucose 6-phosphate, and 10 mM 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 to 0.1 A and 0.1 to 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 a 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 × 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 vivo 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 NaN₃ as sole nitrogen source and the amounts of FeCl₃ indicated in Fig. 2. The replacement of ammonia with nitrate as sole nitrogen source has been previously shown to result in induction of NADPH nitrite reductase activity in *Neurospora* mycelia (29). After 4 hours of incubation in the "induction medium," the mycelia were harvested, and nitrite reductase specific activity measured in mycelia extracts. Fig. 2 demonstrates that induction of nitrite reductase activity is dependent on the presence of iron in the induction medium. Qualitatively similar responses of nitrite reductase activity to iron level were found when either NADH or reduced benzyl viologen replaced NADPH as electron donor for nitrite reduction. Thus, it appears to be the portion of the enzyme which catalyzes nitrite oxidation per se, rather than that portion of the enzyme which is responsible for transferring electrons from NADPH to the nitrite-reducing center (7), which is dependent upon the presence of iron in the growth medium. In order to test for the specificity of the iron dependence of nitrite reductase, mycelial extracts from the experiment of Fig. 2 were assayed for activity of glucose-6-phosphate dehydrogenase, an enzyme which does not contain iron. The activity of this latter enzyme did not require addition of iron to the growth medium.

**Association of a Heme-like Chromophore with Nitrite Reductase Preparations—** Our most highly purified preparations of *Neurospora* nitrite reductase have uniformly exhibited absorption spectra which suggest the presence of a heme component. The spectrum of such a preparation is shown in Fig. 3. Wavelength maxima are observed at 390 and 578 nm; the ratio of absorbivities at these two wavelengths is 3.7. Although the marked instability of *Neurospora* nitrite reduc-
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 hemeproteins, 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 NaN3 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 NaN3; D, 2 mM NADPH + 10 μM FAD + 0.9 mM CO; E, 0.9 mM CO.
Characteristic of enzyme which had been incubated with NaNO, is shown in Fig. 6 (Curve C). Thus, addition of nitrite can prevent recording as soon as possible after addition of reagents and 20 min after addition of reagents to the enzyme, 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 absorption spectrum of the heme-like chromophore by NADPH + FAD is a rapid process compared to CO complex formation, so that the kinetic changes observed in Fig. 7 reflect the latter process alone. In the presence of NADPH and FAD, formation of the CO complex with nitrite reductase exhibits pseudo-first order kinetics with a half-time of 96 s at the CO concentration used. If CO complex formation can indeed be described by the simple equation 

\[ E + CO \rightarrow E\cdot CO \]

then a second order rate constant of 9 M^-1 s^-1 can be calculated for this process.

When enzyme is incubated with NADPH, FAD, and CO under the conditions of Fig. 7 for a time sufficient to permit maximal formation of enzyme-CO complex, and the resulting mixture was passed through a Sephadex G-25 column in order to remove pyridine nucleotide, FAD, and CO from the enzyme, the enzyme species which appears in the column eluate is oxidized, free enzyme rather than CO complex. (This experiment was performed under aerobic conditions so that reduced enzyme could have been oxidized by O_2.) Thus, formation of the CO complex with nitrite reductase is a reversible process. Fig. 6 (Curve C) shows that nitrite can compete effectively with CO for the enzyme, since addition of NaNO_3 together with NADPH, FAD, and CO to nitrite reductase results in the appearance of a species with the absorption spectrum characteristic of enzyme which had been incubated with NaNO_3, NADPH, and FAD in the absence of CO (Fig. 5, Curve C). Although the spectrum shown in Fig. 6 (Curve C) was recorded 7.5 min after addition of reagents to the enzyme, spectra recorded as soon as possible after addition of reagents and 20 min after addition of reagents were indistinguishable from that shown in Fig. 6 (Curve C). Thus, addition of nitrite can prevent complex formation between enzyme and CO. Addition of nitrite to preformed enzyme-CO complex can also reverse the complexation between enzyme and CO, since a slow shift in the absorption spectrum from that characteristic of the CO complex to that characteristic of the nitrite "complex" is observed following addition of NaNO_3 to enzyme preincubated for 15 min in the presence of NADPH, FAD, and CO under the conditions of Fig. 7.

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; ΔΔΔΔ, NADPH + FAD; ΔΔΔΔ, 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; O-O, fraction of enzyme-CO complex formed at time t. ΔA and ΔA are defined in Fig. 7. The ΔAobs./min observed 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 the 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...
together with CO in the preincubation mixture. The data are therefore consistent with a model in which nitrite reductase is able to form a reversible complex with either competitive inhibitor or CO, but not both simultaneously on the same enzyme molecule. It is also apparent that CN, SO, and AsO 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-heme 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 M HCl at 0°C. The chromophore extracted from the nitrite reductase was very unstable in acetone/0.015 M 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 chromophores 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. absorbancy 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 demetalized 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. 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.

Reducases by a scaled down version of the procedure described previously for demetalization 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, 396, 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 demetalized 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.

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
indicates that a complex can be formed between the heme and nitrite (or a reduction product thereof).

2. The similarity in absorption spectra between the enzyme-CO complex and free siroheme-CO indicates that siroheme represents the site of interaction between CO and nitrite reductase. CO is an inhibitor of NADPH-nitrite reductase activity, 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.

3. Nitrite both prevents and reverses the complexation of enzyme heme with CO and CO 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; however, 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 reported to be inhibited by CO (2), although no spectrophotometrically 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 homoproteins 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 pyridine nucleotide as electron donor unless ferredoxin and the exogenous flavoprotein ferredoxin-NADP oxidoreductase are added along with NADPH. The high molecular weight Neurospora nitrite reductase may be considered, then, as a functional 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-
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:

\[
\text{NAD(P)H} \rightarrow \text{FAD} \rightarrow \text{Siroheme} \rightarrow \text{NO}_2^-
\]

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

Acknowledgments—We are indebted to Mr. Phillip Greenbaum and Mrs. Patricia Davis for their excellent technical assistance in certain phases of this work.

REFERENCES
Siroheme: a prosthetic group of the Neurospora crassa assimilatory nitrite reductase.
J M Vega and R H Garrett


Access the most updated version of this article at http://www.jbc.org/content/250/20/7980

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/250/20/7980.full.html#ref-list-1