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Siroheme: A New Prosthetic Group Participating in Six-Electron Reduction Reactions Catalyzed by Both Sulfite and Nitrite Reductases

(heme proteins/biochemical evolution/spinach ferredoxin-nitrite reductase/ *E. coli* NADPH-sulfite reductase)

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ABSTRACT **Ferredoxin-nitrite reductase (EC 1.7.7.1) of spinach, an enzyme that catalyzes the six-electron reduction of nitrite to ammonia, contains siroheme, the new type of prosthetic group recently found in several sulfite reductases (both assimilatory and dissimilatory) that can catalyze the reduction of sulfite to sulfide, also a six-electron reduction. The prosthetic group of sulfite reductase had previously been shown to be an iron-tetrahydroporphyrin of the isobacteriochlorin type (adjacent pyrrole rings reduced) with eight carboxylate side chains. This finding suggests that both types of "multi-electron" reduction processes may share common mechanistic features.**

Biological oxidation-reduction reactions classically involve the transfer of electrons one or two at a time. A limited group of reactions can be classified as "multi-electron" reductions, in that more than two electrons (in fact, as many as six) are transferred to an enzyme-bound substrate before product release from the site of reduction. Multi-electron reduction reactions occupy crucial positions in metabolism, being involved in the utilization of O_2 as terminal electron acceptor by all organisms capable of aerobic metabolism (*cytochrome oxidase:* $O_2 + 4[H\cdot] \rightarrow 2H_2O$), as well as in the biological assimilation of inorganic sulfur (*sulfite reductase:* $SO_3^{2-} + 6[H\cdot] \rightarrow S^{2-} + 3H_2O$) and nitrogen compounds (*nitrite reductase:* $NO_2^- + 6[H\cdot] \rightarrow NH_4^+ + 2OH^-$; and *nitrogenase:* $N_2 + 2H^+ + 6[H\cdot] \rightarrow 2NH_4^+$). For each of these reactions, a single enzyme has been described that can catalyze the entire four- or six-electron transfer without release of inorganic compounds of oxidation state intermediate between substrate and product. It seemed likely that such novel types of redox reactions might be associated with novel types of enzyme prosthetic groups.

The enzymatic reductions of sulfite and nitrite occur as part of two different physiological processes. (*a*) In plants, fungi, and many bacteria, the reductions of sulfite to sulfide and nitrite to ammonia are intermediate steps in the assimilation of sulfate and nitrate, respectively, for the synthesis of sulfur- and nitrogen-containing cellular constituents. (*b*) In some bacteria, the reductions of sulfite and nitrite are large-scale processes associated with anaerobic respiration utilizing sulfate and nitrate as terminal electron acceptors.

Assimilatory sulfite reductases have been purified from various sources, and in all cases these enzymes exhibited absorption spectra indicative of a heme-like chromophore (1). These spectra exhibit characteristic features in having an α-band at 582–589 nm, and a low Soret-to-α band absorptivity ratio. The heme chromophore, which appears to interact directly with sulfite in the reduction process (2), has been isolated from the *Escherichia coli* sulfite reductase and identified as a new type of heme prosthetic group: an iron-tetrahydroporphyrin of the isobacteriochlorin type (adjacent pyrrole rings reduced) with eight carboxylate side chains (3). Murphy *et al.* (3) have proposed that the actual compound is an iron dimethyl-urotetrahydroporphyrin; a plausible isomer, compatible with known biosynthetic pathways, is shown in Fig. 1. This compound has been termed "siroheme" by Murphy and Siegel (4), and has been demonstrated to serve as the prosthetic group for sulfite reductases associated with sulfate respiration as well as sulfate assimilation (4, 5). The sulfite reductases associated with sulfate respiration can also reduce sulfite to sulfide, but in a "leaky" fashion, with release of inorganic sulfur compounds of oxidation state intermediate between sulfite and sulfide (4–7). Three "respiratory" sulfite reductases have been examined (4, 5); despite a diversity of absorption spectra for the native enzymes, each has been found to contain the siroheme prosthetic group.

Assimilatory nitrite reductases, catalyzing the six-electron reduction of nitrite to ammonia, have been identified in various organisms. The enzyme responsible for this activity has been obtained in highly purified form, however, only from plant sources: Chlorella (8) and spinach (9). The natural electron donor for both enzymes appears to be ferredoxin. The Chlorella and spinach nitrite reductases both exhibit absorption spectra indicative of a heme-like component. The low values of the Soret wavelengths (380–390 nm), together with the low Soret-to-α absorptivity ratio (4.0–4.5) and absence of a typical pyridine "hemochromogen" spectrum (8), suggested that these nitrite reductases, like sulfite reductase, might contain a novel type of tetrapyrrole structure, potentially related to siroheme. Indeed, Zumft (8) has noted the similarity in many properties between nitrite and sulfite reductases, and has suggested that they might share a common heme chromophore.

Although in green plants it appears that sulfite and nitrite reduction are catalyzed by different enzymes, it is of interest to note that, in bacteria and fungi, purified sulfite reductases can also catalyze the reduction of nitrite (2, 10) although this reaction does not appear to be physiologically significant due to a high K_M for nitrite. In fact, the product of the NADPH-

Abbreviation: MVH, reduced methyl viologen.

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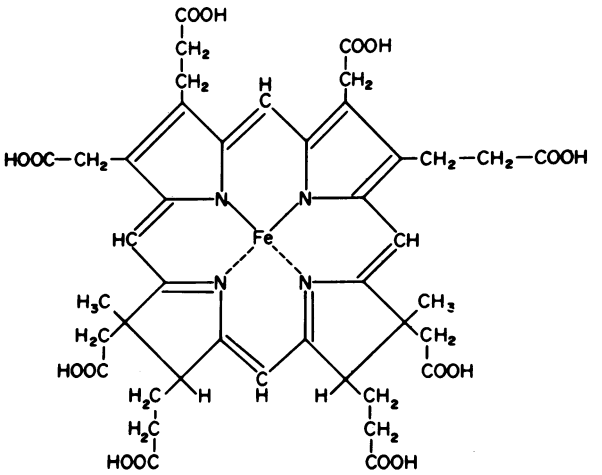

FIG. 1. Postulated structural formula for the siroheme prosthetic group (3).

nitrite reductase reaction catalyzed by *E. coli* sulfite reductase has been identified as ammonia (11). Thus, it is clear that the siroheme prosthetic group can play a role in enzymatic nitrite reduction.

In this paper we report that the heme-like prosthetic group of spinach ferredoxin-nitrite reductase (EC 1.7.7.1; ammonia: ferredoxin oxidoreductase) is identical in its spectral and chromatographic properties to the siroheme prosthetic group of *E. coli* sulfite reductase (EC 1.8.1.2; hydrogen-sulfide: $NADP^+$ oxidoreductase). It is evident then that two of the major multielectron reduction reactions in nature can be catalyzed by enzymes containing the novel siroheme prosthetic group.

EXPERIMENTAL PROCEDURE

Spinach ferredoxin-nitrite reductase was purified by the procedure of Ho and Tamura (9), with the exception that the hydroxyapatite chromatography step was omitted, since this step led to large losses of enzymatic activity with only slight improvement in the purification obtained. The specific activity of our nitrite reductase preparation was 33 units/mg, as compared to 48 units/mg reported by Ho and Tamura for their electrophoretically homogeneous preparation. *E. coli* sulfite reductase was prepared by the method of Siegel *et al.* (1). The heme prosthetic groups were extracted from the nitrite and sulfite reductases with acetone–0.015 N HCl. The hemes were transferred to pyridine, and subjected to demetallization and esterification by described procedures (3). Chemicals were obtained from sources described (1, 3).

Nitrite reductase activity was routinely assayed by the method of Ho and Tamura (9), in which the reduced methyl viologen (MVH)-dependent disappearance of nitrite from the assay mixture is measured. Sulfite and nitrite reductase activities of the purified nitrite reductase preparation were also determined by the technique of Asada *et al.* (12), in which the rate of oxidation of MVH in the presence of either sulfite or nitrite is measured spectrophotometrically. The methyl viologen was reduced with H_2 and platinum asbestos, as described by Siegel *et al.* (2). Concentrations in the 2.5-ml assay mixture were as follows: potassium phosphate buffer, pH 7.7, 0.1 mM; MVH, 0.2 mM; sodium sulfite or potassium nitrite, 1 mM; bovine-serum albumin, 1.33 mg/ml. Measurements were corrected for nonenzymic oxidation and for oxidation of MVH in the absence of either sulfite or nitrite with appropriate controls. The corrections in all cases were small. Protein concentrations were determined by the Zamenhof (13) microadaptation of the biuret method, with prior precipitation of the protein with 10% trichloroacetic acid. Bovine-serum albumin was used as standard.

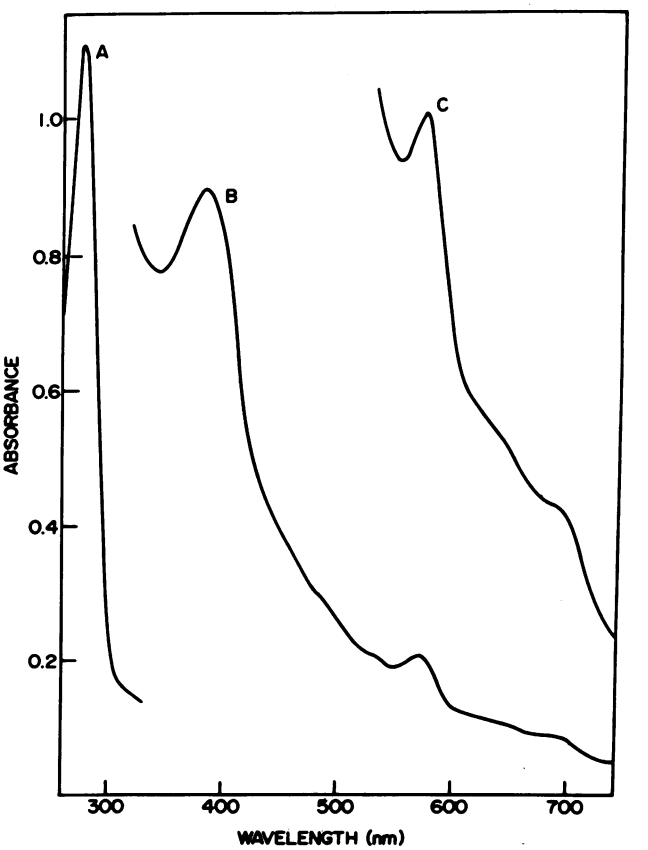

FIG. 2. Absorption spectrum of spinach ferredoxin-nitrite reductase in 50 mM potassium phosphate buffer, pH 7.7. Protein concentrations: *Curve A*, 0.8 mg/ml; *curve B*, 4.0 mg/ml; *curve C*, 19.4 mg/ml.

Absorption spectra were measured at room temperature in silica cuvettes of 1-cm light path with a Cary model 14 spectrophotometer equipped with 0–0.1, 0–0.5, and 0–1.0 A slide wires. Reference cells contained appropriate solvent blanks in all cases. Fluorescence spectra were determined with a Turner Spectro 210 spectrophotofluorometer equipped with constant energy attachment.

RESULTS

Our preparation of spinach ferredoxin-nitrite reductase exhibited an absorption spectrum (Fig. 2) very similar to those reported by Ho and Tamura (9) and Zumft (8) for the spinach and Chlorella nitrite reductases, respectively. Wavelength maxima were observed at 276, 384 (Soret), and 573 (α) nm, with shoulders at approximately 640 and 690 nm. The Soret-to-α band absorptivity ratio, 4.4, is identical to that reported by Ho and Tamura. The enzyme was assayed for the ability to reduce nitrite and sulfite with MVH as electron donor by the procedure of Asada *et al.* (12), originally described by these authors for determination of spinach sulfite reductase activity. Under these conditions, MVH was oxidized at a rate of 45 μmol/min per mg with nitrite as electron acceptor com-

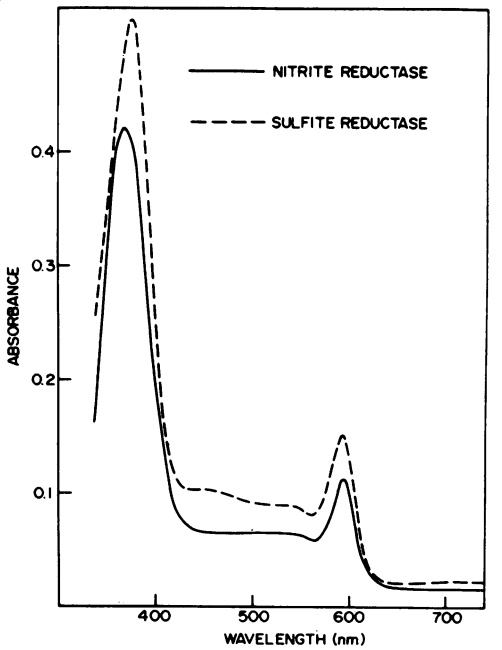

FIG. 3. Absorption spectra of heme chromophores released by extraction of nitrite and sulfite reductases with acetone–0.015 N HCl. The initial protein solutions, diluted with 9 volumes of acetone–HCl, had the following absorbances at their Soret maxima: nitrite reductase, A_{384} = 5.7; sulfite reductase, A_{386} = 4.7.

pared to 0.04 μmol/min per mg with sulfite as acceptor. The ratio of nitrite to sulfite reductase activity then (with both substrates present at 1 mM concentration) is greater than 10^3, a result that indicates little or no contamination of the preparation by sulfite reductase. The absorption spectrum of spinach sulfite reductase with principal absorption maxima at 404 and 589 nm (12) is markedly different from that of

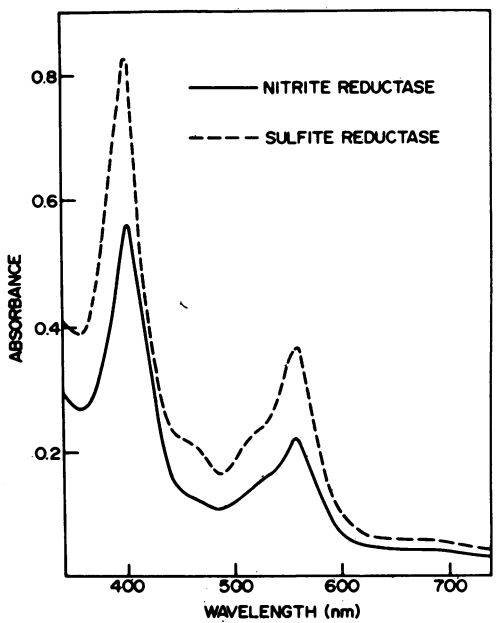

FIG. 4. Absorption spectra of nitrite and sulfite reductase extracted heme chromophores in pyridine. Hemes extracted with acetone–HCl were concentrated, transferred to pyridine, and purified by chromatography on a column of Sephadex LH-20 equilibrated with pyridine, as described (3).

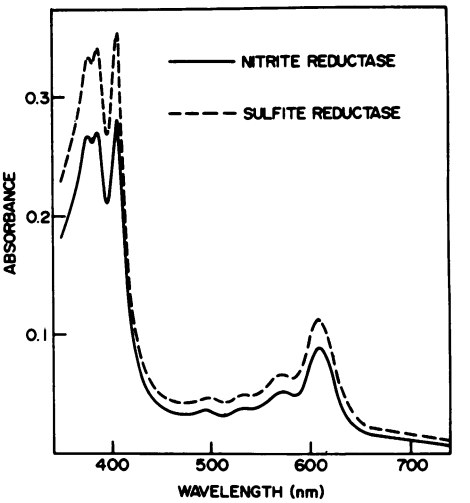

FIG. 5. Absorption spectra of demetallized heme methyl esters in methanol containing 1/20 volume H_2SO_4. The demetallization and esterification procedures were those described in ref. 3.

nitrite reductase. No evidence of absorption peaks at either of these wavelengths was observed in our preparation (Fig. 2).

The heme prosthetic groups were extracted from spinach nitrite reductase and *E. coli* sulfite reductase by treatment of each enzyme with nine volumes of acetone containing 0.015 N HCl at 0°. Spectra of the acetone–HCl extracts, after centrifugation to remove precipitated protein, are shown in Fig. 3. The similarity in these spectra is evident. Upon transfer to pyridine, the extracted hemes from both enzymes exhibited the characteristic siroheme absorption spectrum (Fig. 4), with wavelength maxima at 401 and 557 nm (absorptivity ratio 2.5) and a shoulder at approximately 520 nm.

Iron was removed from the heme prosthetic groups by the procedure described for siroheme (3), and the resulting fluorescent porphyrins were esterified using methanol containing 1/20 volume H_2SO_4. The absorption spectra of the porphyrin methyl esters in an acidic (methanol–5% H_2SO_4) and basic (piperidine) solvent, respectively, are shown in Figs. 5 and 6. The spectra of the porphyrin esters derived from the two

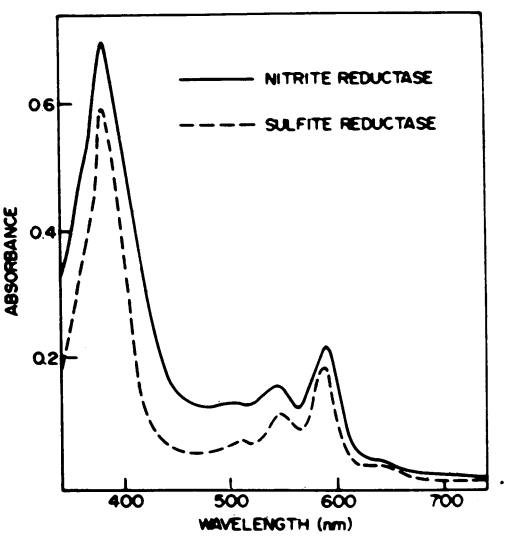

FIG. 6. Absorption spectra of demetallized heme methyl esters in piperidine.

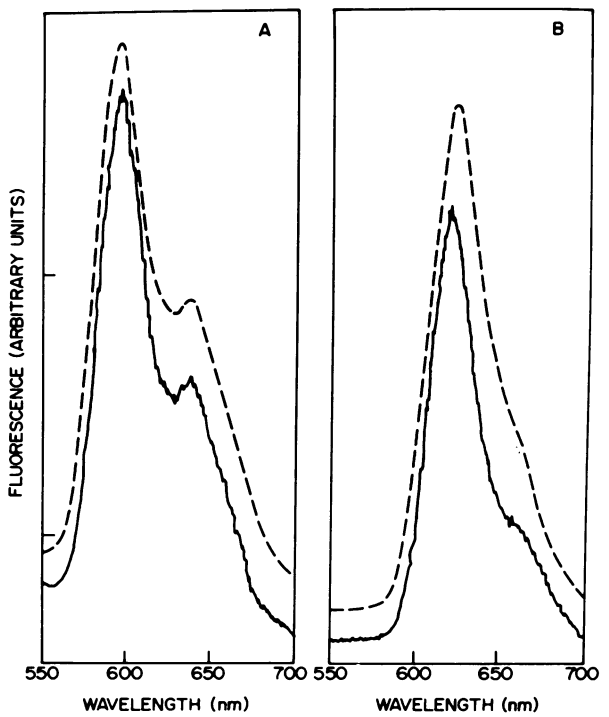

FIG. 7. Fluorescence emission spectra of demetallized heme methyl esters from nitrite and sulfite reductases in (*A*) piperidine and (*B*) methanol containing 1/20 volume H_2SO_4. The excitation bandwidth was 10 nm and the emission bandwidth was 2.5 nm. (*A*) spectra were determined with exciting light at 380 nm. (*B*) spectra were determined with exciting light at 405 nm. *Solid line*, nitrite reductase; *dashed line*, sulfite reductase.

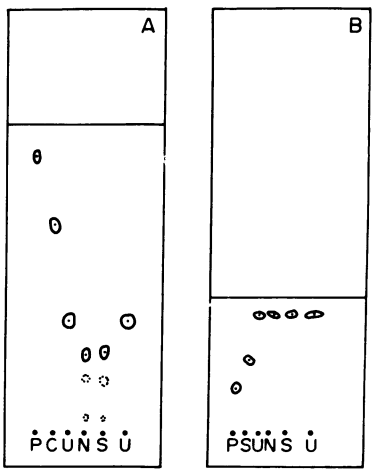

FIG. 8. Thin-layer chromatography of demetallized heme methyl esters from nitrite and sulfite reductases. Esters were applied to the chromatographic plates as concentrated solutions in chloroform. *Plate A* was coated with silicic acid and developed in benzene–ethyl acetate–methanol–butanol, 82:14:3:1. *Plate B* was coated with talc and developed in pyridine–acetic acid–acetone, 1:1:1. Positions of the ester spots were detected by means of their fluorescence. The solvent front is indicated. *P*, protoporphyrin IX dimethyl ester; *C*, coproporphyrin I tetramethyl ester; *U*, uroporphyrin I octamethyl ester; *N*, nitrite reductase tetrahydroporphyrin methyl ester; *S*, sulfite reductase tetrahydroporphyrin methyl ester.

enzymes are virtually identical. Wavelength maxima and absorptivity ratios for siroheme have been described (3, 4). As has been discussed by Murphy *et al.* (3), the absorption spectrum of the neutral porphyrin ester is uniquely characteristic of tetrahydroporphyrins of the isobacteriochlorin type (adjacent pyrrole rings reduced) and is not seen with fully oxidized porphyrins, dihydroporphyrins, or tetrahydroporphyrins of the bacteriochlorin class (opposite pyrrole rings reduced).

Fluorescence activation and emission spectra of the sulfite and nitrite reductase tetrahydroporphyrin esters were also virtually identical to each other, under both acid and neutral conditions. The emission spectra are shown in Fig. 7.

As shown in Fig. 8, the sulfite and nitrite reductase tetrahydroporphyrin methyl esters migrated with identical R_F values in two different thin-layer chromatographic systems previously shown (3) to separate porphyrin methyl esters on the basis of their esterified carboxyl group content. Proto-, copro-, and uroporphyrin methyl esters, containing 2, 4, and 8 carboxyl side chains, respectively, migrate as expected, with uroporphyrin octamethyl ester behaving most like the tetrahydroporphyrin methyl esters.

Zumft (8) has reported that the reduction of nitrite as catalyzed by Chlorella nitrite reductase is inhibited by CO. The absorption spectrum of the complex between spinach nitrite reductase and CO is shown in Fig. 9. This complex exhibited wavelength maxima at 396 (Soret), 543 (β), and 585 (α) nm. The pattern of bands is characteristic of other siroheme containing enzymes (1, 5). It should be noted that the absorption spectrum of the CO complex of the extracted nitrite reductase heme (wavelength maximum at 593 nm for the α band) is identical to that of the CO complex of the siroheme extracted from *E. coli* sulfite reductase (Fig. 9).

DISCUSSION

We have shown previously that siroheme serves as the prosthetic group for enzymes catalyzing the reduction of sulfite to sulfide, a six-electron reduction process (1, 3–5). The results presented in this paper demonstrate the association of siroheme with a second six-electron reduction process, the reduction of nitrite to ammonia as catalyzed by the assimilatory ferredoxin-nitrite reductase of spinach. The presence of this new type of reduced iron-porphyrin compound in enzymes catalyzing two different multi-electron reduction reactions and its absence so far in other enzymes suggests that there may be significant common features in the mechanisms for both of these unusual redox reactions. This possibility is strengthened by the fact that many highly purified sulfite reductases have been shown to possess nitrite reductase activity. Some of this reactivity may be ascribed to the heme; were it due only to similarities in the protein binding sites, competitive inhibition could be expected, but not nitrite reduction. Conversely, if the low level of sulfite-reducing activity found in spinach nitrite reductase represents an activity of that enzyme and not of a contaminant, then the possibility may be entertained that reactivity with sulfite may reciprocally be an inherent property of at least one nitrite reductase.

Although no clear exceptions have yet emerged to the generalization that all adequately studied sulfite reductases contain siroheme as prosthetic group (3–5), this generalization cannot be made for nitrite reductases. Prakash and Sadana (14) have reported that a nitrite reductase that catalyzes the reduction of nitrite to ammonia as part of the process of nitrate respiration in *Achromobacter fischeri* contains a heme *c*

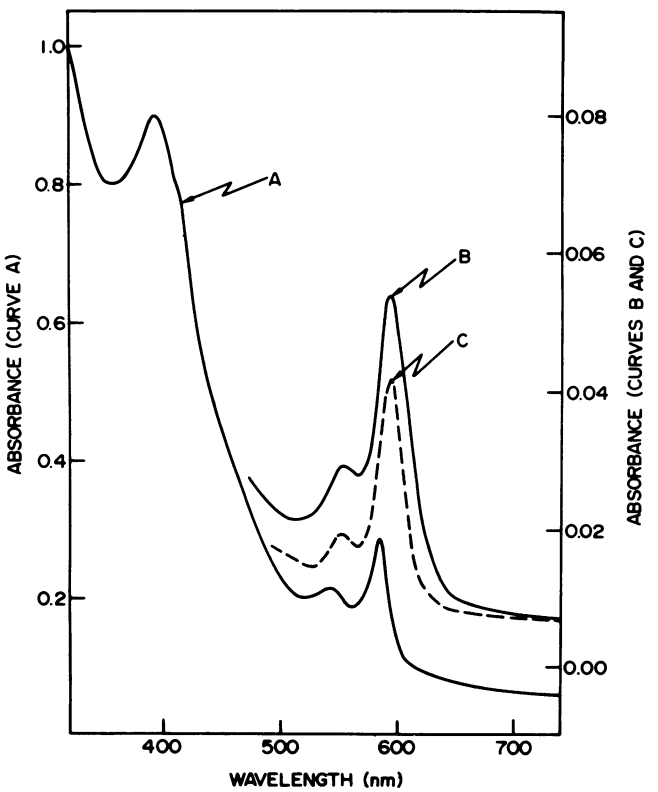

FIG. 9. Absorption spectra of CO complexes of nitrite reductase and the extracted hemes from nitrite reductase and sulfite reductase. The CO complex of spinach nitrite reductase was prepared by anaerobically adding 0.25 ml of enzyme, 19.4 mg/ml, to 0.5 ml of CO-saturated 50 mM potassium phosphate buffer, pH 7.7, and 0.25 ml of 1 mM MVH. After six hr, the MVH was allowed to oxidize by shaking the solution with air, and the absorption spectrum of the enzyme–CO complex (*A*) was recorded. The CO complexes of the extracted hemes of nitrite (*B*) and sulfite (*C*) reductases were prepared by the method described (1), with sodium dithionite as reductant.

prosthetic group as its sole iron-containing moiety. The analytical data of these authors are incompatible with the presence of siroheme in the *Achromobacter fischeri* nitrite reductase. Enzymes involved in the dissimilation of nitrite to gaseous nitrogen products (NO, N_2O, N_2, etc.) do not have the siroheme prosthetic group.

The discovery of siroheme may be significant for our understanding of the evolution of hemes and their roles in respiratory metabolism. Siroheme, associated with the ancient process of dissimilatory sulfate respiration in "sulfate-reducing" bacteria (4), has a structure that suggests that it might have been particularly suited to survive in a primitive photoreducing atmosphere (3). Murphy and Siegel (4) have suggested that this tetrahydroporphyrin derivative may indeed be an ancestral type of heme compound. The recent finding that *Clostridium bifermentans*, an obligate anaerobe previously thought to be devoid of heme compounds, possesses a sulfite reductase with a heme-like absorption spectrum presumably due to siroheme (H. D. Peck, Jr. & J. LeGall, personal communication), lends support to this view. The association of siroheme with reduction processes involved in the metabolism of two of the major elements of the biosphere, nitrogen and sulfur, suggests that this novel heme compound may have played a key role in the evolution of redox metabolism.

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