Vol. 96, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 16, 1980 Pages 278-285

ON THE NATURE OF THE OXIDATION-REDUCTION PROPERTIES OF NITRITE REDUCTASE FROM DESULFOVIBRIO DESULFURICANS

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Received July 12,1980

SUMMARY: Nitrite reductase as isolated from Desulfovibrio desulfuricans shows a complex set of rhombically distorted high-spin ferric heme and low-spin ferric heme resonances at 11°K. These resonances disappear (due to reduction to the diamagnetic ferrous state) on enzymatic reduction with hydrogen, hydrogenase from D. vulgaris and FAD as redox mediator. The addition of nitrite to the reduced enzyme results in reoxidation of nitrite reductase as evidenced by the reappearance of most of the initial signal intensities of high-spin and low-spin ferric heme resonances. Simultaneously an intense and unusual broadened signal appears in the g=2 region, suggesting the formation of a novel heme-nitric oxide signal with the main g-value at 2.08. Confirmation of this heme-nitric oxide complex was demonstrated by reoxidation of reduced enzyme with $^{15}NO_2^{-1}$ instead of $^{14}NO_2^{-1}$ resulting in a decrease from three to two of the hyperfine interaction pattern of nitrogen. Thus nitrite reduction to ammonia by nitrite reductase occurs via a heme-nitric oxide intermediate as reported with spinach nitrite reductase.

INTRODUCTION:

Nitrite reductases can be broadly grouped into two general categories on the basis of function: biosynthetic nitrite reductases which reduce nitrite to ammonia for nutritional purposes and respiratory nitrite reductases which are involved in terminal respiration. The respiratory nitrite reductases can be further subdivided into two types on the basis of function and products: Those catalyzing the six electron reduction of nitrite to ammonia and those reductases catalyzing the one electron reduction of nitrite to nitric oxide which are found in the denitrifying bacteria (1). These groups encompass reductases with quite different redox centers. The biosynthetic nitrite reductase (ammonia ferredoxin oxidoreductase, E.C.1.7.7.1) purified from spinach (2) contains one siroheme and a single Fe_4S_4 center. Reduced NAD, viologen dye or ferredoxin will serve as electron donors. A respiratory nitrite reductase (ammonia forming) has been purified from Achromobacteria fisheri (3) which contains only two c-type heme groups. The respiratory nitrite reductases

0006-291X/80/170278-08$01.00/0

(NO forming) [Nitric oxide (acceptor) oxidoreductase, E.C.1.7.99.3] contain both c and d-type hemes (4) or copper (5). Because reduced NAD or NADP only function as electron donors in the presence of other proteins, artificial electron donors such as reduced viologen dyes, ascorbate and phenazine methosulfate are frequently employed as electron donors in these assays.

Nitric oxide reacts with many heme proteins and produces a distinctive EPR signal in the g=2 region. Such signals have been observed with the respiratory nitrite reductase (cytochrome cd) from Thiobacillus denitrificans (6) and the biosynthetic nitrite reductase from spinach (7). In both cases it has been proposed that a heme-NO enzyme complex is an intermediate in the reduction of nitrite.

A respiratory nitrite reductase (ammonia forming) has recently been isolated from a nitrate respiring strain of Desulfovibrio desulfuricans (8,9) employing reduced methyl viologen as electron donor. It exhibits a typical cytochrome c-type spectrum and contains six c-type hemes with a molecular weight of 60,000. The heme groups are autooxidizable and the reduced form of the enzyme is fully reoxidized by nitrite. Nitric oxide formation has not been detected chemically; however, the enzyme will reduce hydroxylamine to ammonia.

In this communication*, we describe the unusual EPR properties of this hexaheme nitrite reductase and report the formation of a heme-NO EPR signal occurring on the reoxidation of the reduced reductase with nitrite. This latter signal differs from the signal found when nitrite reductase was reacted in the reduced state directly with nitric oxide, but the significance of this difference is unclear.

MATERIALS AND METHODS:

EPR measurements were made with a Varian Model 4051 A spectrometer operating at 100 kcycles modulation and an Air Products Automatic Liquid-Helium Temperature Control Unit. The frequency was measured with a Hewlett-Packard electronic counter (5245L) with a frequency converter plug-in (5255A) and magnetic field intensity with a Ventron Model G-502 precision NMR Gaussmeter. EPR spectra were directly transmitted via the Data General Corporation Nova 2 minicomputer

*These results were presented in part at the Annual Meeting of the American Society for Microbiology, Los Angeles, CA, 1979.

onto a Ball computer magnetic disc system for a permanent record and subsequent handling of experimental data. The spectra presented in Figures 1 and 2 represent the average of at least six scans and the spectra in Figure 3 represent the average of at least 16 scans. Spectra were obtained under nonsaturating microwave power conditions. Other EPR and experimental conditions are found in Figure Legends.

Nitrite reductase was purified according to the procedure of Liu (8,9). Hydrogenase from D. vulgaris was prepared according to van der Westen et al. (10).

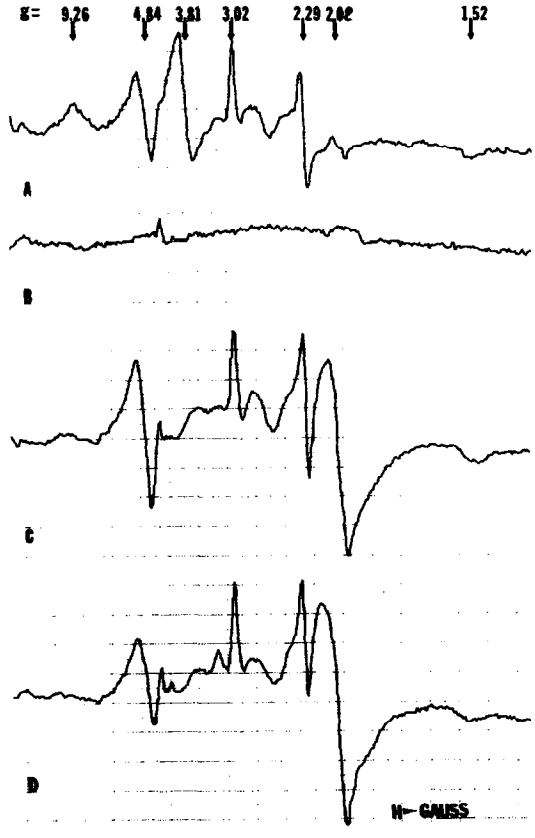

Figure 1. EPR spectra of nitrite reductase from Desulfovibrio desulfuricans. EPR conditions: microwave frequency, 9.189 GHz (frequency-matched EPR tubes); microwave power, 10 mW; modulation amplitude, 5.2 gauss; time constant, 0.1 sec; scanning rate, 1,000 gauss per min; temperature, 11°K and Gain=100 unless otherwise specified.

A. Isolated nitrite reductase (8.9 mg per ml in 100 mM potassium phosphate buffer, pH 7.6).
B. Nitrite reductase, as in A but reduced under anaerobic conditions with hydrogen, 0.5 mM FAD and 0.1 mg hydrogenase from Desulfovibrio vulgaris for 20 min.
C. As B, but then 0.15 M sodium nitrite added under anaerobic conditions and reacted for 1 min.
D. As A, but reduced under anaerobic conditions with excess sodium dithionite in the presence of 0.15 M sodium nitrite for 1 min.

RESULTS AND DISCUSSION:

Isolated Enzyme

The isolated enzyme reveals an extremely complex spectrum (Figure 1,A) over a 5000 gauss scan range. Since the only form of iron detected in nitrite reductase is from heme iron, all EPR signals observed must necessarily arise from heme iron. Ferric signals at g=9.26 and 3.81 were found to respond together during oxidation-reduction measurements and were therefore tentatively assigned to a severely distorted rhombic high-spin ferric heme signal based on the analogy to similar but not identical signals for cytochrome P-450 (11). The ferric heme signal at g=4.84 is quite unusual and the spin state of this species is presently unidentified. The other major signal is that of a low-spin ferric heme species with g-values at 3.02, 2.29, and 1.52.

The spectrum indicates multiple heme species in non-equivalent sites from a minimum of four and possibly six hemes. The enzyme is not completely in the oxidized state as judged by EPR spectroscopy since the addition of nitrite to the isolated enzyme causes a 63% increase in the ferric heme signal at g=4.84 but with no effect on any other signal. The addition of hydroxylamine to the isolated enzyme causes significant effects only when reacted under anaerobic conditions where it was found that the initial signal at g=4.84 declined approx. 50% and smaller decreases in signal intensities were observed at g=9.26 and g=3.81 and at g=3.02. The addition of the product ammonia (added as ammonium chloride) from nitrite reduction to the isolated enzyme causes no effect on any EPR signals.

Reduced Enzyme

It was essential to establish a reduction system which did not involve use of the chemical reductant sodium dithionite since the reaction of dithionite with nitrite generates nitric oxide chemically. It was found that complete reduction of nitrite reductase could be accomplished under anaerobic conditions in the presence of FAD as a redox mediator and hydrogen and hydrogenase from D. vulgaris. Figure 1,B shows that nitrite reductase is completely enzymati-

cally reduced by the latter reducing system as judged by the disappearance of all initial EPR signals due to reduction to the diamagnetic ferrous heme state. All initial EPR resonances detected in the isolated enzyme are thus very likely to be functional components of the enzyme's active site(s).

Reduced Enzyme Reacted with Nitrite or Hydroxylamine

Figure 1,C shows enzymatically reduced enzyme reacted with nitrite under anaerobic conditions. The ferric heme signal at g=4.84 and the low-spin ferric heme signal at g=3.02 have reappeared in full intensity indicating reoxidation to the initial EPR spectrum. A new and intense signal can be seen in the g=2 region suggesting the formation of a heme liganded nitrogen intermediate. Figure 1,D is of nitrite reductase reduced with sodium dithionite and reacted with nitrite; the same EPR spectral pattern is observed as found with enzymatically reduced nitrite reductase reacted with nitrite in Figure 1,C.

Figure 2 shows the five-fold expanded g=2 EPR region of nitrite reductase in order to examine this region in detail. Figure 2,A is of the isolated enzyme and no significant EPR signals are seen in this region. Figure 2,B is of the reduced enzyme ($FADH_2$) reacted with nitrite yielding the unusual and quite broad signal with the main g-value at 2.08. Three hyperfine lines attributable to ^{14}N hyperfine interaction with a splitting constant of 15.8 gauss are observed suggesting that the c-type hemes of nitrite reductase are liganded with a nitrogen-containing ligand. Confirmation that the hyperfine lines are indeed due to heme iron bound to a nitrogen-containing ligand occurred when the reduced enzyme was reacted with $^{15}NO_2^{-1}$. As can be seen in Figure 3 (expanded a further two-fold relative to Figure 2), nuclear hyperfine interaction has decreased from three lines (Figure 3,A; ^{14}N, I=1) to two lines (Figure 3,B; ^{15}N, I=½) indicating that the initial three line hyperfine pattern did indeed arise from a nitrogen ligand system.

The unusual heme-iron nitrogen ligand system was observed by reduction of enzyme with dithionite plus nitrite and very nearly the same EPR spectrum and

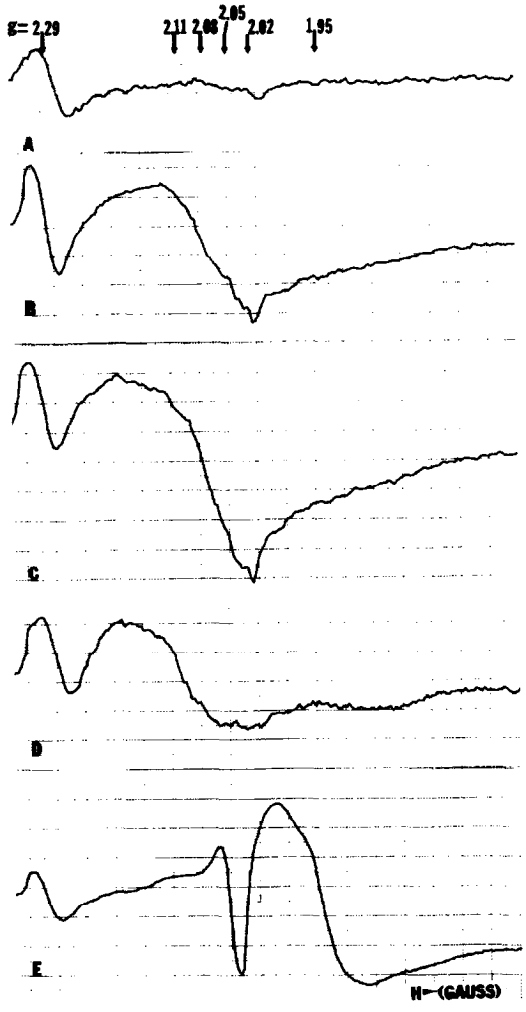

Figure 2. EPR spectra of nitrite reductase from D. desulfuricans. EPR conditions as in Figure 1, except scanning rate is 200 gauss per min and Gain=100 for A-D and Gain=50 for E.

A. As Figure 1,A.
B. As Figure 1,C.
C. As Figure 1,D.
D. As Figure 1,B but then 0.10 M hydroxylamine was added under anaerobic conditions and reacted for 1 min.
E. As Figure 1,B but then reacted with nitric oxide under anaerobic conditions for 1 min.

^{14}N hyperfine interaction pattern as found in Figure 2,B was observed in Figure 2,C.

Figure 2,D is reduced nitrite reductase reacted with hydroxylamine yielding a similar broad EPR spectrum which is however slightly shifted to low-field (major g-value at 2.11). This spectrum indicates that as with nitrite, a

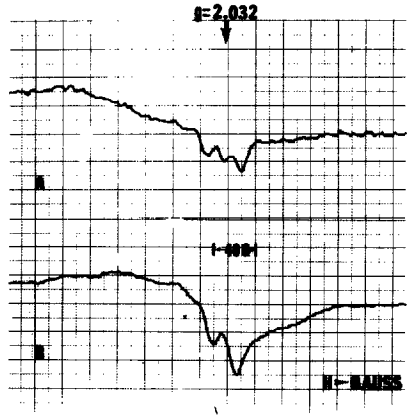

Figure 3. EPR spectra of enzymatically reduced nitrite reacted with $^{14}NO_2^{-1}$ or $^{15}NO_2^{-1}$. EPR conditions as in Figure 2 except that temperature was 26°K, microwave power, 3 mW and scanning rate was 100 gauss per min.

A. As Figure 2,B, i.e. enzymatically reduced enzyme reacted under anaerobic conditions with 0.15 M $^{14}NO_2^{-1}$ for 1 min.

B. As Figure 2,B but enzymatically reduced enzyme reacted under anaerobic conditions with 0.15 M $^{15}NO_2^{-1}$ for 1 min.

similar intermediate involving heme iron and a nitrogen-bearing ligand can be generated from hydroxylamine, presumably by oxidation of hydroxylamine to nitric oxide. It should be pointed out that the addition of the product of nitrite reduction, ammonia, caused no reoxidation or effect on the enzymatically reduced EPR spectrum.

The unusual EPR parameters (broadened signal, relatively unusual g-value and signal shape) observed in this study compared to more typical heme-nitric oxides complexes (12,13) are tentatively attributed to an interaction between the low-spin ferric heme (spin=½ systems) and the ferrous nitric oxide complexes ($\Delta m=1$) as spin coupled induced broadening. Broadened metal-nitric oxide complexes arising from such postulated interactions have been reported by Uiterkamp and Mason (14) and Uiterkamp et al. (15).

Nitrite reductase reacted with nitric oxide

Figure 2,E shows the EPR spectrum resulting when reduced nitrite reductase has been reacted directly under strictly anaerobic conditions with nitric oxide. It is disturbing that the spectrum found in Figure 2,E is not at all similar to that found with the reduced enzyme reacted with nitrite or with dithionite

reduced enzyme reacted with chemically generated nitric oxide. Further experiments are in progress to clarify this point since a rational explanation for this discrepancy can not presently be offered.

In spite of major differences in the chromophores of nitrite reducing enzymes all that have been studied reveal similar heme-nitric oxide signals suggesting a common mechanism for nitrite reduction.

Acknowledgements

These studies were supported by Research Grant No. GM-18895 from the National Institutes of General Medical Sciences to D.V.D. and Contract No. DEAS09-79 ER10499 from the Department of Energy to H.D.P. We thank Mr. Vance Morgan for his valuable assistance during EPR computer data accumulation, Drs. Jack Peisach, Bill Blumberg and Graham Palmer for very helpful discussions and also to Dr. Palmer for a gift of $^{15}NO_2^{-1}$.

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