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Comparative EPR studies on the nitrite reductases from *Escherichia coli* and *Wolinella succinogenes*

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Hexaheme nitrite reductases purified to homogeneity from *Escherichia coli* K-12 and *Wolinella succinogenes* were studied by low-temperature EPR spectroscopy. In their isolated states, the two enzymes revealed nearly identical EPR spectra when measured at 12 K. Both high-spin and low-spin ferric heme EPR resonances with g values of 9.7, 3.7, 2.9, 2.3 and 1.5 were observed. These signals disappeared upon reduction by dithionite. Reaction of reduced enzyme with nitrite resulted in the formation of ferrous heme-NO complexes with distinct EPR spectral characteristics. The heme-NO complexes formed with the two enzymes differed, however, in g values and line-shapes. When reacted with hydroxylamine, reduced enzymes also showed the formation of ferrous heme-NO complexes. These results suggested the involvement of an enzyme-bound NO intermediate during the six-electron reduction of nitrite to ammonia catalyzed by these two hexaheme nitrite reductases. Heme proteins that can either expose bound NO to reduction or release it are significant components of both assimilatory and dissimilatory metabolisms of nitrate. The different ferrous heme-NO complexes detected for the two enzymes indicated, nevertheless, their subtle variation in heme reactivity during the reduction reaction.

Nitrite reductase; EPR; Hexaheme; Nitric oxide intermediate; Dissimilatory ammonia-forming pathway

1. INTRODUCTION

Based on their physiological involvements, ammonia-forming nitrite reductases can be categorized into two distinct types. The biosynthetic nitrite reductases isolated from spinach (ammonia: ferredoxin oxidoreductase, EC 1.7.7.1) contain, for example, one siroheme plus a single [Fe_4S_4*] center per 61 kDa and can utilize reduced NAD, viologen dyes or ferredoxin as electron donors [1,2]. This group of enzymes catalyzes the reduction of nitrite to ammonia which is subsequently assimilated. A different ammonia-forming nitrite reductase is involved in the anaerobic nitrate respiration process. This enzyme, first purified from *Desulfovibrio desulfuricans* (ATCC 27774), was found to contain six *c*-type hemes per molecule of enzyme [3]. Hexaheme nitrite reductases have been purified from *Wolinella succinogenes* [4] and *Escherichia coli* K-12 [5,6] and it is concluded that this multiple-heme cytochrome is the dissimilatory ammonia-forming nitrite reductase in bacteria.

An earlier EPR spectroscopic study revealed that, in the isolated state, *D. desulfuricans* nitrite reductase [7] exhibited a complex set of rhombically distorted high-spin ferric and low-spin ferric heme resonances which disappeared upon reduction of the enzyme. The addition of nitrite to the reduced enzyme resulted in its reoxidation with the concurrent formation of a heme-NO complex exhibiting an intense and broadened signal in the $g = 2$ region. It was suggested that the reduction of nitrite to ammonia catalyzed by the *D.*

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desulfuricans enzyme occurred via a heme-NO intermediate. Here, we describe the EPR properties of the purified *E. coli* and *W. succinogenes* nitrite reductases. Evidence for the formation of heme-NO complexes upon addition of nitrite to the reduced enzymes is presented. Results are discussed with respect to the significance of these heme-NO derivatives in their enzymatic reaction mechanisms.

2. MATERIALS AND METHODS

EPR measurements were made with a Varian E-109 spectrometer interfaced with a Hewlett-Packard HP 9816 microcomputer for data collection and subsequent handling. Measurements at low temperature were performed with an Air Products APD-E automatic temperature controller. The spectra presented in each figure represent the average of at least two scans. Spectra were obtained under nonsaturating microwave power conditions. Other EPR and other experimental conditions are to be found in the respective figure legends.

E. coli K-12, strain CB900 and *W. succinogenes* (VPI 10659) were grown at 37°C under nitrate/nitrite respiring conditions according to Abou-Jaoude et al. [8] and Yoshinari [9], respectively. Hexaheme nitrite reductases were purified from these two organisms based on the procedures of Liu et al. [4,5]. Specific activities of the purified *E. coli* and *W. succinogenes* nitrite reductases were determined to be 517.9 and 766.7 U/mg protein [3], respectively.

3. RESULTS AND DISCUSSION

For the two enzymes studied, EPR measurements were made under the following conditions: as isolated, dithionite-reduced and reduced followed by reaction with nitrite. Results obtained under these conditions with both enzymes are described below.

3.1. *Isolated state*

Both *E. coli* and *W. succinogenes* nitrite reductases, in their isolated states, exhibited complex, but surprisingly similar, spectra over a 5000 G scan range (fig.1). EPR resonances with g values of 9.7, 3.7, 2.9, 2.3 and 1.5 were observed. Since heme

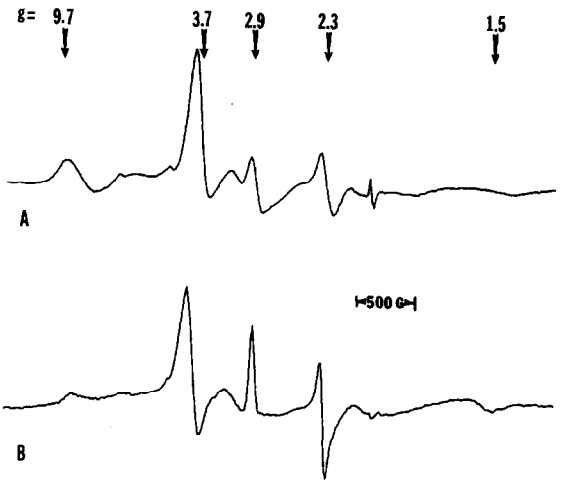

Fig.1. EPR spectra of nitrite reductases from *W. succinogenes* (50 μM in protein, in 100 mM potassium phosphate buffer, pH 7.6), trace A; and from *E. coli* (83 μM in protein and above buffer), trace B. (A) Nitrite reductase from *W. succinogenes*, as isolated. EPR conditions: temperature, 12 K; microwave frequency, 9.163 GHz; scanning rate, 1000 G/min; time constant, 0.3 s; modulation amplitude, 10 G; microwave power, 10 mW.

iron constitutes the only form of iron present in these two enzymes [4,5], all EPR signals detected must necessarily arise from heme iron. Resonances at $g = 9.7$ and 3.7 were found to respond together during oxidation-reduction measurements and were assigned to severely distorted rhombic high-spin ferric signals, which resemble to some extent the EPR signals for cytochrome P-450 [10]. Resonances at $g = 2.9$, 2.3 and 1.5 are assigned to low-spin ferric heme species. Both spectra indicate multiple-heme species in non-equivalent sites, which is consistent with the hexaheme nature of the two nitrite reductases as determined by both heme and iron content analyses [4,5]. The environments about the heme iron atoms appear to be similar in *E. coli* and *W. succinogenes* nitrite reductases as indicated by their nearly identical EPR spectra shown in fig.1. These spectra also resemble to a great extent that of *D. desulfuricans* nitrite reductase [7], the first such enzyme identified in the hexaheme nitrite reductase family.

3.2. *Dithionite-reduced state*

Upon reduction by dithionite, both enzymes ex-

hibited fully reduced spectra when they were analyzed by UV-visible spectroscopy [4,5]. Under the same condition, all initial EPR signals were found to disappear which suggests the reduction of all heme irons to the diamagnetic ferrous state in both enzymes.

3.3. *Reduction followed by reaction with nitrite*

The EPR spectra taken from dithionite-reduced *E. coli* and *W. succinogenes* nitrite reductases following reaction with nitrite are shown in fig.2. The ferric heme signals at $g = 2.9$ and 2.3 have reappeared in nearly full intensity indicating their reoxidation to the initial states. In both cases, however, a new and intense signal is observed in the $g = 2$ region suggesting the formation of a heme-liganded nitrogen intermediate. To determine whether the signal is indeed derived from heme iron bound to a nitrogen-containing ligand, the same experiment was carried out using $^{15}NO_2^-$.

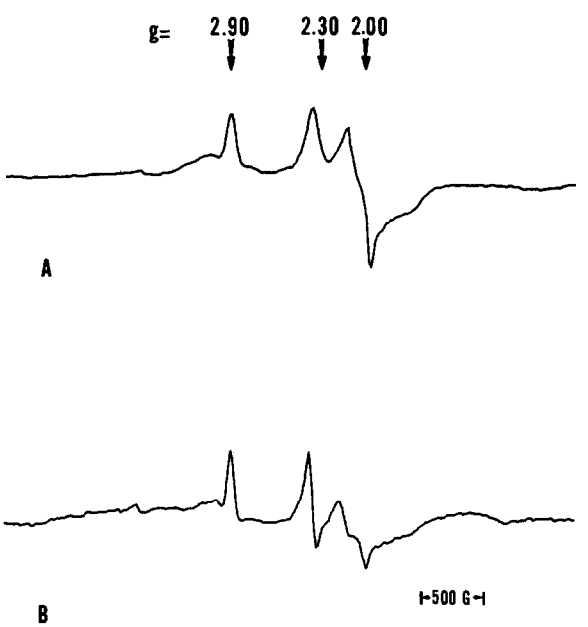

Fig.2. EPR spectra of nitrite reductases from *W. succinogenes* and *E. coli*, reduced with a slight excess of solid sodium dithionite and then reacted with sodium nitrite (final concentration, 10 mM, pH 7.6). Protein concentrations and buffer as in fig.1. (A) Reduced nitrite reductase from *W. succinogenes* reacted with 10 mM sodium nitrite. EPR conditions as in fig.1. (B) Reduced nitrite reductase from *E. coli* reacted with 10 mM sodium nitrite. EPR conditions as in fig.1.

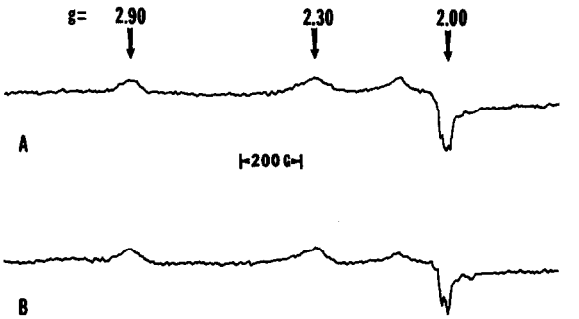

Fig.3. EPR spectra of nitrite reductase from *W. succinogenes*, reduced with sodium dithionite and then reacted with 10 mM $^{14}NO_2^-$ or $^{15}NO_2^-$. Protein concentrations and buffer as in fig.1. (A) Reduced nitrite reductase reacted with 10 mM $^{14}NO_2^-$. EPR conditions as in fig.1 except scanning rate was 250 G/min and microwave power was 50 mW. (B) Reduced nitrite reductase with 10 mM $^{15}NO_2^-$. EPR conditions as in A.

Fig.3 shows the expanded $g = 2$ region of the EPR spectra obtained in these experiments. When the reduced *W. succinogenes* nitrite reductase was reacted with $^{14}NO_2^-$, three hyperfine lines attributable to ^{14}N hyperfine interaction with a splitting constant of 15.8 G were observed (fig.3A). When the enzyme was reacted with $^{15}NO_2^-$, the nuclear hyperfine interaction decreased to two lines (fig.3B). It is thus confirmed that the initial three-line hyperfine pattern indeed arises from a nitrogen ligand system. The same results were obtained when *E. coli* nitrite reductase was used. Similar to that found with *D. desulfuricans* nitrite reductase, this unusual heme iron nitrogen ligand system can be attributed to an interaction between the low-spin ferric heme and the ferrous NO complexes. Broadened metal-NO complexes arising from such postulated interactions have previously been reported by Uiterkamp and Mason [11] and Uiterkamp et al. [12].

Taking together our previous results of studies on *D. desulfuricans* nitrite reductase [7] and the EPR data on *E. coli* and *W. succinogenes* nitrite reductases shown in the present work, it seems clear that the heme groups in these three hexaheme nitrite reductases are in similar environments as suggested by their nearly identical signature spectra in the oxidized state. On the other hand, the presence of a broad $g = 2$ signal in the spectra

observed with these reduced enzymes, followed by reaction with nitrite, seems to indicate that the involvement of an enzyme-bound NO intermediate may be a common denominator in the reaction mechanism of the hexaheme nitrite reductases.

A diheme cytochrome has been purified from *Vibrio* (formerly *Achromobacter*) *fischeri* and identified as a dissimilatory nitrite reductase [16]. A recent study [17] with this reductase revealed an oxidized EPR spectrum very similar to that of the hexaheme nitrite reductases. It is therefore concluded that the nitrite reductase of *V. fischeri* is a hexaheme type of nitrite reductase.

Heme-NO EPR signals appear to be a common feature for all sorts of cytochrome-type nitrite reductases. For example, the biosynthetic nitrite reductase from spinach [2], which catalyzes a six-electron reduction of nitrite to ammonia, also exhibits a heme-NO intermediate. Signals characteristic of heme-NO complexes have been observed in the cd_1 cytochromes of *Thiobacillus denitrificans* [13,14] and *Pseudomonas aeruginosa* [15]. The fate of cytochrome-bound NO (e.g. reduction to ammonia or release as a gas that is subsequently reduced to N_2O) thus constitutes a significant branch-point event in the nitrogen cycle in nature.

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