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ON THE PURIFICATION OF NITRITE REDUCTASE FROM THIOBACILLUS DENITRIFICANS AND ITS REACTION WITH NITRITE UNDER REDUCING CONDITIONS

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SUMMARY: Nitrite reductase (cytochrome cd) from T. denitrificans has been crystallized in high yield in three simple and rapid steps. The spectral absorption ratio at 408 to 280 nm was 1.52. Light absorption spectra in the oxidized and reduced states were virtually identical to those of nitrite reductase from P. aeruginosa. EPR spectroscopy of nitrite reductase at 12° showed a low-spin ferric heme resonance with g-values at 2.52, 2.45 and 1.73 assigned to the d-heme. Reaction of nitrite reductase with nitrite in the presence of the reducing systems [(ascorbate + PMS) or sulfide] resulted in the formation of nitric oxide (confirmed by gas chromatography) which reacted with both c- and d-hemes of nitrite reductase yielding an EPR-detectable enzyme-NO complex with g-values at 2.07, 2.04 and 1.99 and a ^{14}N hyperfine splitting constant of 22.5 gauss. The amount of nitric oxide produced enzymatically with sulfide as electron donor was only 5% of that found when ascorbate plus PMS served as reductant.

To our knowledge the detection of the unique enzyme-NO complex is the first definitive EPR evidence for the mandatory liganding of nitric oxide with pure nitrite reductase during nitrite reduction.

INTRODUCTION:

Cytochrome cd from Thiobacillus denitrificans is a hemoprotein with a c-type and d-type heme moiety (1). The bacteria form cytochrome cd only when grown anaerobically in a medium containing nitrate or nitrite. The enzyme's true biological reaction is the dissimilatory reduction of nitrite to nitric oxide, and in this study it is termed nitrite reductase despite its secondary ability to utilize O_2 as a possible electron acceptor. Nitrite reductase (cytochrome cd) has also been found in Pseudomonas aeruginosa (2), Paracoccus (formerly Micrococcus) denitrificans (3) and Pseudomonas perfectomarinus (4). Nitrite reductase from P. aeruginosa has been extensively studied since it was first crystallized by Okunuki and his coworkers (5) and later with improved purification procedures by Gudat et al. (6) and Parr et al. (7). The enzyme contains two heme c and two heme d moieties with a molecular weight of 120,000 and accepts electrons from the copper protein (azurin) as well as cytochrome c-551.

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A partially purified preparation of nitrite reductase containing a c-type heme was prepared from P. perfectomarinus. EPR spectroscopy at liquid-nitrogen temperatures provided direct EPR evidence for a characteristic hemo-protein-NO complex indicating that nitric oxide was an obligatory and readily EPR-detectable product (4).

In addition to the biochemical significance of nitrite reduction, the agronomic consequences are immense. Crop plants can assimilate nitrate or nitrite from the soil and reduce either ion to ammonia needed for protein and nucleic acid synthesis during growth. But, the instant that nitrite reductase (cytochrome cd) functions during bacterial denitrification, the nitrogen atom in the resultant nitric oxide molecule passes the point of no return as a nutrient. Only after two molecules of nitric oxide are reduced by the bacteria to nitrous oxide, and then dinitrogen, can such atoms regain their potential for fixation and utilization once more as nutrients As the critical event in the Nitrogen Cycle and the specific reaction that initiates the impoverishment of the soil for nitrogen, cd-cytochrome-mediated nitrite reduction thus deserves intense scrutiny.

The current study describes a simplified procedure for the preparation of crystalline nitrite reductase from T. denitrificans with a 408 nm to 280 nm absorption ratio higher than that reported for P. aeruginosa nitrite reductase (cytochrome cd) (2,5-7). Secondly, the interaction of reduced nitrite reductase with nitrite was studied by EPR spectroscopy at liquid-helium temperatures and evidence was obtained for a unique EPR-detectable nitrite reductase-NO complex.

METHODS:

T. denitrificans (Strain RT) was grown as previously described by Schedel et al. (8). A crude cell extract prepared according to (8) was passed onto Amberlite CG-50 and equilibrated with 10 mM potassium phosphate buffer (pH 7.6). Nitrite reductase (cytochrome cd) was adsorbed. Nitrite reductase was then eluted with a potassium phosphate buffer (pH 7.6) gradient. Elution of the enzyme occurred at 160 mM. Nitrite reductase was then diluted two-fold with distilled water and readsorbed on a fresh Amberlite CG-50 column. Elution with the potassium phosphate buffer gradient was repeated exactly as before. Nitrite reductase was crystallized at 60% ammonium sulfate saturation. The yield from 100 g of cell paste was approximately 45 mg.

EPR spectra were obtained for samples at liquid-helium temperatures as previously described (9). EPR conditions are described in figure legends. Light-absorption spectroscopy of oxidized and reduced nitrite reductase was carried out with an Aminco DW-2 dual-wavelength spectrophotometer.

Detection of nitric oxide and other gas products was performed by gas chromatography according to Barbaree and Payne (10).

RESULTS AND DISCUSSION:

Light-Absorption Spectra of Nitrite Reductase

Crystalline nitrite reductase (cytochrome cd) was prepared in high yield in three simple, rapid steps. The light-absorption spectrum of oxidized nitrite reductase exhibited maxima at 280, 408, 525 and 642 nm with shoulders at 291, 329 and 353 nm. The ratio of absorbance at 408 to that at 280 nm was 1.52 which is higher than that reported for nitrite reductase from P. aeurginosa (6,7). On reduction with a slight excess of sodium dithionite, nitrite reductase showed absorbance maxima at 419, 461, 523, 549, 555 and 562 nm with a shoulder at 615 nm. The absorbance maxima in the oxidized and reduced states of nitrite reductase resemble those of nitrite reductase from P. aeruginosa (6) and T. denitrificans (1), except that in the latter case the reduced spectrum shows anomolies above 600 nm, especially a maximum at 695 nm. Sawhney and Nicholas required five steps to prepare an enzyme but reported no 408 to 280 nm absorbance ratio. In the current study spectral characteristics above 600 nm are identical with those of reduced nitrite reductase from P. aeruginosa (6).

EPR Spectroscopy

The EPR species detected in the low-temperature (12°K) EPR spectrum of nitrite reductase is that of the low-spin ferric d-heme with g_z, g_y and g_x values* of 2.52, 2.45 and 1.73, respectively (Fig. 1, I-A). The assignment of the ferric low-spin species observed to the d-heme is based on the studies of Hill and Wharton (11) who removed the d-heme component from nitrite reductase of P. aeruginosa and reconstituted the d-heme to form an active enzyme. The g-values of the latter d-heme system definite ly corresponded to 2.45 and 1.71. Five-fold expansion of the g=2.45 region (Fig. 1, II-A) clearly resolved the g_z and g_y components of the ferric low-spin d-heme of nitrite reductase. The addition of a slight excess of the oxidant potassium ferricyanide had no appreciable effect on either the intensity and the line-shape of the observed EPR signals of the d-heme or the light-absorption spectrum of nitrite reductase, indicating that the enzyme was already maximally oxidized. Quantitation by double in-

* g_z, g_y and g_x are defined as the end maximum, mid-point crossing and end minimum, respectively, of the first derivative absorption.

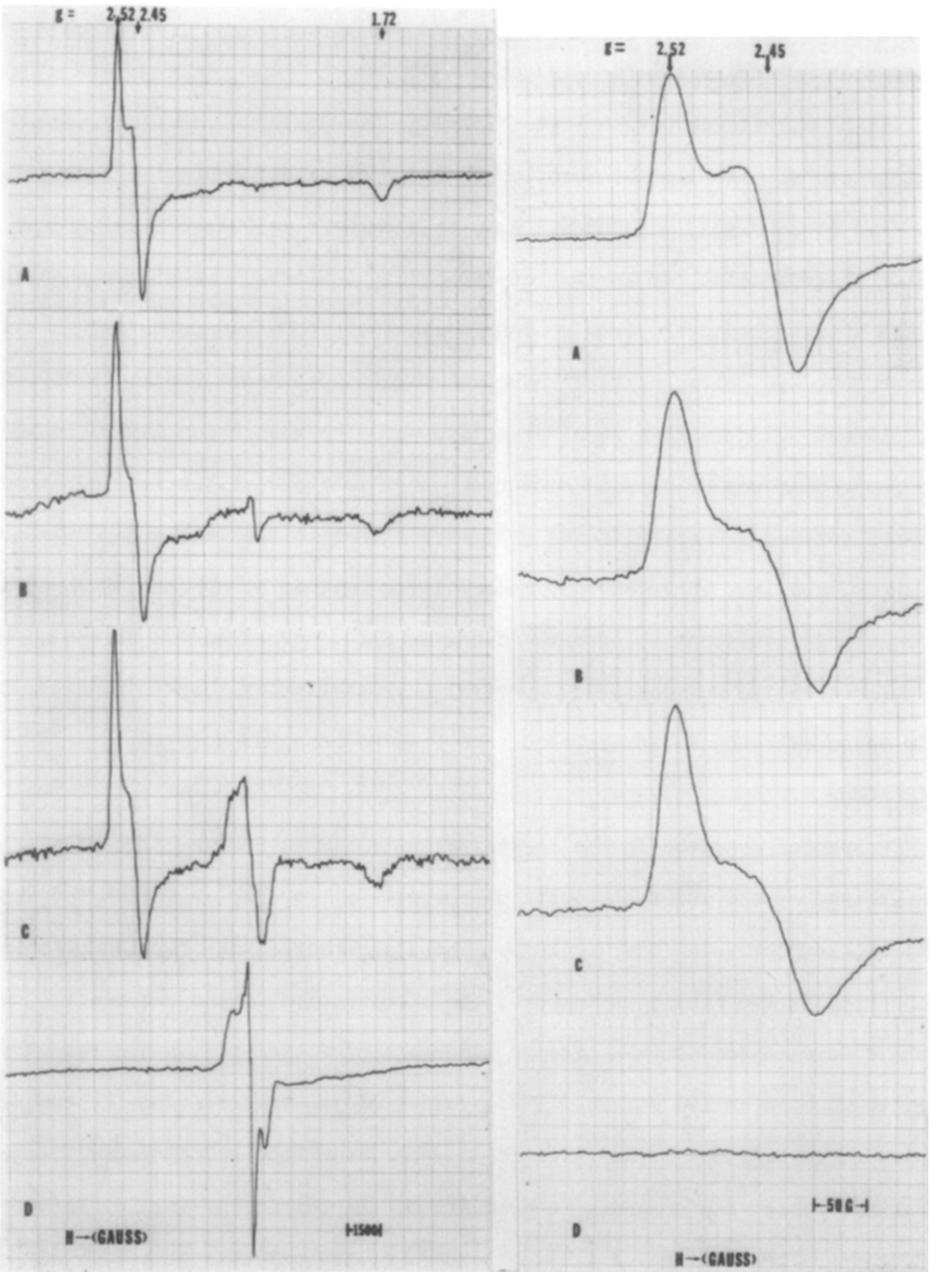

Figure 1. EPR spectra at 12°K of nitrite reductase from T. denitrificans. EPR conditions: microwave frequency, 9.188 GHz; microwave power, 2 mW; time constant, 0.1 sec; scanning rate, 1000 gauss per min; modulation amplitide 5.9 gauss.

I. A. Crystalline nitrite reductase (37 μM) dissolved in 100 mM sodium phos phate buffer (pH 7.6), Gain 100.

tegration of the d-heme low-spin ferric heme resonance with Cu(II)-EDTA as standard (12) recovered one heme, which is consistent with the detection of only the d-heme component of nitrite reductase. The concentration of the enzyme was determined by light-absorption spectroscopy based on the millimolar extinction coefficient of 148 at 408 nm in the oxidized state (13).

The addition of sodium nitrite to nitrite reductase resulted (Fig. 1, I-B and II-B) in a decrease of signal intensity at g_y of 58% of the low-spin ferric d-heme component and the appearance of a very small g=2.00 signal attributable apparently to a small amount of endogenously reduced nitrite reductase which had reacted with nitrite to yield nitric oxide. The nitric oxide formed then reacted rapidly with nitrite reductase to give an enzyme-NO complex. The intensity of this latter complex corresponds to 2.2% of the maximum enzyme-NO complex signal found in the presence of the complete enzymatic nitrite reducing system, i.e., sodium ascorbate plus phenazine methosulfate (PMS). No nitric oxide evolution was detected by gas chromatography under the experimental conditions of Fig. 1, I-B and II-B. These observations indicate that the small amount of enzyme-NO complex formed was due to considerably less than one enzyme turnover.

The addition of ascorbate with sodium nitrite to nitrite reductase (Fig. 1, I-C and II-C) resulted in a further slight decrease in intensity at g=2.45 (final decrease of 64%) of the ferric low-spin d-heme component along with a further increase in the g=2.00 enzyme-NO complex. The intensity of this latter complex now corresponded to 4.2% of that found with the complete enzyme reducing system (see above). When PMS was added along with ascorbate and nitrite to nitrite reductase there was complete loss of the low-spin ferric d-heme EPR absorption due to reduction of the d-heme to

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B. As A but nitrite reductase in the above buffer and at the same enzyme concentration in a different EPR tube (all EPR tubes frequency-matched) reacted with 20 mM sodium nitrite for 1 min, Gain 200.

C. As A but nitrite reductase in a different EPR tube reacted with 20 mM sodium nitrite and 4 mM sodium ascorbate (pH 7.6) for 1 min, Gain 175.

D. As A but nitrite reductase in a different EPR tube reacted with 20 mM sodium nitrite, 4 mM sodium ascorbate and 0.5 mM phenazine methosulfate for 1 min, Gain 100.

II. A-D are identical with I A-D in all respects except that the spectra are expanded five-fold by using a scanning rate of 200 gauss per min.

the low-spin ferrous diamagnetic state, and the maximum appearance of the g=2.00 enzyme-NO complex (Fig. 1, I-D, II-D). The enzyme-NO complex was formed maximally in 1 min but gas chromatographic measurements indicate that the sole product nitric oxide was evolved continuously and in a linear fashion during the 30 min period examined. Nitric oxide was the only product detected in contrast with the studies of Sawhney and Nicholas (1) who found substantial amounts of nitrous oxide along with nitric oxide. The same maximum intensity of the enzyme-NO complex was recorded even when the PMS concentration varied 10-fold, i.e., 0.5 mM and 0.05 mM PMS. The intensity of the enzyme-NO complex signal represented a 46-fold increase over that of the enzyme plus nitrite only. Quantitation by double integration of the unique EPR detectable enzyme-NO complex showed that 2 hemes of the enzyme were liganded with nitric oxide. This very likely means that both the d-heme (the only EPR species observed) and the c-heme are involved in the binding of nitric oxide (the product of nitrite reduction) with nitrite reductase. This explanation is supported by light-absorption spectroscopy (not shown) which indicates that after reaction with nitrite under reducing conditions the c-heme of nitrite reductase was only partially available for reduction to the ferrous state as characterized by appearance of the normal intensities of the α and β peak absorbances. This interpretation is consistent with the results of light-absorption studies (14) which showed that both the c and d-hemes of nitrite reductase from P. aeruginosa bind nitric oxide under reducing conditions.

The addition of sodium sulfide (1) to nitrite reductase resulted in identical EPR spectral changes and led to both the same intensity of the enzyme-NO complex (cf. Fig. 1, I-D and II-D) and the complete loss of the low-spin ferric d-heme signal due to its reduction to the diamagnetic low-spin ferrous state. Although the enzyme-NO complex was formed, also in 1 min, gas chromatographic measurements indicated the continuous evolution of the sole product, nitric oxide, over a 30 min period. The amount of nitric oxide evolved was, however, 5% of that released from the ascorbate-PMS nitrite reducing system.

The ascorbate-PMS or sulfide reduced enzyme-NO complex is expanded five-fold in Fig. 2,A and 2,B. As mentioned above the recovered spin intensities suggested that

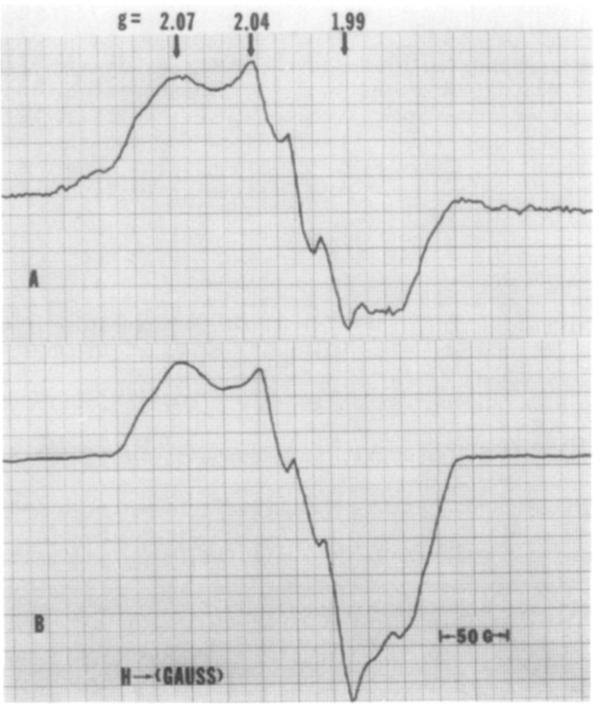

Figure 2. EPR spectra at 26°K of nitrite reductase from T. denitrificans. Nitrite reductase concentration and buffer as in Fig. 1. EPR conditions as in Fig. 1 except microwave frequency is 9.187 GHz and scanning rate is 200 gauss per min.

A. As Fig. 1, I-D, Gain 100.
B. As Fig. 1, I-B, except that nitrite reductase reacted with 20 mM sodium nitrite and 20 mM sodium sulfide (pH 7.6) for 1 min, Gain 125.

both the c and d-hemes of nitrite reductase were liganded to nitric oxide. The EPR spectra in Fig. 2,A & B show two species and a triplet signal that arose from nuclear hyperfine splitting from ^{14}N (I=1) with a hyperfine coupling constant of 22.5 gauss. All three EPR parameters (g-values of 2.07, 2.04 and 1.99, line shape and nitrogen hyperfine splitting) are strikingly similar to those for other hemoprotein-nitric oxide complexes (15-18). The characteristic EPR parameters may be diagnostic for the interaction of the product nitric oxide (fron nitrite reduction) with a nitrite reductase system. These results also indicate that during nitrite reduction the enzyme exists as the enzyme-NO complex and continuously releases nitric oxide from the active site. These results are consistent with our previous observations with the partially purified nitrite reductase from P. perfectomarinus (4).

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