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The bacterial nitrate reductases: EPR studies on nitrate reductase A from *Micrococcus denitrificans*

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SUMMARY

EPR studies on nitrate reductase indicate at 80 °K a signal at g=1.985 and g=2.045 attributable to Mo(V). At 15 °K a signal is observed at g=2.016 due to Fe(III) in an unknown symmetry. The reaction of nitrate reductase with nitrate, nitrite or azide results in changes of the Mo(V) signal. Quantitation of the Mo(V) signal intensity accounts for 15% of the chemical molybdenum content. A similar quantitation of the Fe(III) signal intensity indicates equimolarity with enzymatically reducible g=1.9-type resonance.

Enzymatic reduction of nitrate reductase and nitrate with sodium dithionite *plus* benzyl viologen or molecular hydrogen *plus* hydrogenase results in the disappearance of the initial Mo(V) and Fe(III) signals and the appearance of a temperature-sensitive iron-sulfide absorption with g-values at 2.057, 1.947 and 1.881 when measured at 15 °K. Chemical reduction of nitrate reductase with sodium dithionite also results in loss of the initial Mo(V) and Fe(III) signals but gives a more complex iron-sulfide signal consisting of two different types of iron-sulfide systems. Quantitation of both types of iron-sulfide system indicated they are present in approx. equal concentrations. These two iron-sulfide systems may differ in redox potential.

These studies support the involvement of iron and molybdenum in nitrate reductase.

Nitrate reductase A ($FMNH_2$:nitrate (chlorate) oxidoreductase A) is a membrane-bound enzyme which has a respiratory function in the anaerobic life of many bacteria.

Taniguchi and Itagaki[1] reported that the nitrate reductase in *Escherichia coli* had a molecular weight of $1 \cdot 10^6$ and contained 40 atoms of iron and 1 atom of molybdenum per mole.

Recently Lam and Nicholas[2] purified from *Micrococcus denitrificans* a nitrate reductase which contained molybdenum; however, they did not estimate the amount. Forget[3] solubilized nitrate reductase A by alkali-acetone treatment of particles from *M. denitrificans* and purified the enzyme 50-fold. The enzyme was found to be highly labile, did not contain flavin or heme and was found to exhibit a brown color. The light-absorption spectrum was found to increase progressively from 600 to 280 nm revealing a weak maximum at 410 nm. This type of spectrum is in agreement with the presence of non heme iron. Chemical determinations of nitrate reductase revealed maximally approx. 8 atoms each of iron and labile sulfide as well as 0.4 atom molybdenum per mole (mol. wt 160 000). The reaction of sodium dithionite with nitrate reductase was found to result in a decrease of absorbance in the optical spectrum. The loss of absorbance could be partially restored by the addition of nitrate.

The presence of iron and molybdenum in highly purified preparations of nitrate reductase raised the strong possibility of their involvement in the mechanism of action of the enzyme. We were prompted then to undertake the first EPR studies of a homogenous preparation of nitrate reductase in order to obtain evidence for the functional role of iron and molybdenum. The results obtained indicate a clear involvement of both metals in enzymatic action. A preliminary report of these studies has appeared elsewhere[4].

Nitrate reductase A was prepared from *M. denitrificans*, strain 4 (Delft collection) by a procedure previously described[3], except that the Sephadex G-200 gel filtration was conducted on a column of greater height (5 cm × 160 cm) and the second calcium phosphate gel step was omitted. These modifications resulted in a preparation of nitrate reductase with a specific activity of 73.3 μmoles nitrate reduced per min per mg protein. The protein was essentially homogenous by analytical disc electrophoresis. The molybdenum content of nitrate reductase used in these studies was determined by the dithiol method[5].

EPR spectroscopy was carried out as previously described[6,7]. EPR measurements of molybdenum absorptions were determined at 80 °K with an automatic variable temperature system while iron-sulfide resonances were determined at 15 °K using a helium transfer system involving a rapid flow of cold helium gas from heated liquid helium. Temperature measurements (10 to 40 °K) were determined with a calibrated Cryocal thermocouple mounted directly beneath the sample. Further EPR conditions are detailed in figure legends. Quantitation of EPR signal intensities was determined by double integrations using a cupric-EDTA standard and integrated intensities were corrected for g-value dependence on transition probability by the method of Aasa and Vänngard[8].

Isolated nitrate reductase revealed at 80 °K an asymmetric EPR signal at $g_\perp = 1.985$ and $g_\parallel = 2.045$ (Fig. 1A). Based on its presence by chemical analysis, observed g-values, EPR microwave power saturation behavior and temperature dependence the signal may be attributed to Mo(V) a monomeric state. Quantitation of the Mo(V) signal

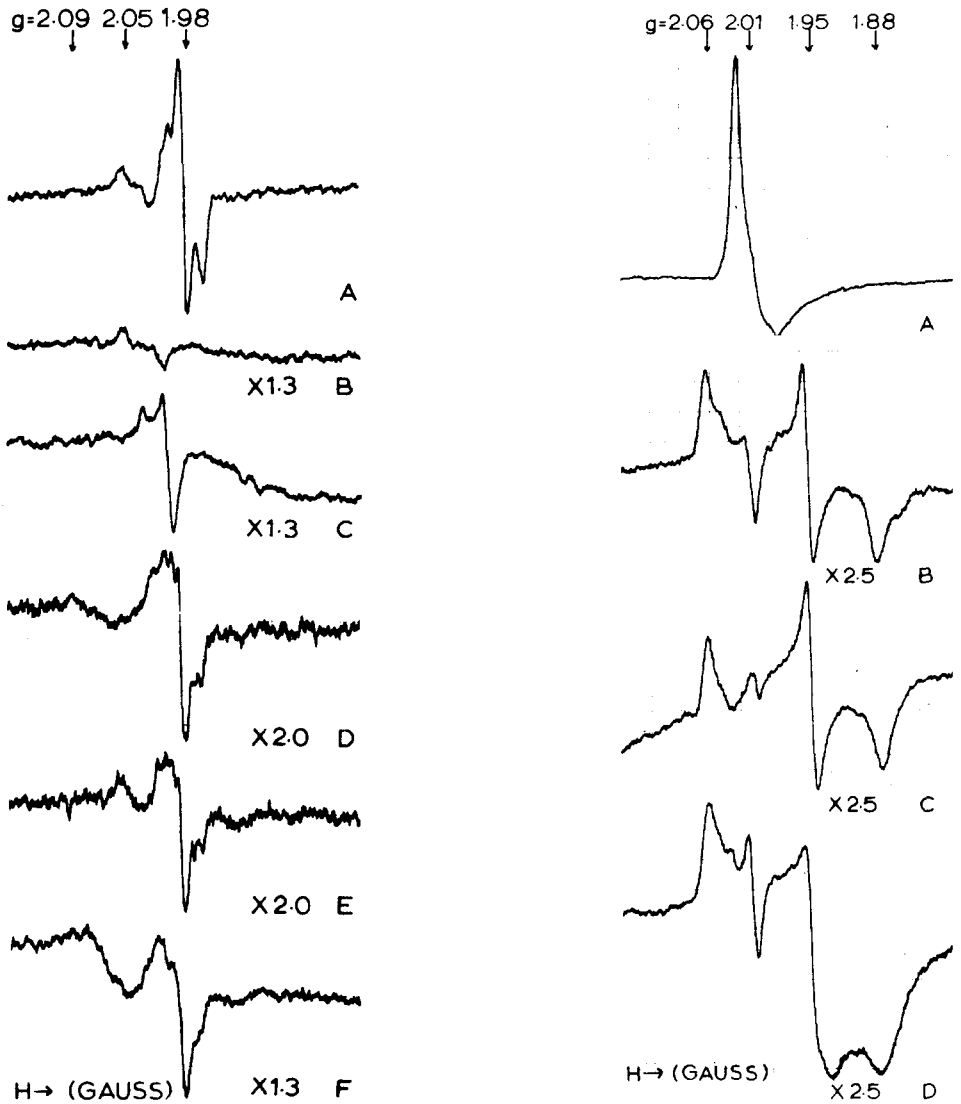

Fig. 1. EPR spectra of nitrate reductase in 100 mM potassium phosphate buffer (pH 7.4). Reactions were carried out in the type of anaerobic quartz tube described previously[6, 7] and after repeated cycles of vacuum evacuation and helium unless otherwise indicated. Final concentrations of reactants are given in each instance: A, nitrate reductase as isolated, enzyme at 53 μM, frequency 9.371 GHz, under helium; B, after addition of 0.9 mM sodium nitrate, 1.1 mg/ml partially purified hydrogenase from *D. gigas*, enzyme at 43 μM and under hydrogen for 20 min at 0 °C, frequency 9.239 GHz; C, after reaction with sodium dithionite (slight excess) for 5 min at 25 °C, enzyme at 53 μM, frequency 9.242 GHz; D, after reaction with 0.9 mM sodium nitrate for 25 min at 25 °C, enzyme at 48 μM, frequency 9.236 GHz; E, after reaction with 1.9 mM sodium nitrite for 25 min at 25 °C, enzyme at 48 μM, frequency 9.239 GHz; and F, after reaction with 0.8 mM sodium azide for 25 min at 25 °C, enzyme at 49 μM, frequency 9.241 GHz. EPR conditions: temperature, 80 °K; time constant, 0.3 s; modulation amplitude, 5.9 G; microwave power, 7 mW; scanning rate, 400 gauss/min.

Fig. 2. EPR spectra of nitrate reductase at 15 °K. Different anaerobic quartz tubes were used but final concentration of enzyme and reactants is similar to those used in Fig. 1 with similar reactions: A, as in Fig. 1A, frequency 9.239 GHz; B, as in Fig. 1B, frequency 9.236 GHz; C, nitrate reductase at 47 μM *plus* slight excess sodium dithionite, 0.9 mM sodium nitrate and benzyl viologen (1 μg/ml) under helium, reacted for 5 min at 25 °C, frequency 9.242 GHz. EPR conditions as in Fig. 1 except that temperature was at 15 °K and microwave power, 0.8 mW.

intensity accounts for 15% of the chemical molybdenum content. The spin recovery for the molybdenum resonance is in the range observed for analogous molybdo-iron-sulfide proteins[9].

At 15 °K a new type of resonance was observed in the isolated enzyme (Fig. 2A) with a g-value at 2.016. This signal is assigned to non-heme ferric ion but of unknown symmetry. The g=2.016 signal is temperature sensitive and disappears at about 40 °K. At 15 °K the Mo(V) signal is not observed because of the intensity of the ferric signal. Also at the lower temperature the microwave power used to observe the ferric signal results in saturation of the Mo(V) signal with a subsequent loss of signal intensity. Quantitation of the ferric ion signal intensity accounts for approx. 12 μM iron based on a low-spin ferric configuration. This value is approx. equimolar with the amount of enzymatically reducible g=1.9-type resonance. The temperature-sensitive signal in the g=2 region has been noted in the oxidized state of homogenous iron-proteins such as clostridial-type ferredoxins[10], ferredoxin from *Desulfovibrio gigas* (D.V. DerVartanian and J. LeGall, unpublished studies) and adenylyl sulfate reductase from *Desulfovibrio vulgaris* (D.V. DerVartanian, G. Michaels and H.D. Peck, Jr.,unpublished studies). On the other hand this temperature-sensitive signal has not been reported in many bacterial, plant and mammalian-type ferredoxins as well as succinate dehydrogenase[11]. A possible explanation for the presence of this temperature-sensitive ferric signal is that it is derived from a contaminating iron system strongly but non-specifically bound during purification. If, however, the ferric resonance is not due to a contamination, it would be interesting to consider whether the interaction of two iron-sulfide systems of different redox potentials could theoretically give rise to the observed resonance.

The reaction of nitrate reductase with nitrate, the reduction product, nitrite or a competitive inhibitor, azide,results in an alteration of the Mo(V) signal at g=1.985, when measured at 80 °K (Figs 1D-1F). Fig. 1D shows the alteration of the Mo(V) signal on reaction of nitrate with nitrate reductase. The $g_{\perp}$ component at g=1.985 is not changed but the signal shape has been altered and $g_{||}$ has shifted to g=2.090. Fig. 1E represents the EPR changes occurring when nitrate reductase has reacted with nitrite. A similar alteration is noted in signal shape but there is no change in g-values. Fig. 1F is obtained when azide has reacted with nitrate reductase. As in Figs 1D and 1E the shape of the Mo(V) signal has been altered but $g_{||}$ is now shifted to 2.077. It is possible that reaction of each of these compounds with nitrate reductase has altered the ligand field surrounding the molybdenum system or has resulted in direct ligand binding to molybdenum.

Both chemical and enzymatic reduction methods were used to ascertain the functional role of molybdenum and iron in nitrate reductase. Chemical reduction of nitrate reductase with sodium dithionite results in the loss of the initial Mo(V) signal and the appearance of a new type of resonance at $g_{\perp}$=1.999 and $g_{||}$=2.023 (Fig. 1C) presumably arising from Mo(III) in a different ligand environment. A decline of resonance absorption was noted in the g=1.9 region but was too broad to be resolved at 80 °K. However, measurements at 15 °K (Fig. 2D) revealed a complex g=1.9 type of reduced iron-sulfide absorption. This resonance could be assigned to at least two different iron-sulfide systems

of approx. equal concentration: Type I exhibited g-values at 2.057, 1.947 and 1.881 and Type II consisted of approx. similar g-values but could be readily distinguished by additional details at g=2.031 and 1.926 (Figs 1D–1F). Chemical reduction also resulted in the loss of the initial ferric signal with only a weak residual signal observed at about g=2.009 (Fig. 1F). Gutman *et al.*[12] recently reported that four iron-sulfide centers of differing redox potential could be observed in complex forms of NADH dehydrogenase by EPR spectroscopy. The possibility exists that the two types of iron-sulfide systems found in nitrate reductase may also differ in redox potential. This suggestion is supported by the observation that enzymatic methods of reducing nitrate reductase lead only to Type I iron-sulfide absorption.

The enzymatic reduction methods involved reduction of nitrate reductase and nitrate either in the presence of sodium dithionite *plus* benzyl viologen or molecular hydrogen and a partially purified preparation of hydrogenase from *D. gigas* (we are grateful to Dr. J. LeGall for this preparation). Since sodium dithionite *per se* is able to reduce the iron-sulfide and molybdenum components of nitrate reductase it seemed especially important to use a reduction system avoiding the chemical reductant. The use of hydrogenase proved very useful in this regard. Both enzymatic methods of reduction lead to identical results (Figs 1A–1E): at 80 °K almost complete disappearance of the Mo(V) signal at g=1.985 (Figs 1A and 1B), and at 15 °K disappearance of the initial ferric signal at g=2.016 (Figs 2A–2C) and appearance of Type I iron-sulfide resonance (Figs 2B and 2C). The amount of Mo(V) signal disappearing in the dithionite-benzyl viologen reducing system is similar except in addition a radical signal (not shown) is observed at g=2.00 presumably attributable to a radical form of benzyl viologen. It is quite clear that only Type I iron-sulfide absorption is observed with each enzymatic reduction method. Quantitation of the signal intensity present in Type I iron-sulfide resonance accounts for approx. 14 μM. A similar determination for the iron-sulfide resonances obtained on chemical reduction of nitrate reductase (Type I *plus* Type II) results in a recovery of 29 μM iron. It is apparent then that the amounts of Type I and II iron-sulfide resonances are of approx. equal concentrations. The enzyme concentration in the enzymatic reduction studies was approx. 44 μM which would correspond to 8 × 44 μM or 352 μM iron. Based on an analogy to the bacterial ferredoxins which yield on reduction a temperature-sensitive complex g=1.9 signal[13] and which require a multiplication factor of four for equivalence of EPR spin intensity with iron content, a value of 4 × 14 μM = 56 μM iron would be recovered in enzymatically appearing g=1.9-type resonance in nitrate reductase. This value in turn accounts for 16% of the chemical iron content. Including both Type I and II EPR signal intensities and the factor of four, approx. 32% of the chemical iron content would be recovered in nitrate reductase. Because of the similarity of the light-absorption spectrum and temperature sensitivity of the g=1.9 resonances to those of the clostridial ferredoxins, the use of the factor of four of the preceding calculations is probably valid. Although the recovery of spin intensity in the g=1.9 resonances of nitrate reductase is of the order of complex metalloproteins[11] it is lower than that reported for the clostridial ferredoxins using analogous calculations[13]. The possibility of interaction between the paramagnetic

centers of iron and molybdenum in nitrate reductase leading to a decrease of spin intensity may be a consideration. It is interesting to reiterate that the amount of iron recovered in the initial ferric signal at g=2.016 which disappears on reduction is of the same approx. concentration as found for Type I iron-sulfide resonance appearing on enzymatic reduction. However, if the ferric signal was due to a high-spin configuration or was due to a contaminant iron system the relationship noted would be merely fortuitous.

Inactivation of nitrate reductase by prolonged ageing results in a decline of enzymatic activity, decline and change in line shape of the initial Mo(V) signal and the appearance of a quite broad temperature-sensitive resonance located at g=2.64. This broad resonance is not observed at 77 °K but is observed below 40 °K (Fig. 3A). Enzymatic

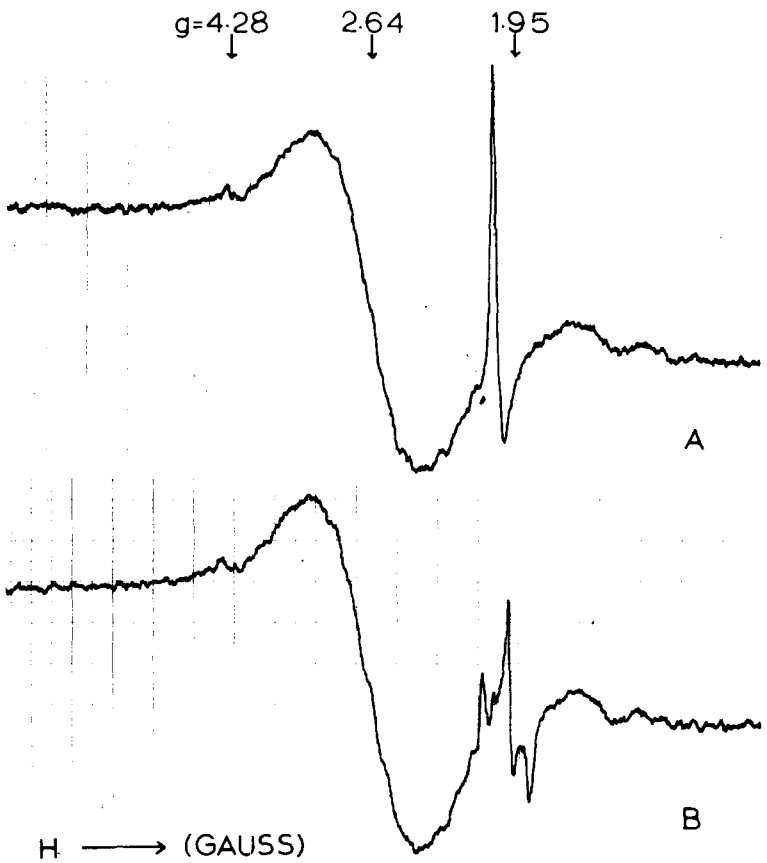

Fig. 3. EPR spectra of nitrate reductase at 15 °K: A, enzyme as isolated and at 53 μM but with a specific activity of 16.7 μmoles nitrate reduced per min per mg protein, frequency 9.248 GHz; B, enzyme of Fig. 3A but reduced as in Fig. 2C, frequency 9.241 GHz. EPR conditions as in Fig. 1 except that the temperature was at 15 °K, the scanning rate at 1000 gauss/min and the microwave power at 0.8 mW.

reduction of this less active preparation results in decline of the ferric signal at g=2.016 and appearance of Type I iron-sulfide absorption (Fig. 3B). The initial broad signal was not found to be altered on enzymatic reduction (Fig. 3B). Part of the iron previously liganded with sulfide has apparently undergone a drastic change in binding (as evidenced by loss of labile sulfide) and/or spin state.

Based on the equilibrium-type measurements on enzymatically reduced nitrate reductase, these studies support the functional role of molybdenum and iron in nitrate reductase. The low values for molybdenum obtained by chemical analysis probably are due to the inherent lability of nitrate reductase. In this regard Fewson and Nicholas[14] reported the addition of nitrate and NADH to their complex form of nitrate reductase (containing cytochrome) resulted in a weak signal at g=1.97 which was attributed to Mo(V). The involvement of molybdenum in nitrate reductase has been strongly suggested by model studies of Guymon and Spence[15]. These workers studied at low pH the reduction by Mo(V) of nitrate in tartrate buffer, observed an unequivocal Mo(V) EPR signal and proposed that monomeric Mo(V) was an active species in nitrate reduction. Our studies indicate that iron as well as molybdenum are functional components of nitrate reductase.

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