ELECTRON PARAMAGNETIC RESONANCE STUDIES ON MEMBRANE-BOUND RESPIRATORY NITRATE REDUCTASE OF *KLEBSIELLA AEROGENES*

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1. Introduction

Respiratory nitrate reductase of several organisms contains molybdenum, iron and acid-labile sulfide [1–4]. Electron paramagnetic resonance (EPR) studies on the isolated enzymes from *Klebsiella aerogenes*, *Micrococcus denitrificans* and *Escherichia coli* have confirmed the presence of molybdenum and iron–sulfur groups and have also shown that iron–sulfur groups participate in the intramolecular electron transfer to nitrate [4–7]. These EPR studies on isolated nitrate reductases and also studies of molybdenum model complexes have not given conclusive evidence for a specific mode of action of molybdenum in the reduction of nitrate to nitrite [4–8]. It is possible that the isolation of the enzyme from the membranes leads to modification of the active site. This would explain some discrepancies in the results obtained with isolated nitrate reductases [4–7].

Therefore, to avoid isolation artifacts we have performed EPR studies on membrane vesicles from *K. aerogenes*. These membrane preparations have a high nitrate reductase content (11% total protein) and have the additional advantage that NADH, instead of artificial electron donors, can be used as reducing agent. It is shown, that only a small part of the molybdenum present in nitrate reductase can be detected by EPR as a Mo(V) signal. Experiments in the presence of D_2O show that molybdenum in this membrane-bound enzyme interacts with a proton. No signal attributable to Mo(III) can be detected. These observations suggest that molybdenum in nitrate reductase of *K. aerogenes* acts as a Mo(IV)–Mo(VI) redox-couple.

2. Methods

K. aerogenes (strain S45) was grown in minimal medium as in [9]. After cell disruption, the membrane vesicles were isolated by centrifugation (100 000 × *g*, 1 h) and washed with 65 mM sodium–potassium phosphate buffer, (pH 7.0) containing 50 mM NaCl. Samples were prepared by suspending the vesicles in the same buffer to a final protein concentration of about 45 mg/ml, and were stored in liquid nitrogen.

Samples in D_2O (EGA-chemie, FRG) were prepared by incubating the vesicles in D_2O at 4°C. After 16 h the suspension was centrifuged and the pellet was resuspended in D_2O to the original protein concentration (45 mg/ml). In the molybdenum-substitution experiments the ^{95}Mo metal was oxidized by roasting it in the presence of air, the formed oxide was dissolved in concentrated NaOH and added to the culture medium (5 mg/ml).

Nitrate reductase activity was tested as in [9]. Protein was measured by the method in [10], using bovine serum albumin as a standard. Molybdenum content was determined by means of proton-induced X-ray fluorescence [11].

Nitrate reductase content of the membrane preparations was determined by uniform labeling. *K. aerogenes* was grown in the presence of [^{3}H]amino acids for 2 generations, membranes were isolated and dissolved by sodium dodecylsulphate (SDS). Membrane proteins were analysed by SDS gel electrophoresis according to the method in [12]. Gels were sliced and assayed for radioactivity. Determination of

nitrate reductase content was based on the amount of radioactivity found in its large subunit (M_r 150 000) versus total radioactivity on the gel, with appropriate correction for contribution of the small subunit (M_r 57 000). EPR spectra were obtained with a Varian model E-9 spectrometer, equipped with a helium-flow system [13]. Temperature, magnetic field and microwave frequency were determined as in [14].

3. Results

By means of uniform ^{3}H-labeling of *K. aerogenes* proteins it was found that 11% total membrane protein is nitrate reductase. Membrane preparations from cells grown under conditions inducing synthesis of nitrate reductase contained 6.3 μg Mo/100 mg protein whereas membrane preparations from cells grown under conditions where no nitrate reductase is formed (cf. [9]) contained 0.6 μg Mo/100 mg protein. The difference in Mo-content between these membrane preparations will be due to nitrate reductase. Based on these values and the known mol. wt (200 000 [15]) of the enzyme it can be calculated that nitrate reductase contains 1.1 atom Mo/enzyme molecule. This content is close to that of the isolated enzyme from *E. coli* as reported [16,17].

The EPR spectrum at 77°K of oxidized membrane vesicles containing respiratory nitrate reductase shows several signals with *g*-values ranging from 2.04–1.97 (fig.1A). The *g* 1.99 and *g* 1.97 lines disappear upon reduction by NADH (fig.1B) and they reappeared upon reoxidation by nitrate (not shown). The signals with *g*-values of 2.04, 2.02 and 2.00 were largely unaffected by reduction by NADH or reoxidation by nitrate. Figure 2 shows more detailed spectra of the *g* 1.99 and *g* 1.97 lines of samples in H_2O and D_2O, respectively.

A rhombic type of signal split into a doublet is observed with g_y 1.989 and g_x 1.968 (fig.2A). This signal with a splitting of 0.8–1.0 mT possibly originates from a Mo(V) centre interacting with a proton. The splitting magnitude increased to 1.1–1.2 mT when 10 mM nitrate was added (not shown). EPR spectra of particles prepared in the presence of D_2O clearly show the disappearance of the splitting observed (fig.2B). This splitting, therefore, must be due to interaction of Mo with an exchangeable proton.

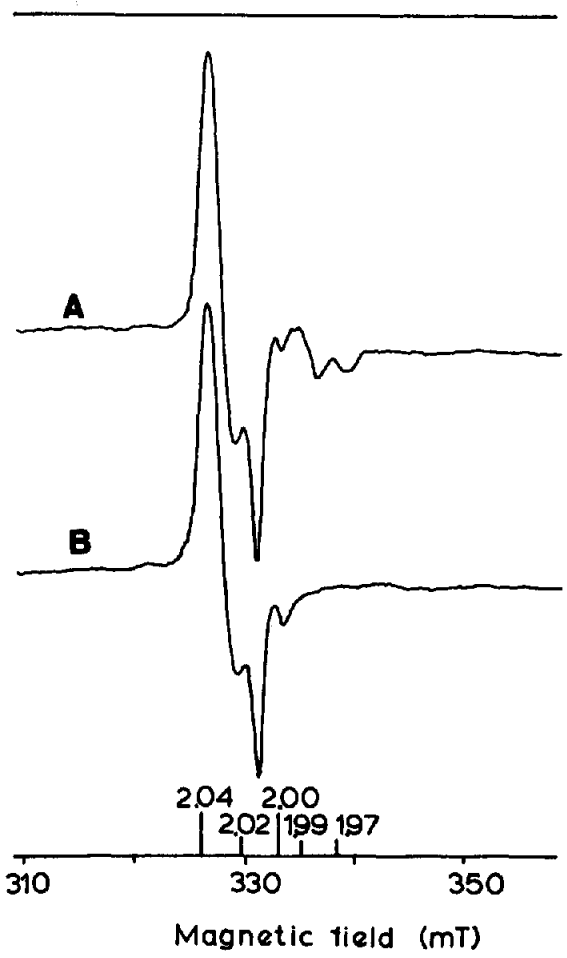

Fig.1. EPR spectra of membrane vesicles of *K. aerogenes*, induced for respiratory nitrate reductase at 77°K. (A) Membranes vesicles as isolated in 0.07 M phosphate buffer (pH 7.0). Protein concentration 44 mg/ml; estimated nitrate reductase concentration 24 μM. (B) As in (A) but sample was reduced by 10 mM NADH at 20°C for 10 min. EPR conditions: frequency, 9.309 GHz; time constant, 0.3 s; modulation amplitude, 1.0 mT; microwave power, 5 mW; scanning rate, 25 mT/min.

Because of severe interference with other signals at lower fields, we were not able to obtain complete resolution of the g_z component. The *g*-values and splittings recorded here are almost identical to those of isolated nitrate reductase from *E. coli* [7].

In order to assign the signal with lines at *g* 1.99 and *g* 1.97 definitely to a molybdenum centre, *K. aerogenes* was grown on a medium containg ^{95}Mo.

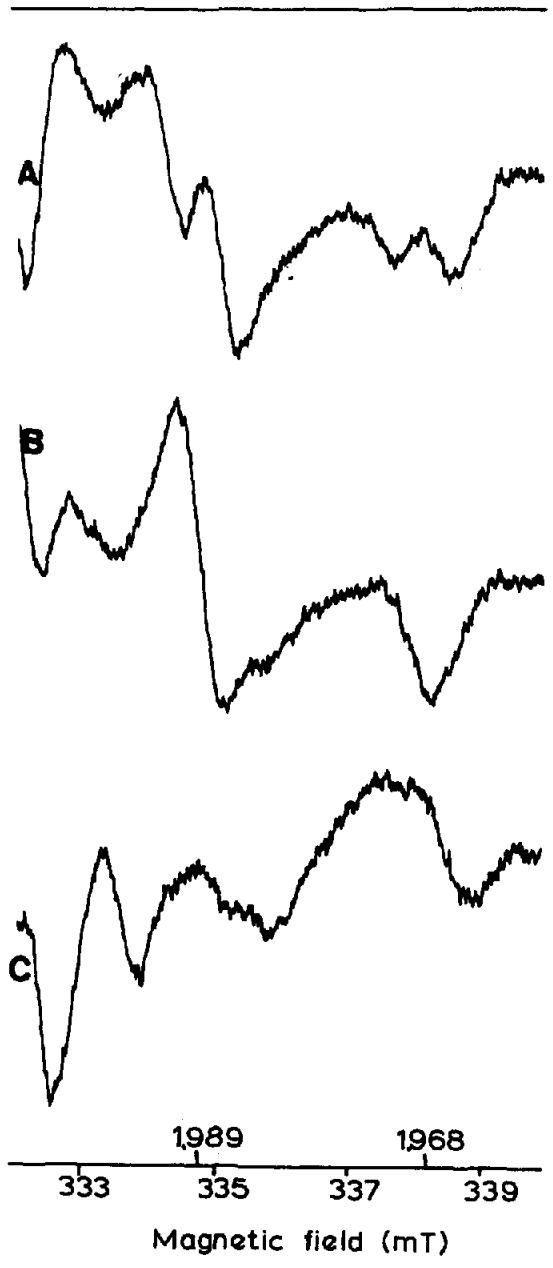

Fig.2. EPR spectra of membrane vesicles (cf. fig.1) as isolated, at 77°K. (A) Membrane vesicles as in fig.1A. (B) Membrane vesicles in D_2O. Protein concentration 40 mg/ml, estimated nitrate reductase concentration 22 μM. (C) Membrane vesicles substituted with ^{95}Mo. Protein concentration 55 mg/ml; estimated nitrate reductase concentration 30 μM. EPR conditions: frequency, 9.314 GHz; time constant 1 s; modulation amplitude, 0.5 mT; microwave power, 5 mW; scanning rate, 1.25 mT/min.

^{95}Mo (I = 5/2) splits all molybdenum signals into 6 smaller hyperfine lines. This was used to identify the molybdenum signals of xanthine oxidase [18]. As expected, substitution of the membrane vesicles with ^{95}Mo caused the g 1.989 and g 1.968 lines to disappear (fig.2C), but due to very low signal intensity, we were unable to assign the resulting hyperfine lines and to resolve the spectrum in detail. Other signals were not affected by ^{95}Mo substitution. This result clearly

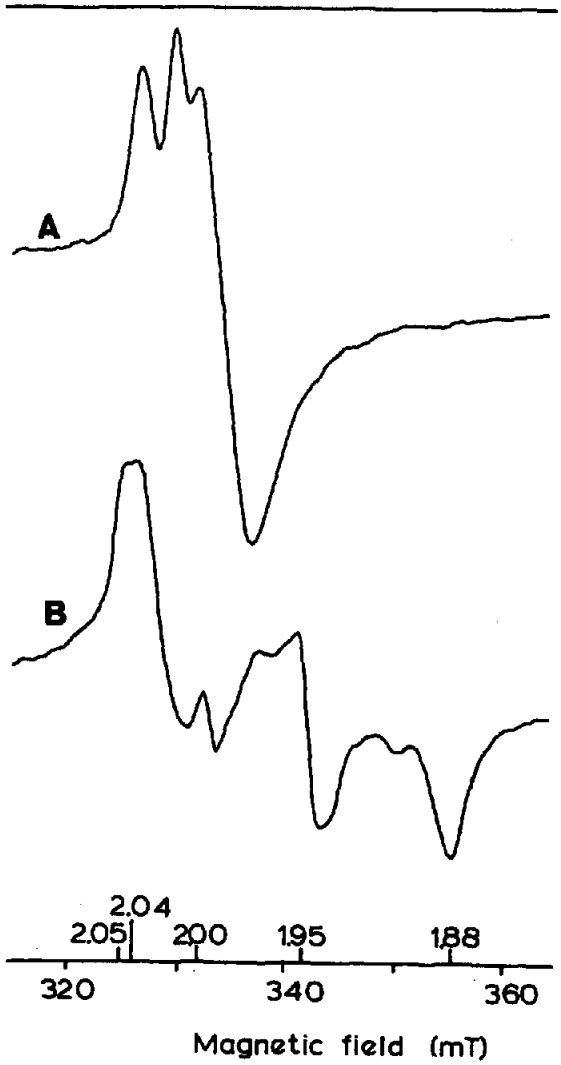

Fig.3. EPR spectra of membrane vesicles at 14°K. (A) Membrane vesicles as in fig.1A. (B) Membrane vesicles as in fig.1B. EPR conditions as in fig.1, except that microwave power was 100 mW.

shows that the lines at g 1.99 and g 1.97 are due to Mo(V). Double integration of the molybdenum signal shown in fig.2B and comparison with a $Cu(ClO_4)_2$ standard [19,20] showed that the signal intensity accounts for only 2% of the molybdenum present in nitrate reductase. EPR spectra taken at 14°K show that signals at 2.04 and 2.00 (cf. fig.1) are still detectable but in addition a signal at g 2.02 is observed which is only detectable at temperatures below 25°K (fig.3A). This signal disappears upon reduction by NADH (fig.3B) and may be due to a high-potential iron–sulfur cluster identical to that present in isolated nitrate reductase from *K. aerogenes*, *M. denitrificans* and *E. coli* [4–6]. In the spectrum of reduced membranes a rhombic signal is observed with g-values of 2.05, 1.95 and 1.88 (fig.3B), originating from an iron–sulfur centre which is also present in isolated nitrate reductase from *K. aerogenes* [4]. Reoxidation of both centers occurred upon addition of nitrate. Like at 77°K the signals at g 2.04 and at g 2.00 are also at 14°K not affected by reduction or reoxidation.

4. Discussion

All EPR signals shown in this paper are related to respiratory nitrate reduction since none of these signals is observed in EPR spectra of membrane vesicles from *K. aerogenes* grown under conditions where no nitrate reductase is formed. Furthermore, the abundance of nitrate reductase in the membranes makes it likely that the signals belong to the respiratory nitrate reductase itself. This conclusion is supported by the fact that most of the signals present in the membrane fraction are also observed in isolated nitrate reductase, although changes in shape of the Mo-signal have occurred (cf. [4]). These observations simplify the interpretation of our results and emphasize the usefulness of membrane preparations in the study of the properties of nitrate reductase.

We were able to detect a Mo(V) signal in oxidized membrane vesicles from *K. aerogenes* attributable to nitrate reductase which is nearly identical to the one observed for the isolated *E. coli* nitrate reductase [7]. In their preparations another species was present only giving rise to a Mo(V) signal under mild reduction and reoxidation conditions. They suggest that the latter signal is due to a 'transient' Mo(V) between Mo(IV) and Mo(VI), comparable to the 'very fast' and 'fast' molybdenum signals of xanthine oxidase [21]. We have not been able to detect such a 'transient' signal which may be due to electron-transfer reactions in our membrane system. The Mo(V) signal observed by us accounts for only 2% of the molybdenum present in nitrate reductase (1 atom Mo/enzyme molecule). Upon reduction by NADH it disappears completely and no signal attributable to Mo(III) is observed under reducing conditions. This demonstrates that most of the molybdenum present in nitrate reductase remains in an EPR-undetectable valence state. Other investigators also report low spin recoveries (9–15%) of the Mo(V) signal in isolated nitrate reductases [5–7]. DerVartanian and Forget explain this by assuming an intense spin coupling between molybdenum and iron–sulfur centers, a hypothesis based on the observation that the molybdenum signal is not saturated with microwave powers up to 30 mW. We did not find proof of such interaction, the molybdenum signal observed in membrane vesicles was 30% saturated at 25 mW.

Since nitrate reductase contains only 1 atom Mo/molecule enzyme, also the existence of an antiferromagnetically spin–spin coupled Mo dimer [22] which might account for the observed diamagnetism is unlikely. It could be argued that Mo in nitrate reductase does not participate in electron transfer reactions and stays in a diamagnetic redox state. However in view of the known redox function of Mo in enzymes like xanthine oxidase, sulfite oxidase and aldehyde oxidase [21] this is not probable. In addition, inorganic molybdenum complexes easily reduce nitrate to nitrite [8].

An explanation more consistent with our findings is that molybdenum in the respiratory nitrate reductase acts as a Mo(IV)–Mo(VI) redox couple of which the components are not detectable by EPR. The Mo(V) signal we observe may therefore originate from a modified form or minor form of the enzyme which for instance reduces nitrate only to nitrogen dioxide.

Since ^{95}Mo substitution caused no alteration in the signals at g 2.04 and g 2.00, these signals can not be attributed to molybdenum [4–7]. It is conceivable that these signals originate from iron. To our knowledge no report has appeared yet in the literature describing similar EPR signals attributable to non-heme iron proteins.

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