Vanadium(V) is reduced by the 'as isolated' nitrogenase Fe-protein at neutral pH

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Orthovanadate has been investigated in the presence of the nitrogenase Fe-protein. Electron paramagnetic resonance (EPR) spectra demonstrate that vanadium(V) is reduced by the reduced Fe-protein to vanadium(IV) which then probably binds to the nucleotide binding site in place of the Mg^{2+} which is normally present. In contrast, the oxidized Fe-protein is unable to reduce vanadate. In this case vanadate has potential for use as a phosphate analogue where it acts as transition state mimic for hydrolysis.

It is generally assumed that the nitrogenase Fe-protein is the specific reductant of the nitrogenase MoFe-protein at which dinitrogen is converted to ammonia.[1] The catalytic centre is a [7Fe-9S-Mo-homocitrate] cluster with an as yet unidentified interstitial atom.[2] In the alternative nitrogenases the molybdenum atom is replaced by either vanadium or iron.[3,4] The α_2-dimeric Fe-protein contains a [4Fe-4S] cluster that transfers electrons in an ATP-dependent process to the MoFe-protein. MgATP binding to the Fe-protein changes the redox-potential of the [4Fe-4S] cluster by -120 mV from -300 mV to -420 mV[5] which presumably facilitates the transfer of electrons to the P-cluster, a [8Fe-7S] cluster in the MoFe-protein, before they are transmitted to the FeMo-cofactor. The [4Fe-4S] cluster is located at the hinge between the two Fe-protein subunits and close to the interface of the Fe-protein–MoFe-protein interaction region.[6]

Orthovanadate, the oxyanion form of pentavalent vanadium, is like the more commonly used aluminium fluoride generally considered to be a good phosphate analogue in phosphoryl transfer reactions where it acts as a transition state mimic of the penta-coordinate intermediate owing to its trigonal bipyramidal coordination geometry.[7] In ATPases and GTPases it approximately occupies the position of the terminal phosphate group of ATP or GTP in the metal–nucleoside-diphosphate-bound protein. The other transition state analogue, AlF_x, uses fluoride ligands instead of the oxygen atom environment in vanadate (and phosphate) and the Fe-protein–MoFe-protein complex with AlF_4^- bound has been analyzed by X-ray crystallography.[8] On the other hand, oxovanadium(IV) or VO^{2+} is able to substitute for the Mg^{2+}-ion normally bound to the nucleotide in the nitrogenase Fe-protein.[9] Thus, vanadium is assumed to bind at different sites and in different environments depending on the oxidation state of the metal.

In the present study we investigated the Fe-protein from *Azotobacter vinelandii* (Av2) in the presence of orthovanadate

$(VO_4)^{3-}$. However, in this case it is shown that vanadate can not be used as transition state analogue with the reduced Fe-protein (see below).

Dithionite is frequently used as a reducing agent in many nitrogenase preparations. Because it is potentially also capable to reduce vanadate,[10] dithionite was avoided by adding vanadate to strictly dithionite-free Av2 preparations.[11] The electron paramagnetic resonance (EPR) spectra are shown in Fig. 1. The spectra for Av2 alone in HEPES buffer solution (pH 7.4) and that in the additional presence of MgADP show the typical rhombic signal from the paramagnetic [4Fe-4S]$^{1+}$ cluster of the reduced Fe-protein with inflections at $g = 2.05$, 1.94 and 1.86. Intriguingly, after addition of $(VO_4)^{3-}$ in the form of EPR-silent Na_3VO_4, the spectrum shows features typical of a signal from the cationic form of vanadium, *i.e.* one that is consistent with paramagnetic $[V(IV)O]^{2+}$ which has an unpaired electron spin $S = 1/2$. The hyperfine coupling with the ^{51}V nuclear spin with $I = 7/2$ typically
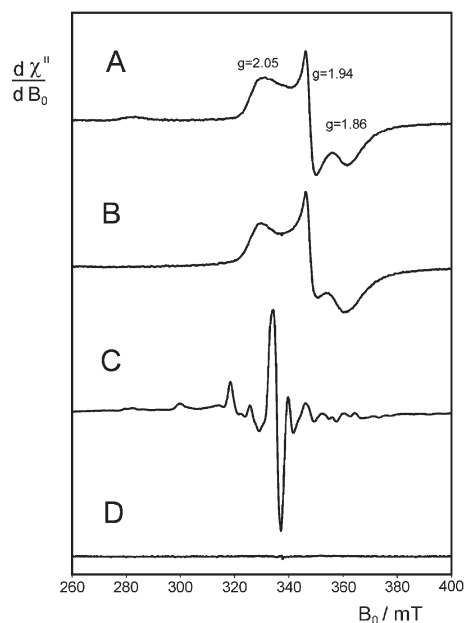

Fig. 1 Frozen solution X-band EPR-spectra of the nitrogenase Fe-protein from *Azotobacter vinelandii* (Av2) in 25 mM HEPES buffer solution (pH 7.4). (A) is the spectrum of Av2 alone, (B) of the Av2·MgADP complex, (C) of the Av2·MgADP complex in the additional presence of sodium orthovanadate (Na_3VO_4), and (D) is the spectrum of Na_3VO_4 alone. Spectra are corrected for a small cavity impurity. Spectrometer conditions: Temperature, 10 K; modulation amplitude, 0.2 mT at 100 kHz; microwave power, 10 mW; microwave frequency, 9.44 GHz.

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gives rise to an eight-line spectrum in solution that is split into parallel and perpendicular components in powder-type samples, as the symmetry of the spin centre is generally close to axial.[12] The presence of a prominent VO^{2+} EPR-spectrum requires that vanadium(V) must be reduced to vanadium(IV). Since the preparations contained no exogenous reductant, it suggests that the Fe-protein itself is able to reduce vanadium(V). In fact, the EPR spectrum does not exhibit a [4Fe-4S] cluster signal underlying the VO^{2+}-signal as was observed when vanadyl is added to Fe-protein preparations,[13] indicating that the [4Fe-4S] cluster of the Fe-protein has been oxidized. A control was also conducted for inorganic vanadate free in solution in order to test whether $(VO_4)^{3-}$ might already be reduced before Av2 was added. However, no EPR-signal is observed (Fig. 1, trace D) consistent with EPR-silent, diamagnetic V(V) in the initial solution.

In order to confirm the above results, we also conducted experiments with oxidized Av2. The comparison of the reduced Fe-protein with that after oxidation with indigo carmine is shown in Fig. 2. The oxidized Fe-protein is no longer able to transfer electrons to vanadate so that the pronounced EPR-spectrum that originates from the unpaired spin of the VO^{2+}-ion is no longer detected. The remaining signal probably arises from some residual VO^{2+} that might be reduced by the incompletely oxidized Fe-protein. It is noteworthy, that with the oxidized Fe-protein, where vanadate is not reduced, it is possible that vanadate adopts the transition state structure as observed for other phosphoryl transfer proteins.
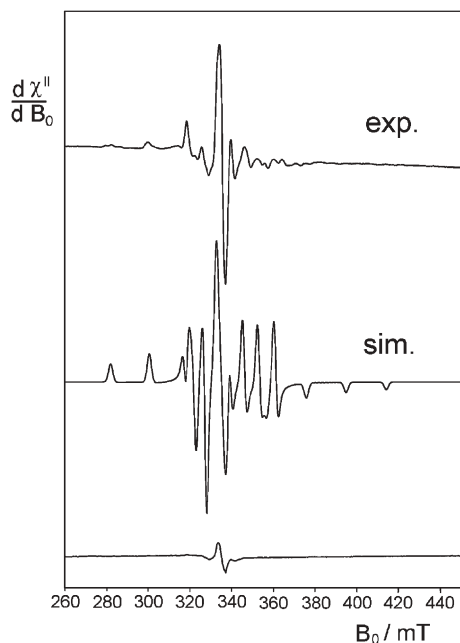

Fig. 2 Comparison of the frozen solution X-band EPR-spectra of reduced and oxidized Av2. The top spectrum is for the Av2$^{red.}$·MgADP complex in the additional presence of Na$_3$VO$_4$, and the bottom spectrum for Av2$^{ox.}$·MgADP with Na$_3$VO$_4$. The experimental parameters are those of Fig. 1. The middle spectrum is the simulation for ^{51}V with simulation parameters for the g-tensor components of $g^{xx} = g^{yy} = 1.980$, and $g^{zz} = 1.935$ and for the hf-tensor components of $A^{xx} = A^{yy} = 57.0 \times 10^{-4}$ cm^{-1}, and $A^{zz} = 171.0 \times 10^{-4}$ cm^{-1} and a Lorentzian linewidth of 2.0 mT.

Spectral simulations for vanadium have been carried out for ^{51}V with a natural abundance of >99.7% (cf. Fig. 2);[14] the other vanadium isotope has been neglected. These demonstrate that the spin Hamiltonian parameters of vanadate after reduction by Av2$^{red.}$·MgADP are similar to those of Kp2 after replacement of the Mg^{2+} by VO^{2+} at neutral pH.[9] Thus inorganic vanadate (Vi) is not bound as a transition state analogue in place of a phosphate ion but is readily reduced by the Fe-protein and then presumably replaces the divalent metal bound to the nucleotide which is normally Mg^{2+}. Therefore vanadium is bound to reduced Av2 as the VO^{2+}·ADP complex instead of the MgADP·Vi complex. Consequently, vanadate can not be used as a transition state mimic in conjunction with the nitrogenase Fe-protein; this is likely to be also true of other redox-enzymes. Thus only AlF$_x$ can serve as a transition state mimic because aluminium does not undergo redox-changes; it only exists as Al(III) under physiologically relevant conditions.[15]

In the native Fe-protein–MoFe-protein complex, electron transfer only occurs when it is associated with an ATP-induced conformational rearrangement in the Fe-protein.[16] It is interesting to note that the electron transfer from the Fe-protein to vanadate occurs without the need for a conformational rearrangement. This raises the question whether a conformational rearrangement is an absolute requirement for electron transfer between the protein components. It could be that the conformation change is primarily required for protein–protein association.

These experiments are particularly interesting with regard to the alternative nitrogenase containing vanadium instead of molybdenum in the FeX-cofactor. For the Mo-containing nitrogenase there is still some controversy about the oxidation state composition of the FeMo-cofactor. However, the Mo is generally assumed to be Mo(IV) in the resting state enzyme.[17] On the other hand, purely on the basis of the isoelectronic similarity, Lovell, Noodleman and coworkers[18] argued that the oxidation state of V in the FeV-cofactor of the resting state enzyme is +3. However, we have no indication that the Fe-protein is able to reduce vanadate beyond the +4 oxidation state level.

There is even less certainty about the other catalytically relevant oxidation states of FeMo-cofactor. It has recently been argued that the reduction of the reduced FeMo-cofactor to the super-reduced state is associated with the Mo-atom, while the semi-reduced and reduced states are supposedly accessible by electron transfer to the delocalized iron–sulfur core of the FeMo-cofactor.[19] The present results are compatible with this notion for V in the FeV-cofactor and suggests that the X-atom in FeX-cofactor may take part in redox changes when electrons are transferred to the cofactor. We propose that the resting state oxidation level of vanadium is +4 which might only be reduced to +3 in the super-reduced state of the FeV-cofactor.

Finally, it is generally assumed that electron transfer to the FeX-cofactor proceeds via the MoFe-protein P-cluster. Remarkably, the P-cluster is not required for the reduction of inorganic vanadate.

In conclusion, to our knowledge this is the first report of a protein capable of directly reducing V(V) apart from the physiologically relevant, non-enzymatic reductants such as glutathione, catechol, NADPH or ascorbic acid. Furthermore, these results demonstrate that the transition state mimic orthovanadate is critically dependent on the oxidation state of the Fe-protein. It

can not be used as a classical transition state analogue in conjunction with the reduced Fe-protein because it is reduced to vanadium(IV), which then presumably occupies the divalent metal binding site while the oxidized Fe-protein does not reduce V(V) and vanadate can be used in this case as such a transition state mimic.

Notes and references

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