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Spectroscopic studies of partially reduced forms of *Wolinella succinogenes* nitrite reductase

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Reductive titrations of the dissimilatory hexa-haem nitrite reductase, *Wolinella succinogenes*, with methyl viologen semiquinone ($MV^{\cdot}$) and sodium dithionite, have been followed at room temperature by absorption, natural (CD) and magnetic circular dichroism (MCD) spectroscopies and at liquid helium temperature by electron paramagnetic resonance (EPR) and MCD spectroscopies. The nature of the reduced enzyme depends on the reductant employed. At room temperature a single high-spin ferous haem, observed by MCD after reduction with $MV^{\cdot}$, is absent from dithionite reduced samples. It is suggested that a product of dithionite oxidation becomes bound with high affinity to the reduced state of the enzyme causing the ferrous haem to become low-spin. The site occupied is likely to be the substrate binding haem. The course of the titration with $MV^{\cdot}$ at room temperature shows the reduction of high-spin ferric to high-spin ferrous haem. Since the EPR spectrum reveals the presence of an unusual high-low spin ferric haem pair in the oxidised state we propose that the active site of the enzyme is a novel haem pair consisting of one high (5-coordinate) and one low-spin (6 coordinate) haem, magnetically coupled and possibly bridged by a histidinate ligand.

Nitrite reductase; Electron paramagnetic resonance; Magnetic circular dichroism; Hemeprotein; *Wolinella succinogenes*

1. INTRODUCTION

The nitrite reductase from *Wolinella succinogenes* reduces nitrite to ammonia in a dissimilatory process [1]. It contains 6 c-type haems bound to a single polypeptide of 63000 Da [2]. Similar enzymes containing 6 c-haems have been identified in *E. coli* [3], *Desulfovibrio desulfuricans* [4] and *Vibrio alginolyticus* [5]. Previous magnetic circular dichroism (MCD) studies [6] of *W. succinogenes* nitrite reductase as isolated have shown that all the haems in the enzyme are ferric, one of them being high-spin and weakly exchange coupled to a low-spin haem. This coupled centre is responsible for the electron paramagnetic resonance (EPR) signals observed at the unusual g-values of 10.4 and 3.7 [6]. The dithionite reduced form of the enzyme, however, was found to possess only low-spin haems with slightly less than one haem per enzyme molecule remaining ferric. This 'non-reducing' haem was attributed a very low redox potential [7].

In this paper we report the results of reductive titrations of *W. succinogenes* nitrite reductase, using the semiquinone form of methyl viologen ($MV^{\cdot}$) and sodium dithionite. Redox changes were monitored at room temperature by absorption, circular dichroism (CD) and magnetic circular dichroism (MCD) and at liquid helium temperature by electron paramagnetic resonance (EPR) and MCD spectroscopies. This work shows that a product of dithionite oxidation, probably sulphite, becomes tightly bound to the active site of the enzyme in the reduced state. The nature of the reduced enzyme resulting from the reductase titration is different at ambient and liquid helium temperature suggesting that reducing equivalents are redistributed between the haem groups by freezing to 10 K, the temperature at which EPR spectra were measured.

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2. MATERIALS AND METHODS

All reagents were purchased from Sigma. *W. succinogenes* was grown in 150l culture as previously described [6]. Nitrite reductase was purified as outlined [8]. EPR absorption and MCD spectra were recorded as reported earlier [6]. Reductive titrations of oxidised nitrite reductase were carried out using either methyl viologen semiquinone ($MV^{\cdot}$) or sodium dithionite as the reductant. The semiquinone was prepared in an electrochemical cell by reduction at a mercury electrode. After each addition of a sub-stoichiometric quantity of reductant, the spectrum was recorded. The titration was continued until the addition of reductant gave no further spectral change.

3. RESULTS

The room temperature MCD spectra of a sample of nitrite reductase reduced with a two-fold excess of $MV^{\cdot}$ and another reduced with dithionite are shown in Fig. 1. The MCD signals in the visible region (450–580 nm) are dominated by low-spin ferrous haems. The dithionite reduced sample contains more low-spin ferrous haem. The Soret region (360–460 nm) of the $MV^{\cdot}$ reduced sample contains a peak at 435 nm

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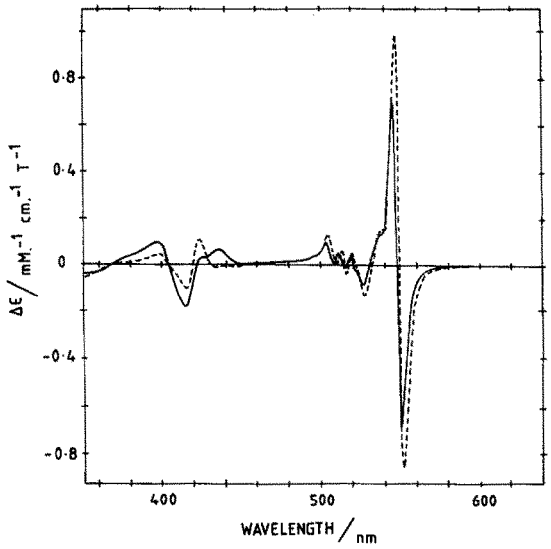

Fig. 1. Room temperature MCD spectra of nitrite reductase reduced with excess methyl viologen semiquinone (——) and dithionite (– – –). Enzyme concentration 9.1 μM, 100 mM Hepes, pH 7.5, 0.05% Triton X-100, pathlength 2 mm.

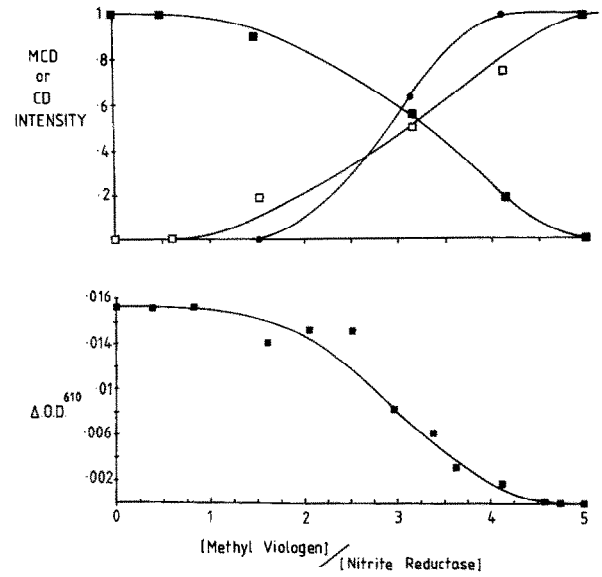

Fig. 2. Reductive titration of nitrite reductase with methyl viologen semiquinone monitored by CD, MCD and absorption spectroscopy. (a) CD and MCD intensity. The signal intensity is expressed as a fraction of the maximum intensity observed. (●) Reduced Soret band CD, (□) MCD signal at 435 nm, (■) MCD signal from high-spin ferric haem at 615 nm. (b) Absorption intensity at (■) 610 nm. Enzyme concentration 9.1 μM, pathlength 2 mm, buffer conditions as for Fig. 1.

assigned to high-spin ferrous haem. Its magnitude suggests the presence of not more than one haem in this state [9]. The appearance of this signal at 435 nm in the MCD spectrum during the titration with MV· is shown in Fig. 2.

The CD spectrum (not shown) in the Soret band of the dithionite and MV· reduced enzyme is unusually intense and biphasic with a peak at 414 nm and a trough at 423 nm [6]. The CD spectrum is abolished in the presence of 1% (w/v) SDS suggesting it is characteristic of the native form of the enzyme. The intensity and biphasic shape likely arises from an exciton interaction between a pair of haems [6]. The appearance of this CD feature on reduction is also plotted in Fig. 2.

The reductive titration by MV· was followed by absorption spectroscopy at room temperature in the visible region. A band at 610 nm assigned to high-spin ferric haem is lost as reduction proceeds (Fig. 2b). The room temperature MCD spectrum also reveals this band at 615 nm and its disappearance on reduction has been followed (Fig. 2a).

Fig. 2 clearly shows that the high-spin ferric haem group is reduced as 1–5 equivalents of MV· are added. The course of disappearance of the MCD at 615 nm and the absorption peak at 610 nm are in close agreement. As this haem is reduced one high-spin ferrous haem is generated as monitored by the appearance of the MCD peak at 435 nm. Fig. 2 shows that this haem is about 50% reduced after 3 equivalents of MV· have been added.

The MCD spectra obtained at room temperature during the reductive titration of nitrite reductase with sodium dithionite are shown in Fig. 3. At no point is a ferrous high-spin haem signal at 435 nm observed. The weak negative trough at ~620 nm which arises from high-spin ferric haem is abolished on addition of dithionite. The reductive titration of nitrite reductase was also followed by absorption spectroscopy in the visible region. The loss of the high-spin band at 610 nm and the increase in the α-band is shown in Fig. 6. Continued addition of dithionite beyond stoichiometric levels caused further reduction of the enzyme.

Fig. 5 shows the EPR signals observed at several stages during the reductive titration of resting nitrite reductase by MV·. A plot of the intensity changes of the major EPR signals against the molar ratio of MV·/nitrite reductase is shown in Fig. 6. The intensities of derivative signals were estimated by measuring the peak-to-trough distance while absorption shaped peaks intensities were obtained by cutting and weighing chart paper. The plot therefore gives an indication of the changes in the concentration of a given paramagnet but no conclusion can be drawn about the amounts of one paramagnet relative to another. The signals at g = 10.4 and 3.72 are lost first and together. This supports the assignment of this pair to the same centre. There is a slight lag before the signals at g = 4.8 and 3.21 decrease. Both sets of signals are completely lost after the addition of 3 reducing equivalents. The signal at g_z = 3.08, arising from a low-spin haem is clearly apparent only after the addition of 3 electron equivalents. However, since this region of the EPR spectra is con-

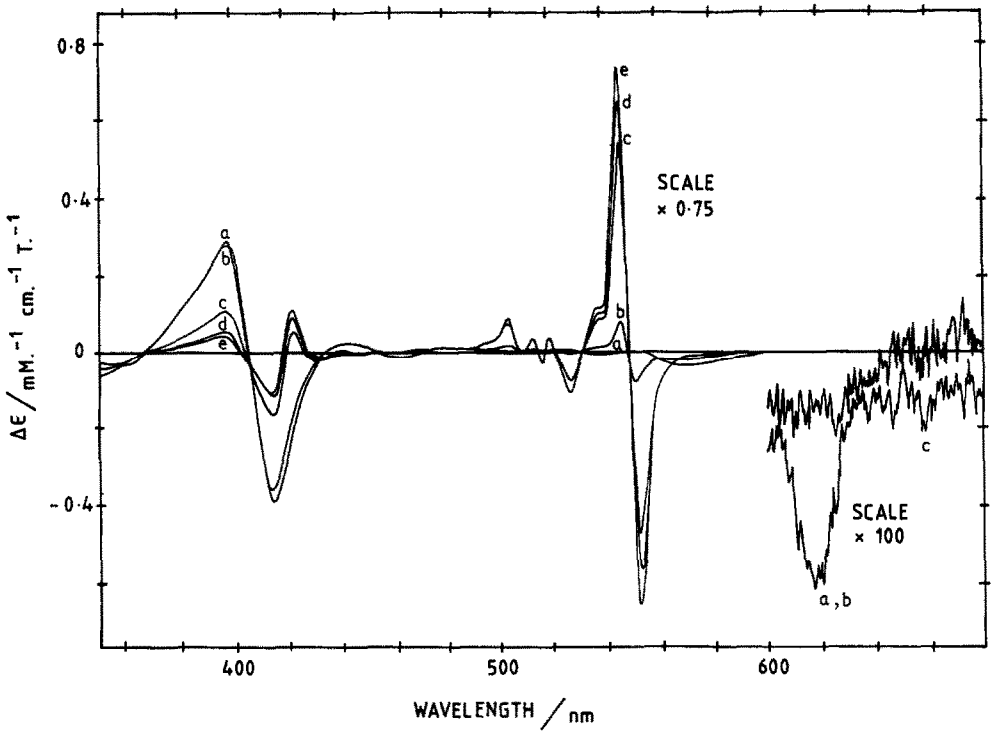

Fig. 3. Room temperature MCD spectra of nitrite reductase as a function of partial reduction with dithionite. Molar ratio $SO_2^{\cdot-}$/nitrate reductase (a) 0, (b) 1, (c) 5, (d) 25, (e) solid dithionite addition. Enzyme concentration 10 μM, pathlength 2 mm, buffer as for Fig. 1.

gested with signals in the early stages of the titration it is not possible to follow the early growth of this g-value. The low-spin signal at $g_z = 2.92$ decreased in two distinct steps after an initial lag. A signal at $g = 2.49$, observed clearly only after the addition of 3 electrons and lost after reduction by 5 electrons, may be the g_z component of a low-spin ferric haem appearing as one or other of the coupled signals are lost. This signal is not plotted in Fig. 6 since its intensity variation cannot be followed.

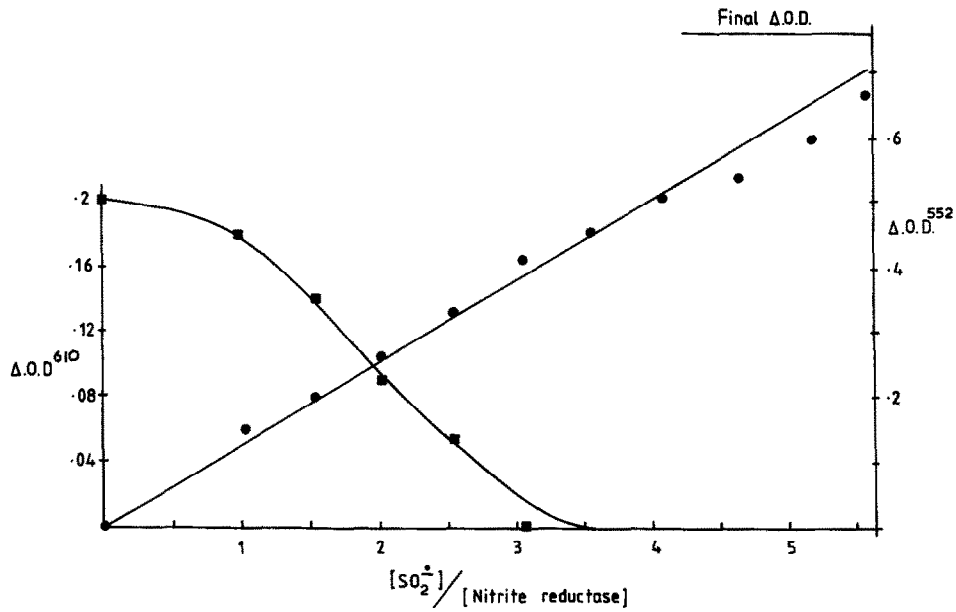

Fig. 4. Reductive titration of nitrite reductase with sodium dithionite monitored by change in absorption spectrum. (■) 610 nm, (●) α-band, 552 nm. Enzyme concentration 8.9 μM, pathlength 10 mm, buffer as for Fig. 1.

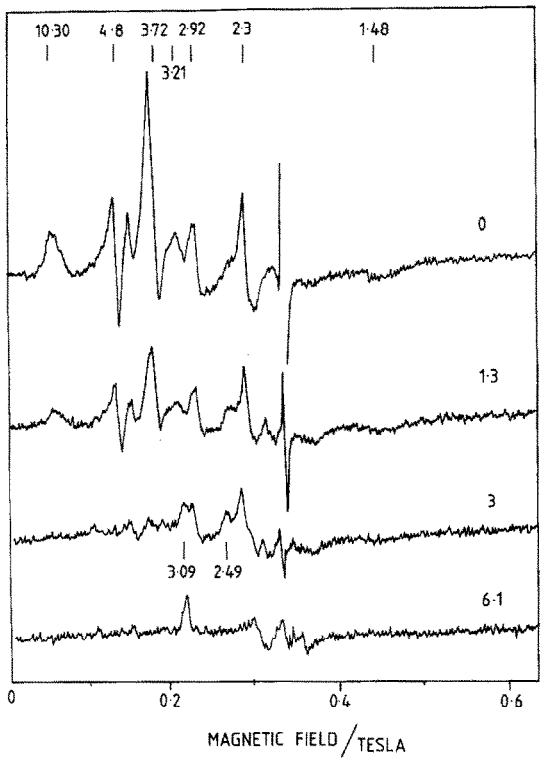

Fig. 5. EPR spectra at intermediate levels of reduction of nitrite reductase by methyl viologen semiquinone. The ratio of methyl viologen semiquinone:nitrite reductase for each sample is shown alongside each spectrum. Enzyme concentration 111 μM, 100 mM Hepes, pH 7.5, 0.5% Triton X-100, temperature 10 K, microwave frequency 9.4 GHz, power 2.02 mW, gain 5×10^5.

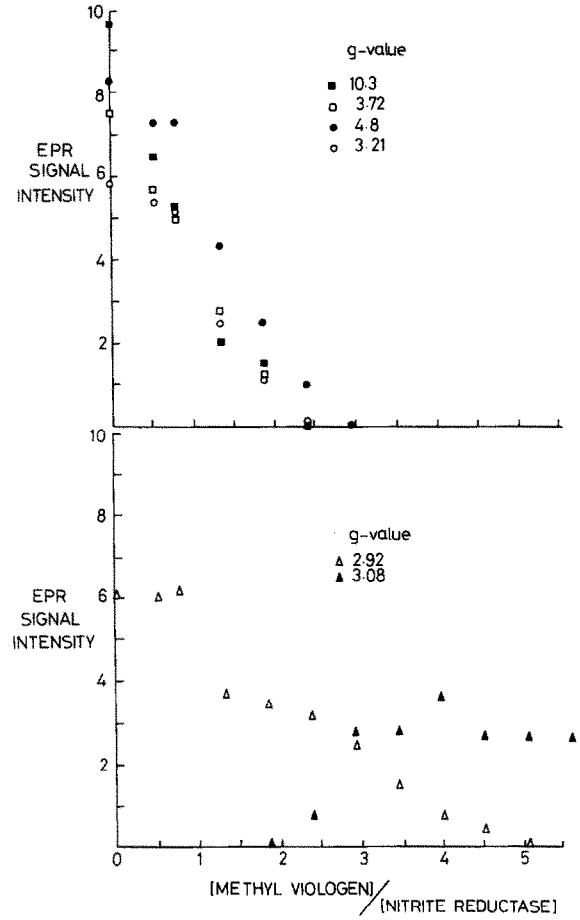

Fig. 6. Variation in EPR signal intensity of nitrite reductase, *W. succinogenes*, as a function of reduction by methyl viologen semiquinone. The intensity of derivative signals was estimated by measuring the peak-to-trough distance and of absorption shaped peaks by cutting out and weighing chart paper. Conditions as for Fig. 5.

4. DISCUSSION

The reductive titrations of the enzyme observed at room temperature by MCD and absorption spectroscopy (Fig. 2) show that the reduction of the high–low-spin pair is not complete until 5 electrons have been added and, importantly, the reductive phase occurs over the addition of ~4 electrons. Clearly the added electrons are being spread over more than one centre. The change in the CD spectrum takes place on addition of two electrons. Since the CD arises from exciton coupled haems we assign this to the strongly interacting pair characterised by g = 4.8, 3.2.

Reduction of the enzyme with sodium dithionite leads to distinct differences compared with $MV^{\cdot}$. No high-spin ferrous signal appears in the room temperature MCD spectrum at any stage during reduction with dithionite. Either dithionite or one of its oxidation products (such as bisulphite) binds to the high-spin haem in the ferrous state causing it to become low-spin. This implies that the reduced enzyme has a high affinity for the ligand. It would not be surprising if the nitrite reduction site were able to bind a variety of anions of similar charge and size. The presence of dithionite affects the redox potential of the high-spin haem. A comparison between Figs 2 and 4 shows that the high-spin haem is completely reduced in the presence of dithionite after addition of 3 electrons whereas a total of 5 electrons needs to be added to the enzyme in the absence of dithionite or its product.

This is the first report of high-spin ferrous haem in this enzyme. We have also observed the presence of this spin-state after low-temperature photolysis of the CO inhibited form of the enzyme.

An unusual feature of the dithionite titration was the ability of increasing quantities of dithionite to cause further reduction. Mayhew [10] has shown that the redox potential of dithionite increases with increasing concentration. Therefore, increasing levels of dithionite might have been expected to decrease the level of reduction. However, it is likely that the binding of dithionite to the active site enables the 'non-reducing' haem to be slowly reduced.

The EPR signals at g = 10.3, 3.72 have been assigned [6] to an exchange coupled high- and low-spin ferric

haem pair. There is only one high-spin ferric haem, in the oxidised state of the enzyme [6]. Thus these signals should be reduced in parallel with the ferric high-spin haem signals observed at room temperature by MCD and absorption spectroscopy. This is not found. The low temperature EPR signals are completely lost after addition of 3 electron reducing equivalents whereas the reduction of the high-spin ferric haem, monitored by room temperature MCD, takes place during the addition of between 1 and 5 reducing equivalents. The formation of high-spin ferrous haem occurs as the high-spin ferric haem is titrated away leading to the conclusion that one haem remains high-spin throughout the redox cycle of the enzyme when $MV^{\cdot}$ is the reductant.

It is only possible to reconcile these two sets of observations if the distribution of added reducing equivalents is different in the enzyme at 10 K, and at room temperature. One explanation is that the reduction potentials of the different sets of haem change to a different extent on cooling the protein. Alternatively, added reducing equivalents may re-equilibrate to the special pairs on cooling to 10 K if electron transfer between the haem groups is rapid. Either process will lead to a re-distribution of added electrons amongst the metal centres on cooling the enzyme. Cooling of proteins is also known to lead to pH changes [11].

Reduction of the exchange-coupled high–low-spin ferric pair apparently leads to the generation of an EPR silent state since no new signals appear which are correlated with the disappearance of the g = 10.3, 3.72 features. An exchange coupled high–low-spin ferrous pair (S_1 = 2, S_2 = 0) will undoubtedly be EPR-silent. On the other hand, partial reduction of the low-spin–low-spin ferric haem pair with g = 4.8, 3.21 should lead to the formation of an EPR-detectable low-spin ferric haem as one of the haems is reduced to the diamagnetic low-spin ferrous state. The low-spin ferric haem at g = 3.09, 2.3, 1.48 which appears rather late in the titration is a candidate although the point at which the signals begin to appear is difficult to determine with precision since this spectral region is congested. The signal at g = 2.49 may well arise from a low-spin ferric haem and is more likely to be one of the haems belonging to the coupled pair with g = 4.8, 3.21 in the oxidized state. If the two haems are strongly interacting, it is quite reasonable that the two reduction steps Fe^{III}-Fe^{III} ⟶ Fe^{III}-Fe^{II} ⟶ Fe^{II}-Fe^{II} are separated by a large redox potential, the second being more negative than the first. The quantitation of the electron equivalents titrated into the protein are in reasonable agreement with this assignment. After 3 electrons are added, about 3 low-spin ferric haem signals remain, namely, half the low-spin haems at g = 2.92, which integrate to ~2 in the oxidized state, the non-reducing haem at g = 3.08 and the signal at g = 2.49. The overall electron count is good. After the addition of 5 electrons, one low-spin ferric haem remains and the other 5 haems are in the reduced state.

5. CONCLUSIONS

The reductive titrations show that sodium dithionite or one of its oxidation products binds to a single reduced haem group of the enzyme causing it to switch from high- to low-spin. Methyl viologen semiquinone, $MV^{\cdot}$, does not induce this spin-state change. The EPR spectrum of this enzyme is very complex in the oxidised state but can be assigned to a pair of interacting high-low spin ferric haems (g = 10.4, 3.7), a second pair of interacting haems, both low-spin (g = 4.8, 3.2), and two magnetically isolated haems (g_2 = 2.92). The disappearance of their signals on reduction with $MV^{\cdot}$ has been followed. This titration confirms that the pairs of signals at 10.4, 3.7, and 4.8, 3.2 belong to different centres. A new low-spin ferric signal at g = 3.08 appears during the course of reduction and persists in the presence of excess $MV^{\cdot}$. We have been unable to identify unambiguously the counterpart of this haem in the oxidised state of the enzyme. The course of reduction of the high-spin ferric haem monitored by EPR spectroscopy at 10 K and by absorption and MCD spectrum at room temperature is different, the high-spin haem being reduced at low temperature after the addition of only two electrons. This suggests that reducing equivalents can be redistributed amongst the haem centres on cooling the enzyme to 10 K.

These results overall show that the unusual high–low-spin haem pair, characterised by the g-values of 10.4, 3.7 in the oxidised state, persists in the reduced state in an EPR-silent form, also as a high–low-spin ferrous pair capable of ligating dithionite or one of its oxidation products. This suggests a structure for the active site of this enzyme consisting of one 5-coordinate and one 6-coordinate ferrous haem weakly coupled by a magnetic exchange. One possible model of this centre is a histidinate-bridged pair of haems, hist Fe(II) $hist^{-}$ Fe(II), with one haem being able to coordinate substrate.

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