Azide Binding to the Diferrous Clusters of the R2 Protein of Ribonucleotide Reductase from Escherichia coli

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Ribonucleotide reductases provide the only biosynthetic pathway for the generation of deoxyribonucleotides.1 The Escherichia coli enzyme is a 1:1 complex of R1 and R2 proteins;2 the X-ray structure of the latter establishes the presence of two nonheme (μ-oxo)diiron(III) clusters in close proximity to Tyr122,3 which must be oxidized to its radical form to elicit enzyme activity.4,5 It is the reaction of the differrous form (R2,d) with O2 that generates the tyrosyl radical.6,7 However, the coordination environment of the diiron clusters in R2,d is less well characterized than that of the diferrous form (R2met). It is clear that the strong antiferromagnetic coupling of the diferrous clusters8 is significantly decreased upon reduction, but there is some disagreement on the nature of the metal-metal interaction.6,8 The NMR resonance for the imidazole N–H protons of the coordinated histidines shifted from 24 ppm in R2met to 57 ppm in R2,d; furthermore, the temperature dependence of the 57-ppm peak suggested a weak antiferromagnetic coupling of ca. 10(10) cm−1 (H = JS, S).7 On the other hand, preliminary EPR studies on R2,d in our laboratory revealed the presence of a low-field EPR signal arising from a ground state; such signals are usually associated with an integer spin system, which in this case might be associated with a ferromagnetically coupled diferrous center. More recently, a multifield saturation magnetization study of R2,d found J = −0.6(4) cm−1.9 Since fitting the temperature dependence of an Fe(II)-shifted NMR signal requires a number of simplifying assumptions and the bulk susceptibility measurements assume a homogeneous sample, we have taken advantage of the higher resolution of the EPR method to reconcile these conflicting observations. We have used integer spin EPR spectroscopy with azide as a probe to examine the magnetic properties of R2,d and its complexes with azide and report here the first spectroscopic evidence of exogenous ligand binding to the diferrous clusters of the R2 protein.

The EPR spectrum of R2,d9 with H21H (Figure 1) exhibits a signal at g = 14.410 whose temperature dependence indicates that it arises from a ground-state doublet. This, together with the signal position and line shape, indicates that the signal originates from a ferromagnetically coupled diferrous cluster. Similar signals have been observed for reduced methane monoxygenase,11 deoxyhemerythrin azide12 (deoxyHrN3), and diferrous model complexes.13 However, the signal intensity for R2,d is 20-fold weaker than that for deoxyHrN3. Anaerobic addition of 40 mM NaN3 to R2,d elicits a new EPR signal at g = 17.0 (Figure 1), which is also from a ground-state doublet, but now with an intensity comparable to that of deoxyHrN3. The g = 14.4 EPR spectrum of R2,d is unchanged in the presence of 1.0 M NaCl, ruling out ionic strength effects as the cause of the new signal at g = 17.0. These observations suggest that the majority of the diferrous sites in R2,d do not give an EPR signal at low temperature and that azide binding converts these EPR-silent centers into EPR-active ferromagnetically coupled sites.14 This is consistent with the finding from NMR measurements of antiferromagnetically coupled diiron(II) clusters.7 It is also consistent with the magnetization data of Figure 2 of ref 8 (note the general decrease in the value of χT as the temperature is lowered). However, the interpretation that the diiron cluster is weakly ferromagnetically coupled10 is questionable for two reasons. First, the simulations of the magnetization data required physically unrealistic g-values. Second, the minor ferromagnetic component observed here may affect the interpretation.

EPR titrations with azide show an increase in the intensity of the g = 17.0 signal up to 70 mM azide (Figure 1).15 Further addition of azide results in a shift of the signal to higher field; at 500 mM azide, the signal is found at g = 14.7 (Figure 1). Control samples were prepared under argon, containing 2 mM Fe(NH4)2(SO4)2 in 25 mM HEPES buffer, pH 7.6, and 5% glycerol (same as protein buffer), with 0, 20, or 700 mM azide; from a ferromagnetically coupled diferrous cluster. Similar signals have been observed for reduced methane monoxygenase,11 deoxyhemerythrin azide12 (deoxyHrN3), and diferrous model complexes.13 However, the signal intensity for R2,d is 20-fold weaker than that for deoxyHrN3. Anaerobic addition of 40 mM NaN3 to R2,d elicits a new EPR signal at g = 17.0 (Figure 1), which is also from a ground-state doublet, but now with an intensity comparable to that of deoxyHrN3. The g = 14.4 EPR spectrum of R2,d is unchanged in the presence of 1.0 M NaCl, ruling out ionic strength effects as the cause of the new signal at g = 17.0. These observations suggest that the majority of the diferrous sites in R2,d do not give an EPR signal at low temperature and that azide binding converts these EPR-silent centers into EPR-active ferromagnetically coupled sites.14 This is consistent with the finding from NMR measurements of antiferromagnetically coupled diiron(II) clusters.7 It is also consistent with the magnetization data of Figure 2 of ref 8 (note the general decrease in the value of χT as the temperature is lowered). However, the interpretation that the diiron cluster is weakly ferromagnetically coupled10 is questionable for two reasons. First, the simulations of the magnetization data required physically unrealistic g-values. Second, the minor ferromagnetic component observed here may affect the interpretation.

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a solution of 2 mM Fe(NH\(_4\))\(_2\)(SO\(_4\))\(_2\), 700 mM azide, plus 0.1 mM R\(_{2\text{red}}\) in the same buffer was also examined. EPR spectra of these four samples did not reveal signals like those of Figure 1 but did show varying intensities of a \( g \approx 9 \) resonance typical of mononuclear Fe\(^{2+}\).\(^{16}\) Thus, for the range of azide concentrations considered here, the EPR signals of Figure 1 cannot be ascribed to an adventitiously bound Fe\(^{2+}\) species.

Computer simulations of the resonances for R\(_{2\text{red}}\) and R\(_{2\text{red}}\) plus 500 mM azide indicate a weak ferromagnetic exchange of \(-3 < J < 0 \text{ cm}^{-1}\). The positions of these two resonances (\( g < 15 \)) cannot be achieved for exchange energies greater than the zero-field energy (\( |\mu| > |D| \)). A similar case has been observed for a phenoxy-bridged diiron(II) complex.\(^{13b}\) However, these positions can be theoretically predicted for weak exchange coupling (\( |J| < |D|/3 \)). Thus, since the zero-field energy of Fe(II) is typically \( |D| \approx 10 \text{ cm}^{-1} \), we have \( |J| < 3 \text{ cm}^{-1} \). For R\(_{2\text{red}}\) plus 40 mM azide, the resonance occurs above \( g = 16 \). Since this position is consistent with either weak or strong exchange coupling,\(^{12}\) the range can be confined only to \( J < 0 \) on the basis of the temperature dependence of the signal.

The above data constitute the first spectroscopic evidence of exogenous anion binding to the diiron clusters of R\(_2\). The implications of our observations on the diiron cluster structure in R\(_{2\text{red}}\) are illustrated in Figure 2. As suggested by the crystal structure of the Mn(II)-reconstituted R\(_2\),\(^{17}\) most of the diiron centers in R\(_{2\text{red}}\) are probably bridged by Glu115 and Glu238, which mediate the observed weak antiferromagnetic coupling.\(^{3}\) A similar exchange coupling is observed in a bis(p-carboxylato)-diiron(II) model complex.\(^{18}\) However, a fraction of molecules (roughly 10\%) have ferromagnetically coupled sites which give rise to the \( g = 14.4 \) signal found in R\(_{2\text{red}}\).

The binding of one azide converts most, if not all, of the diiron clusters to ferromagnetically coupled sites with a \( g = 17 \) resonance. The signal shift from low to high azide concentrations indicates a change in the electronic structure of the diiron(II) unit and suggests that a second azide may bind. The availability of binding sites on both iron centers of the diiron cluster is consistent with the coordination numbers inferred from circular dichroism data.\(^{19}\) Given the possibility of a four-coordinate iron in the cluster, the second azide could bind to either iron site, but charge considerations lead us to prefer the binding configuration shown in Figure 2.

The similarity of the \( J \) values estimated for deoxyHrN\(_3\),\(^{12}\) R\(_{2\text{red}}\), and R\(_{2\text{red}}\)(N\(_3\))\(_2\) suggests that the three clusters are structurally related. An aqua bridge has been proposed for deoxyHrN\(_3\) to rationalize the observed ferromagnetic coupling.\(^{20,21}\) We thus propose the presence of an aqua bridge in the diferrous forms of R\(_2\) with EPR-active centers; indeed, the crystal structure of the Mn(II)-reconstituted R\(_2\)\(^{17}\) indicates the presence of a coordinated water that may be converted into the aqua bridge. We have considered the possibility that a bridging azide could explain the ferromagnetic exchange coupling and the EPR signals observed here and in previous studies of deoxyHrN\(_3\).\(^{12}\) However, we have observed similar or identical integer spin EPR signals from both R\(_{2\text{red}}\) and deoxyHr in the absence of azide. Thus, the ferromagnetic exchange interaction does not require exogenous ligands like azide.

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