Differential Iron(II) Affinity of the Sites of the Diiron Cluster in Protein R2 of *Escherichia coli* Ribonucleotide Reductase: Tracking the Individual Sites through the O_2 Activation Sequence

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Received January 30, 1997

The R2 subunit of ribonucleotide reductase (RNR) from *Escherichia coli* contains an oxo- and carboxylato-bridged diiron(III) cluster and an adjacent tyrosyl radical (Figure 1).[1,2] The iron−radical cofactor, which is essential for RNR catalytic activity, assembles spontaneously upon mixing of the R2 apoprotein, Fe^{2+}, and O_2.[3] When carried out with excess Fe^{2+} or in the presence of a reductant such as ascorbate, the assembly reaction proceeds as follows: ApoR2 binds Fe^{2+}, forming a diiron(II) complex that reductively activates O_2. A single intermediate, which contains the one-electron-oxidized (formally Fe(IV), Fe(III)) cluster **X,** accumulates and decays when **X** oxidizes tyrosine residue 122 by one-electron to give the stable radical and product diiron(III) cluster.[4−7]

The first step of the cofactor assembly reaction is complex: apoR2 binds (at least) two Fe^{2+} ions, and an as yet uncharacterized conformational change is rate-limiting for formation of the O_2-reactive diiron(II) cluster.[8] Few details are available regarding the kinetics or thermodynamics of Fe^{2+} binding. Despite this absence of data, several reports have stated or (to our reading) implied that a great preference exists for Fe^{2+} binding to R2 in dinuclear fashion, which indicates cooperativity between the two sites that the cluster comprises.[9−13] In contrast, our earlier evidence suggested that Fe^{2+} is bound by R2 in both mononuclear and dinuclear fashion when the two are complexed at low Fe^{2+}/R2 ratios (<2),[14,15] and more recent results from the laboratory of J. Stubbe support this conclusion.[16] Mononuclear Fe^{2+} binding could arise from a lack of positive cooperativity (or even negative cooperativity) between the sites, a significant difference between the affinities of the sites, or both. In this work, we have used a two-iron-isotope (^{56}Fe and ^{57}Fe) reaction protocol and Mössbauer spectroscopy to demonstrate that the individual sites of the cluster do, in fact, have significantly different affinity for Fe^{2+}. Furthermore, we have exploited this differential affinity to track each site through the O_2-activation sequence.

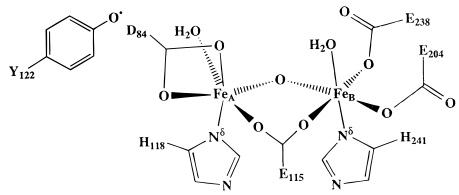

Figure 1. Schematic representation of the R2 cofactor showing the ligand sphere of each iron. It is adapted from figures in refs 1 and 2, but is not derived from the crystallographic coordinates.

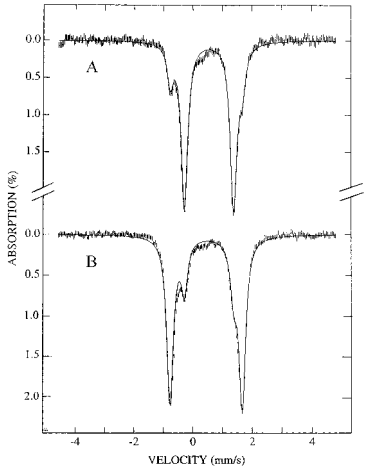

Figure 2. Mössbauer spectra of R2 samples prepared by sequential mixing (A) first with $^{57}Fe^{2+}$ and then with $^{56}Fe^{2+}$ or (B) first with $^{56}Fe^{2+}$ and then with $^{57}Fe^{2+}$. Both spectra were acquired at 4.2 K with a magnetic field of 500 G applied parallel to the incident γ beam. The solid line plotted over the data in A is the result of summing the reference subspectra of site 1 and site 2 of the diiron(III) cluster with coefficients of 18 and 82%, respectively, of the total iron absorption. The solid line plotted over the data in B is the result of summing the reference subspectra of site 1 and site 2 of the diiron(III) cluster with coefficients of 76 and 23%, respectively, of the total iron absorption.

It is known that formation of the O_2-reactive Fe(II)−R2 complex and subsequent reaction with O_2 are fast (rate constants of 5−10 s^{-1} and 60−80 s^{-1}, respectively, at 5 °C)[7,8] relative to dissociation of the Fe(II)−R2 complex (~0.05 s^{-1} as reported by formation of the colored Fe(II)−ferrozine complex).[16,17] We therefore anticipated that any Fe^{2+} bound in mononuclear fashion after anaerobic complexation with a limiting quantity of one isotope might be "trapped" (by reaction with O_2) in a heteroisotopic cluster following addition of an O_2-saturated solution of a second isotope. In this event, any difference in occupancy of the sites in the anaerobic complexation (i.e., differential affinity) would be evident in the Mössbauer spectrum of the R2 product, in which only ^{57}Fe is detected and the two sites of the cluster are well resolved ($\Delta E_Q = 2.41$ mm/s and $\delta = 0.45$ mm/s for site 1; $\Delta E_Q = 1.62$ mm/s and $\delta = 0.55$ mm/s for site 2).[7] Indeed, the Mössbauer spectrum of R2 precomplexed with 0.5 equiv of $^{57}Fe^{2+}$ and then trapped by addition of 3 equiv of $^{56}Fe^{2+}$ and excess O_2 (Figure 2A) shows 4−5-fold greater intensity from site 2 than from site 1.[18] Conversely, the spectrum of R2 precomplexed with 2.3 equiv of $^{56}Fe^{2+}$ and then trapped by addition of 1.1 equiv of $^{57}Fe^{2+}$ and excess O_2

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S0002-7863(97)00319-3 CCC: $14.00

(Figure 2B) shows 3-fold greater intensity from site 1.[19] The simplest interpretation of these results is that site 2 is preferentially occupied and site 1 preferentially *un*occupied upon complexation with low ratios of Fe^{2+}/R2. This interpretation implies that site 2 has a greater affinity for Fe^{2+} than site 1 and that the cooperativity between the sites is small with respect to this difference in affinity.

We previously suggested that Fe_A, by virtue of its more asymmetric coordination sphere resulting (in part) from the unique bidentate aspartate 84, is associated with the higher ΔE_Q (2.41 mm/s) and lower isomer shift (0.45 mm/s) observed for the diiron(III) cluster (i.e., that Fe_A is site 1).[6] This assignment and our current observations would imply that the Fe_B site has greater affinity for Fe^{2+} than the Fe_A site. Recent X-ray crystallographic studies on the R2 protein from mouse[13] are consistent with this conclusion. The authors found that, at the low pH employed for crystallization (4.7), the mouse R2 had only partial occupancy of the Fe_B site and no Fe bound at the Fe_A site. When this crystal was infused with Fe^{2+}, binding was seen only to the Fe_B site, implying that this site has sufficient affinity at pH 4.7 to retain infused Fe^{2+}, while the Fe_A site lacks sufficient affinity.[13] Thus, the rank order of Fe^{2+} affinities observed for the mouse R2 sites (at pH 4.7) matches that which we have deduced for the *E. coli* R2 sites (at pH 7.6).

We also previously speculated, on the basis of correlations in isomer shifts values, that Fe_A is the site of the intermediate cluster **X** now known to have an oxidation state intermediate between Fe(III) and Fe(IV),[20] while Fe_B is the pure Fe(III) site of **X**.[6] The differential Fe^{2+} affinity of the sites demonstrated herein suggested an experimental test of this hypothesis. R2 was precomplexed in the absence of O_2 with 0.7 equiv of $^{57}Fe^{2+}$, mixed with an O_2-saturated solution which delivered 5.5 equiv of $^{56}Fe^{2+}$, and freeze-quenched after the mixture was allowed to react at 5 °C for 0.35 s.[21] The Mössbauer spectrum of this sample (Figure 3) has three major components. An apparent doublet comprising 21 ± 1% of the total absorption area (dashed line above data) has Mössbauer parameters consistent with either an Fe(II)−R2 complex or Fe(II) ions in solution.[6] A second doublet (18 ± 1% of absorption area) has parameters consistent with site 2 of the diiron(III) cluster (dashed line over data).[7] Site 1 of the cluster is much less intense or absent (<5% of absorption area), in qualitative agreement with the experiment of Figure 2A. Most importantly, the third and predominate component (45 ± 1%) is paramagnetic in nature and corresponds to the site of **X** with partial Fe(IV) character (solid line over data), while the pure Fe(III) site of **X** (solid line above data to show position of features) is much less intense (∼5% of absorption area).[22] It is clear from this analysis and the data of Figure 2 that, in contrast to our previous speculation,[6] the Fe site with partial Fe(IV) character in **X** becomes site 2 of the diiron(III) cluster. Thus, on the basis of the above argument that site 2 of the diiron(III) cluster is Fe_B, we propose that the pure Fe(III) site of **X** is Fe_A and that the Fe site of **X** with partial Fe(IV) character is Fe_B. This assignment places the more oxidized Fe ion of **X** farther from Y122, consistent with the notion that Y122 oxidation by **X** occurs by electron and proton transfer steps rather than direct hydrogen atom abstraction.

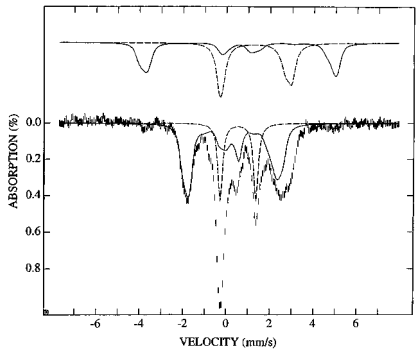

Figure 3. Mössbauer spectrum of an R2 sample freeze-quenched 0.35 s after initiation of the assembly reaction by the sequential-mixing ($^{57}Fe^{2+}$ then $^{56}Fe^{2+}$) procedure. The spectrum was acquired at 4.2 K with a magnetic field of 500 G applied parallel to the incident γ beam. The reference spectra plotted over the data are the following: solid line, site 2 of **X** (45% of total iron absorption); dashed line, site 2 of the diiron(III) cluster (18%). The reference spectra plotted above the data are the following: solid line, site 1 of **X** (20% shown to illustrate the position of these features); dashed line, Fe^{2+} (21%).

The tentative conclusion that Fe_A is the pure Fe(III) site of **X** also suggests a possible mechanism by which the R2 protein may direct the outcome of its O_2 reaction. Perhaps the unique coordination sphere of Fe_A[23] has evolved specifically *to prevent* this site from accessing an oxidation state in excess of +3. This adaptation would preclude formation in R2 of a species analogous to compound **Q** of methane monooxygenase (MMO), the diiron(IV)-like intermediate that is thought to effect methane hydroxylation in this related diiron enzyme.[25−28] Preclusion of a diiron(IV)-like species would, presumably, prevent undesired two-electron oxidation (e.g., self-hydroxylation) of the R2 protein. A corollary of this speculation is that the O−O bond of the presumptive peroxodiiron(III) intermediate in R2 should undergo cleavage only reductively (by injection of the exogenous electron needed to form **X**), in contrast to the peroxodiiron(III) intermediate observed in MMO, which undergoes O−O bond cleavage (to give **Q**) without change in the net oxidation state of the cluster.[29,30] We are currently attempting to use site-directed mutagenesis to block injection of the exogenous electron, characterize the precursor(s) to intermediate **X**, and thereby test this prediction.

Acknowledgment. This work is supported in part by the National Institutes of Health grants GM 55365 (J.M.B.) and GM 47295 (B.H.H. and D.E.E.). We thank Dr. Alice S. Pereira for her assistance in making Figure 3.

JA970319S

(18) A 0.84 mL aliquot of O_2-saturated 2.4 mM $^{56}Fe^{2+}$ in 5 mN H_2SO_4 was injected forcefully at ambient temperature (27 °C) into an equal volume of O_2-free solution containing 0.79 mM R2 and 0.40 mM $^{57}Fe^{2+}$ in 96 mM Na−Hepes buffer (pH 7.6), while the latter was gently vortexed. The volume was reduced in a Centricon 30 microconcentrator (Amicon) to ∼0.5 mL, and the sample was transferred to a Mössbauer cell and frozen for analysis.

(19) A 0.57 mL aliquot of O_2-saturated 1.0 mM $^{57}Fe^{2+}$ in 6 mN H_2SO_4 was injected forcefully at ambient temperature (27 °C) into an equal volume of O_2-free solution containing 0.92 mM R2, 2.1 mM $^{56}Fe^{2+}$, and 2.4 mM sodium ascorbate in 79 mM Na−Hepes buffer (pH 7.6), while the latter was gently vortexed. Na_2EDTA was added to 5 mM to chelate unbound Fe, the sample was diluted 10-fold with O_2-free 100 mM Na−Hepes buffer (pH 7.6), the volume was reduced in Centriprep 30 and Centricon 30 concentrators (Amicon) to ∼0.4 mL, and the sample was frozen in a Mössbauer cell for analysis.

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(21) An O_2-free solution of 1.15 mM R2 and 0.8 mM $^{57}Fe^{2+}$ in 96 mM Na−Hepes buffer (pH 7.6) was mixed at 5 °C with an equal volume of O_2-saturated 6.4 mM $^{56}Fe^{2+}$ in 5 mN H_2SO_4. The apparatus and procedure for preparing freeze-quenched Mössbauer samples have been described in ref 6.

(22) The quoted area ratios for Fe(II), **X**, and diiron(III) cluster are consistent with previously published analyses of samples quenched at this time under similar reaction conditions.

(23) It should be noted that, in the recently reported crystal structure of the diiron(II) form of R2, the two sites of the cluster have a more symmetrical disposition of their carboxylate ligands, as E238 bridges the Fe ions and D84 appears to be only monodendate at Fe_A.[24] Thus, a presumption of this discussion is that the site asymmetry in the precursor to **X** is more similar to that of the diiron(III) cluster.

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