spectra. Previously published model compound data were used in analyzing the protein EXAFS data over the k range 2-12.5, following the procedure previously employed in the analysis of data from form C.[5b] EPR spectra taken before and after exposure to synchrotron radiation did not reveal any changes in these samples during data collection and were used to ascertain the "purity" of the samples. The form A sample had 80% of the enzyme poised in form A, while the form B sample contained 85% of the enzyme poised in form B. The X-ray absorption spectra reported have not been corrected for sample composition, since performing this correction did not lead to significantly different results. Edge spectra monitored as a function of exposure time did not show any significant changes.

Nickel K-edge X-ray absorption spectra obtained for H_2ase poised in forms A, B, and C are compared in Figure 1. Forms A and B show small but discernable peaks at 8331 eV, which are assigned to 1s → 3d transitions.[5f,10] The absence of a peak or shoulder near 8338 eV in both spectra and the small relative areas of the 1s → 3d peaks (0.010 (5) eV and 0.020 (5) eV, respectively) indicate that the Ni sites in both complexes are six-coordinate.[5f,10a] No 1s → 3d peak is observed in the spectrum obtained from the 0.3 mM sample of form C, although an examination of the edge structure is most consistent with a site composed of five non-hydrogen ligands in a distorted trigonal-bipyramidal arrangement.[10a] The reason that no preedge feature is observed may lie in the increased noise apparent in the spectrum obtained from the more dilute sample of form C.

The most striking result shown in Figure 1 is that within the precision of the experiment (±0.2 eV) the edge energies of the three forms do not change. The edge energy obtained from a number of Ni complexes in various oxidation states has been shown to reflect the charge on the Ni center.[10a,11] The observation that the edge energies in forms A, B, and C do not vary indicates that the electron density on the Ni center does not change during reductive activation.

The charge residing on a metal center in a complex is determined by the oxidation state of the metal and the ability of the ligands to reduce the charge on the metal through covalent interactions. The possible effect of changes in the Ni ligand environment that might occur during reductive activation can be addressed by using EXAFS analysis. Figure 2 shows EXAFS spectra of the Ni center in forms A, B, and C. Fits of the Fourier filtered spectra from forms A and B were obtained by analyzing all possible combinations of N(O) and S(Cl) donors with a coordination number of six, as determined from the edge analysis (vide supra), and are compared with the results obtained previously for form C. In all three cases there are 2 ± 1 S(Cl) donors at ca. 2.2 Å and 3 ± 1 N(O) donors at ca. 2.0 Å. The largest difference arises from the presence of long Ni-S(Cl) interactions in form A (Ni-S(Cl) = 2.40 Å) and in form B (Ni-S(Cl) = 2.50 Å). The absence of a long Ni-S interaction in an active form of the enzyme (form C) suggests that it is one possible site of protonation in the heterolytic cleavage of H_2.

Reductive activation of H_2ase by H_2 has been monitored by EPR.[4b] The disappearance of the EPR signals associated with forms A and B, followed by the appearance of the signal associated with form C and its eventual disappearance under continued exposure to H_2, has been interpreted in terms of a number of schemes based on Ni-centered redox chemistry.[4a,b,12] These schemes employ formal Ni oxidation states from III to 0 to interpret the appearance and disappearance of the EPR signals. On the basis of XAS studies of complexes with well-established metal-centered redox chemistry, edge shifts of 2-3 eV and 1-2 eV would be expected for the oxidations of Ni(I) to Ni(II) and Ni(II) to Ni(III), respectively.[5f,10a,13] In the absence of major changes in the ligand environment,[10a] the lack of a significant shift in the edge energy observed between oxidized and reduced forms is a strong indication that no real change in oxidation state of the Ni occurs during reductive activation of the H_2ase. Further, no change in the Ni-S bond length associated with two S-donor ligands present in all three forms of the enzyme is detected. This is in contrast to a decrease of 0.14 Å in the average Ni-S distance upon oxidation of a six-coordinate Ni(II) model complex to Ni(III).[14] Thus, mechanisms invoking different oxidation states for forms A, B, and C and implying significant changes in electron density at Ni are inconsistent with these XAS results.

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Novel Diferric Radical Intermediate Responsible for Tyrosyl Radical Formation in Assembly of the Cofactor of Ribonucleotide Reductase[1]

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Ribonucleotide reductases (RNRs) play an essential role in DNA biosynthesis, catalyzing the conversion of NDPs to dNDPs.[2,3] *Escherichia coli* RNR, the prototype for the mammalian and herpes viral reductases, is composed of two subunits, B1 and B2. The homodimeric B1 subunit binds both the NDP substrates and the dNTP and ATP allosteric effectors and contains the cysteine residues which are essential for substrate reduction. The B2 subunit, also a homodimer, contains the dinuclear iron cluster-tyrosyl radical cofactor which is required for activity. The mechanism of assembly of this unusual cofactor in B2 has been the subject of ongoing investigation in several laboratories.[4-7]

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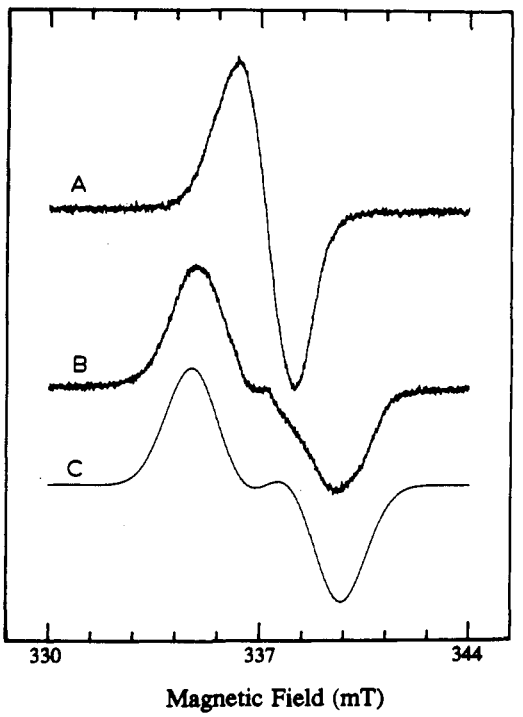

Figure 1. EPR spectra of the intermediate (X) formed in the reconstitution of B2-Y122F. Apo B2-Y122F (100 μM) in 100 mM HEPES buffer, pH 7.7, was mixed with an equal volume of 220 μM (A) $^{56}Fe^{+2}$ or (B) $^{57}Fe^{+2}$ in 5 mM HCl. Both samples were quenched after reacting for 1 s at 5 °C. The spectra were recorded at 40 K on a Brüker ER 200D-SRC spectrometer equipped with an Oxford Instruments ESR 910 continuous-flow cryostat. Other experimental conditions were as follows: microwave frequency, 9.43 GHz; microwave power, 0.2 mW; modulation field, 0.4 mT; modulation frequency, 100 kHz; receiver gain, 1.25×10^5. (C) Simulation of spectrum B using the same magnetic hyperfine A values for the two iron sites as obtained from the Mössbauer analysis.

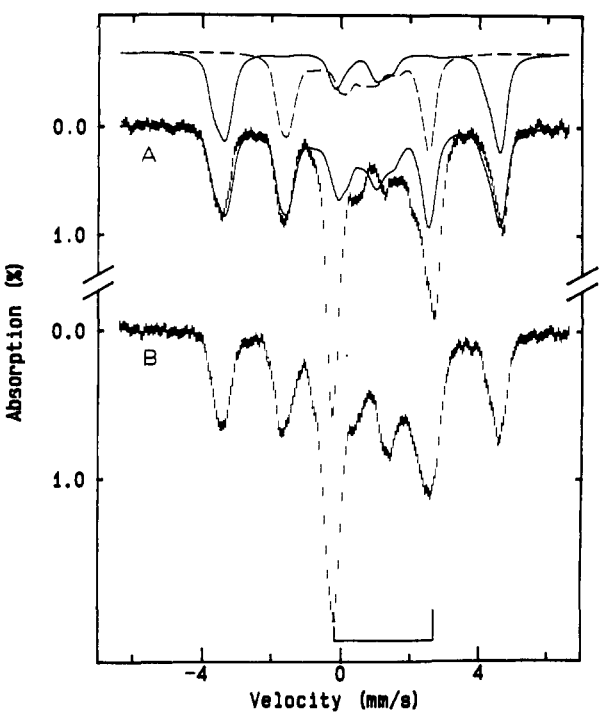

Figure 2. Mössbauer spectra of the reconstitution of (A) B2-Y122F and (B) B2-wt. Apo B2-Y122F (1.54 mM) or apo B2-wt (1.48 mM), in 100 mM HEPES, pH 7.7, was mixed with an equal volume of 4.6 mM $^{57}Fe^{2+}$ or 5.0 mM $^{57}Fe^{2+}$ solution, respectively, each containing 5 mM ascorbate. All solutions were equilibrated under an atmosphere of pure O_2 resulting in an oxygen concentration of ca. 1 mM. Both samples were quenched after reacting for 310 ms at 5 °C. The spectra were acquired at 4.2 K with an external field of 50 mT applied parallel to the γ beam. The *solid line* on spectrum A is a simulation using the parameters quoted in the text. Theoretical spectra for the two iron sites are also shown at the top of spectrum A: (—) site 1; (---) site 2.

It has been suggested by a number of investigators that the oxidation of phenols to phenoxyl radicals catalyzed by the heme-iron-dependent peroxidases might serve as a model for the tyrosyl radical generating reaction in B2, which is mediated by the dinuclear iron cluster.[4,5] In peroxidases, the one-electron oxidation of substrate is effected by either of two high-valent iron–oxo intermediates, compound I (an iron(IV)–oxo porphyrin π-cation radical intermediate) or compound II (an iron(IV)–oxo porphyrin intermediate).[8]

Recently we began investigating the mechanism of conversion of the apo B2 subunit of *E. coli* RNR, ferrous ion, and O_2 to native B2.[9] Stopped-flow absorption spectroscopy and rapid freeze–quench EPR spectroscopy were used to identify two intermediates in this reaction. On the basis of kinetic data, it was proposed that both intermediates can oxidize tyrosine 122 to the stable tyrosyl radical (•Y122) of the cofactor.[10] This communication reports the use of rapid freeze–quench Mössbauer spectroscopy[11] to structurally characterize one of these intermediates responsible for production of •Y122. In contrast to expectations based on the heme-iron peroxidase reaction, this intermediate is not a high-valent iron–oxo species.

Our current working hypothesis for the mechanism of reconstitution of B2 in the presence of excess reductant (excess Fe^{2+} or ascorbate) is shown in Scheme I.[12] As demonstrated by rapid freeze–quench EPR spectroscopy, an intermediate (X) rapidly accumulates prior to the production of •Y122. (X) exhibits an isotropic $g = 2.00$ EPR signal with a peak-to-trough separation of 2.2 mT. Generation of (X) with $^{57}Fe^{2+}$ results in marked hyperfine broadening (peak-to-trough separation of 4.4 mT), establishing that (X) is an iron-containing center.[10]

In order to structurally characterize (X), we have made use of a mutant protein, B2-Y122F, in which Y122 has been replaced by phenylalanine.[10] Due to the absence of the oxidizable Y122, this mutant protein allows trapping of 1 equiv of (X), free from both •Y122 and product diferric cluster. The EPR spectra of (X) formed in the reconstitution of B2-Y122F with $^{56}Fe^{2+}$ (A) or with $^{57}Fe^{2+}$ (B) are shown in Figure 1. These spectra are identical to the corresponding spectra obtained with the wild-type protein (B2-wt),[10] except for the lack of any contribution from •Y122.

The Mössbauer spectrum of ^{57}Fe-reconstituted B2-Y122F quenched at 310 ms is shown in Figure 2. The spectrum consists of a central quadrupole doublet (indicated by the bracket) and a magnetic component with resolved absorption peaks at −3.9, −1.8, 2.7, and 5.0 mm/s. The quadrupole doublet has Mössbauer parameters ($\Delta E_Q = 3.20$ mm/s and $\delta = 1.30$ mm/s) consistent either with the diferrous cluster of B2 or with high-spin ferrous

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(12) Mixing apo B2 with limiting Fe^{2+} (2–3 molar equiv) in the presence of O_2 results in the rapid appearance of a transient absorption band centered at 565 nm. Nonlinear least-squares fitting of the 565-nm absorbance versus time curve is consistent with the species giving rise to this transient having a molar absorption coefficient (ϵ_{565}) of 1500 M^{-1} cm^{-1}. The λ_{max} and molar absorptivity of this intermediate are similar to those of a μ-peroxodiferric model complex (Menage, S.; Brennan, B. A.; Juarez-Garcia, C.; Münck, E.; Que, L., Jr. *J. Am. Chem. Soc.* **1990**, *112*, 6423–6425). On the basis of this similarity, we have tentatively proposed that the 565-nm transient observed in the reconstitution of B2 with limiting Fe^{2+} is due to a μ-peroxodiferric intermediate.

Scheme I

$$2Fe^{2+} + O_2 + \text{B2-Y122} \longrightarrow Fe^{3+}\ O^-\text{—}O^-\ Fe^{3+}\ \text{(B2-Y122)}$$

$$\xrightarrow{e^-} \text{X (B2-Y122)} \longrightarrow Fe^{3+}\ O^{2-}\ Fe^{3+}\ \text{(B2-Y122}^{\bullet}\text{)}$$

ions in solution.[13] The magnetic Mössbauer component must be associated with (X), since no EPR signal attributable to iron is observed other than the $g = 2.0$ ($S = {}^1/_2$) signal due to (X). This component can be interpreted as a superposition of two spectral components of equal intensity, each originating from an iron site (top of Figure 2A). It can be analyzed by the following spin Hamiltonian with $S = {}^1/_2$ (eq 1),

$$\hat{H} = \beta\vec{S}\cdot\tilde{g}\cdot\vec{H} + \vec{S}\cdot\tilde{A}\cdot\vec{I} + \frac{eQV_{zz}}{4}\left[I_z^2 - I(I+1)/3 + \frac{\eta}{3}(I_x^2 - I_y^2)\right] - g_n\beta_n\vec{H}\cdot\vec{I} \quad (1)$$

and using the following parameters: for site 1, $\Delta E_Q = -1.0$ mm/s,[14] $\eta = 0.5$, $\delta = 0.55$ mm/s, and $|A/g_n\beta_n| = 52$ T; for site 2, $\Delta E_Q = -1.1$ mm/s,[14] $\eta = 0.7$, $\delta = 0.45$ mm/s, and $|A/g_n\beta_n| = 24.5$ T. The solid line plotted over the experimental spectrum (Figure 2A) is the theoretical simulation resulting from this analysis. Good agreement between experiment and theory is observed. By using the same A values, the corresponding EPR spectrum can also be simulated. Again, excellent agreement is observed between experiment (Figure 1B) and theory (Figure 1C). This agreement establishes unambiguously the association of the magnetic Mössbauer component with the $g = 2.0$ EPR signal due to (X). The isomer shifts and the isotropic A values used in the simulations suggest that (X) is composed of two high-spin ferric ions ($S = {}^5/_2$). Since two $S = {}^5/_2$ ions cannot by themselves couple to form the observed $S = {}^1/_2$ system spin, a third constituent with half-integer spin is required. We propose that this constituent is either an oxygen radical or an amino acid radical, and we designate the intermediate, (X), a *diferric radical species*.

Having characterized this novel diferric radical species in B2-Y122F, a similar experiment was performed using B2-wt. The resulting Mössbauer spectrum (Figure 2B) is identical to that observed with B2-Y122F, except that the total amount of (X)/B2 is less (0.9 equiv compared to 1 equiv in B2-Y122F) and ~0.1 equiv of the product diferric cluster is also present. As alluded to above, (X) formed in B2-wt is kinetically competent to produce •Y122, resulting in the product diferric cluster.[10]

The detailed structure of the diferric radical intermediate, (X), remains to be established. A key question in this regard is the identity of the radical constituent. The observation of (X) with both B2-wt and B2-Y122F eliminates •Y122 as a candidate. Since the large ^{57}Fe hyperfine coupling observed in (X) suggests that the radical is ligated to either one or both ferric ions, the recent report of the X-ray structure of B2[15] defines defines the remaining possibilities. One possible candidate is an oxyl or hydroxyl radical derived either from O_2 or from H_2O. Experiments using $^{17}O_2$ and $H_2{}^{17}O$ to test this hypothesis are in progress.

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(14) The rapid-quench Mössbauer samples were frozen in isopentane which has a melting temperature of 112.7 K and is present in the packed samples. To obtain the ΔE_Q value for a paramagnetic iron center, the general practice is to increase the temperature until the electronic relaxation rate is fast in comparison with the nuclear precession time (10^{-7} s) and collapse the Mössbauer spectrum into a quadrupole doublet. However, at temperatures just below 112 K, the electronic relaxation rate of the diferric intermediate (X) is such that its Mössbauer spectrum is broad and featureless. Consequently, the value of ΔE_Q is chosen within the range for high-spin ferric ions and through comparison of a series of theoretical simulations with experimental data, which suggest a value of less than 1.5 mm/s.

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Photoelectrochemical Behavior of C_{60} Films

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Fullerene allotropes of carbon can now be produced in quantities suitable for conventional reaction and structural studies.[1] Of particular relevance from these advances is the rich electrochemical behavior (three successive, reversible reduction steps) that has been shown for both C_{60} and C_{70} molecules in solution[2,3] and the redox activity[4] of C_{60} films, CH_2Cl_2-cast onto microelectrodes. We have found that solvent-cast films of C_{60} on noble-metal electrodes show the photovoltaic response typical of n-type semiconductors in liquid junction cells. This search was initiated when experiments on sublimed films bridging metals indicated the existence of a photoconductive response linear in bias voltage and excitation intensity.[5] Photovoltaic behavior of organic molecular solids has an extensive history.[6] We demonstrate the photovoltaic character of C_{60} liquid junctions through voltammetry of cast film-rotating disk electrodes (RDE) under chopped light, measurements of photovoltage and photocurrent dependence on irradiance from an Ar laser, and by excitation spectra of the photocurrent response.

Solvent-cast films (from benzene solution) of C_{60} are quite insoluble in most nonaromatic organic solvents, and we have used acetonitrile solutions with 0.3 M tetra-*n*-butylammonium perchlorate [(TBA)ClO_4/MeCN] as supporting electrolyte in all cases. Jehoulet, Bard, and Wudl[4] have shown that their cast films have redox activity in MeCN solution when the TBA cation is present. Our results in the course of this work are consistent with their description of the hysteresis of the reduction-reoxidation of such films. Very significantly, for the photoelectrochemical behavior we demonstrate, the C_{60} electrode is stable to anodic reaction over a region of more than 2 V, up to +1.3 V vs the ferrocene/ferrocinium couple.[4] Thus, for n-type behavior, photooxidation of a redox species in solution has an ample potential window to compete successfully with substrate reaction, a relatively unusual situation for n-type semiconductors of solar-matched band gaps.[7,8]

Films of about 50-100 monolayers (estimated as in ref 4) were cast onto a Pt RDE of about 0.2-cm diameter, sheathed in Teflon (Pine Instrument Co.), from dilute (ca. 0.1 mM) benzene solution. Benzene was allowed to evaporate at ambient temperature leaving a pale yellow-brown film whose uniformity was not otherwise controlled. Variability from this simple procedure occurs only in the quantitative photocurrent sensitivity observed, not in any other effects described. The electrode, after coating, was run in 0.3 M (TBA)ClO_4/MeCN containing 1-100 mM iodide (as TBA salt).[9] No obvious changes occurred in the film during experi-

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