Heterogeneity of the Local Electrostatic Environment of the Tyrosyl Radical in *Mycobacterium tuberculosis* Ribonucleotide Reductase Observed by High-Field Electron Paramagnetic Resonance

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Abstract: Ribonucleotide reductase (RNR) is a radical enzyme that catalyzes de novo biosynthesis of deoxyribonucleotides. The catalytically required tyrosyl free radical in class I RNRs is produced in conjunction with a μ-oxo-bridged diferric center in protein R2, 35 Å away from the substrate-binding site in protein R1. High-field EPR at 285 GHz was applied to probe the environment of the tyrosyl radical in both native and reconstituted samples of protein R2-2 of class Ib RNR from *Mycobacterium tuberculosis*. Two distinct peaks (2.0080, 2.0092) of the g_x component were observed in the spectrum from freshly purified native R2-2 protein as well as from ferrous iron/oxygen reconstituted apoprotein R2-2. The $g_x = 2.0092$ peak was relatively stable, whereas the $g_x = 2.0080$ peak decayed after freezing−thawing and storage. The two peaks corresponding to total *g*-anisotropies of 0.007 and 0.006, respectively, are interpreted in terms of a non-H-bonded and a weakly H-bonded fraction of the radicals. The hydrogen bond may be provided from a well-ordered water molecule in the vicinity of the radical-iron site, based on a model study of the protein. This first direct observation of conformational heterogeneity of the local tyrosyl radical environment may be important for understanding the role of the radical in the proposed long-range radical transfer through a chain of H-bonded residues from the radical site in protein R2-2 to the catalytic site in protein R1. The enzyme may become activated by connection of a complete H-bonded chain to the radical.

Introduction

Ribonucleotide reductase (EC 1.17.4.1, RNR) catalyzes the reduction of ribonucleotides to deoxyribonucleotides and is essential for DNA biosynthesis.[1−3] The RNR enzyme from the important pathogen *Mycobacterium tuberculosis* (*M. tuberculosis*) is classified as Ib.[1] It has a diferric-tyrosyl radical cofactor in its smaller protein R2-2 component[4] and the substrate-binding site in its larger protein R1 component.[5,6] Enzyme activity requires both components and is allosterically regulated. The tyrosyl radical in the class I RNR[1] is of neutral, phenoxyl type[7] and is formed in a complex reconstitution reaction of apoprotein R2 (metal free) with ferrous iron and molecular oxygen by loss of an electron and a proton at the target tyrosine residue.[2] The importance of the fate of the lost proton from the hydroxyl terminal of the tyrosine is emphasized by the recent proposal of a coupled electron−proton transfer (radical transfer)[8−11] taking place in the catalytic reaction and also in the radical formation reaction.[12−14]

The radical transfer is proposed to involve a number of fully conserved H-bonded residues, spanning a distance of about 35 Å between the substrate binding site in protein R1 and the iron-radical site in protein R2.[8−10] The proposal is further supported by a systematic biochemical study on site-directed mutants along the transfer pathway, in both *Escherichia coli* (*E. coli*) and mouse RNRs.[2,3,12] Cutting off the H-bond transfer chain at any point generally causes enzymatic inactivation, even if some of the point mutants are able to form a seemingly proper complex

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with protein R1.[3] In EPR experiments of the inactivation reaction of *E. coli* RNR by a substrate analogue 2′-azido-2′-deoxycytidine 5′-diphosphate (azidoCDP), the tyrosyl radical was found to decay and simultaneously a transient azidoCDP-involved radical species located at a cysteine in the substrate binding site of protein R1 was detected.[15,16] There is accumulating evidence to support the proposal that the function of the tyrosyl radical in R2 is to generate a thiyl radical on a cysteine of R1.[2]

High-field EPR gives detailed information on the Zeeman *g*-anisotropy, and it has been applied to measure accurate principal *g*-value tensor components of the tyrosyl radicals in RNR and photosystem II (PSII).[17−23] The presence of an H-bond to the phenoxyl group of a tyrosyl radical influences the *g*-anisotropy, based on the theory of Stone.[24] However, high-frequency EPR studies of purified protein R2 from three prokaryotes, *E. coli* (class Ia), *M. tuberculosis*, and *Salmonella typhimurium* (*S. typhimurium*) (class Ib), showed no evidence for the crucial link of this H-bonded chain connecting to the tyrosyl radical.[6,18,19] On the other hand, the tyrosyl radical in R2 proteins from *Arabidopsis thaliana (A. thaliana)*, herpes simplex virus (HSV), and mouse are clearly H-bonded,[20−22] judging from high-field EPR and supported by electron nuclear double resonance (ENDOR) observations in HSV and mouse proteins.[14]

In the present study, the conformational heterogeneity of the tyrosyl radical in freshly prepared *M. tuberculosis* R2-2 protein is demonstrated by high-field EPR. The tyrosyl radical spectra show two peaks of the g_x component, interpreted as a weakly H-bonding and a non-H-bonding state of the tyrosyl radical, respectively.

Materials and Methods

Preparation of Native Tyrosyl Radical Containing Proteins. The expression, fermentation, and protein purification of protein R2-2 from *M. tuberculosis* have been described previously.[5,6] The main purification steps were streptomycin sulfate precipitation and DE52 and FPLC Mono Q anion exchange chromatography. To get freshly prepared R2-2 protein, every effort was made to shorten the time of purification from the crude extracts after an X-press step to break the cells. It lasted 2−3 days at 4 °C, including desalting, anion-chromatography, and ultra-dialysis. The purification procedures of R2 protein from *E. coli* were described elsewhere.[25] The purified protein was packed into the high-field EPR containers (typically 0.2−0.6 mM, 400 μL each), frozen in liquid nitrogen, and kept in dry ice until they were measured at 5−15 K.

Reconstituted Protein R2-2. Apoprotein was purified from the overexpressed cells grown on iron-depleted amino acid minimum medium, which contained 45.4 mM phosphate buffer (pH 7.5), 7.57 mM $(NH_4)_2SO_4$, 610 μM L-leucine, 406 μM $MgSO_4$, 50 μM EDTA, 5 μM $CaCl_2$, 0.6 μM $ZnCl_2$, 60 μM $CuSO_4$, 0.6 μM $MnCl_2$, 0.75 μM $CoCl_2$, 5.9 μM thiamine dichloride, 2% glucose, and 100 μg/mL ampicillin. All had been autoclaved except the metal ion solutions that were filtered through 0.22 μm Millex-GS membranes (Millipore) prior to the medium preparation. All glassware was washed with 0.1 M sulfuric acid and double distilled water. The cells grew in this medium up to an optical density of $A_{640} = 1.2$ to 1.4 after induction by isopropyl thiogalactopyranoside. Apoprotein prepared in this way contained only trace amounts of the radical which were detected by 9.6 GHz continuous wave electron paramagnetic resonance (cw-EPR).

The subsequent reconstitution reaction was accomplished by mixing equal volumes of apoprotein and anaerobically prepared $(NH_4)_2Fe(SO_4)_2$ solution for 60 s in the presence of air at room temperature. An identical preparation in normal EPR tubes for 9.6 GHz measurements at 5−15 K was done in most cases to check the radical formation during the reconstitution reaction as well as to calibrate *g* values.

High-Field EPR Experiments and Determination of *g*-Values. High-field, high-frequency (up to 285 GHz) EPR spectra of tyrosyl radical containing samples were recorded at 5−15 K with maximum 3 G modulation amplitude in the High Magnetic Field Laboratory (CNRS-MPI, Grenoble, France). The EPR system has been previously described.[26] Three principal **g**-tensor components and the *g*-value anisotropy ($\Delta\mathbf{g} = g_x - g_z$) were directly obtained from the well-resolved high-field spectra of the tyrosyl radical. The absolute *g*-values were further calibrated by comparing the g_{av} (the average value of three principal *g* components) with that precisely determined by 9.6 GHz EPR using a Bruker 035M NMR gaussmeter. The 9.6 GHz EPR measurements were carried out using a Bruker E-300 spectrometer at low temperature as described elsewhere.[6]

Atomic Coordinates of a Molecular Model of Protein R2-2. The model was previously built[6] based on the crystal structure of the highly related protein $R2_F$ of *S. typhimurium*.[7] The model was improved by adapting all known water molecules inside protein $R2_F$ of *S. typhimurium.*

Results

High-Field EPR Reveals Two Peaks of the g_x Component from Reconstituted Radical Containing Protein R2-2. All previous high-field EPR spectra of tyrosyl free radicals in RNR had only one single resolved g_x component. We found here that the freshly reconstituted protein R2-2 had two partially resolved peaks of the g_x component. Figure 1A shows an EPR spectrum at 285 GHz of *M. tuberculosis* R2-2 containing freshly produced tyrosyl radical by reconstitution of apoprotein *M. tuberculosis* R2-2 and ferrous ions (60 s reaction time). The two g_x peaks correspond to $g_x = 2.0092$ and 2.0080, respectively, with about equal intensities. The 2.0092 peak was previously reported in native R2-2 and attributed to a non-H-bonded radical population.[6] The new peak at $g_x = 2.0080$ was observable in all the spectra recorded at 5, 10, and 15 K.

No significant difference of the g_x component separation could be observed after performing the reconstitution reaction with stoichiometric amounts of either $3Fe^{2+}$ or $6Fe^{2+}$ per R2-2. The presence (up to 25%) or absence of glycerol or a moderate pH variation (for example pH 6.8 instead of 7.5) gave similar proportions of the two peaks of g_x in the spectra (data not shown).

The New Peak of the g_x Component at 2.0080 Is Observable Also in Native Protein R2-2 when Freshly Prepared. In the native form of the *M. tuberculosis* protein R2-2 that was freshly purified from anion-exchange chromatography and had never been thawed after freezing, two peaks of the g_x component were clearly visible, although the $g_x = 2.0080$ peak was relatively smaller in amplitude (Figure 1B). The relative intensity of the two peaks varied somewhat from one preparation to the

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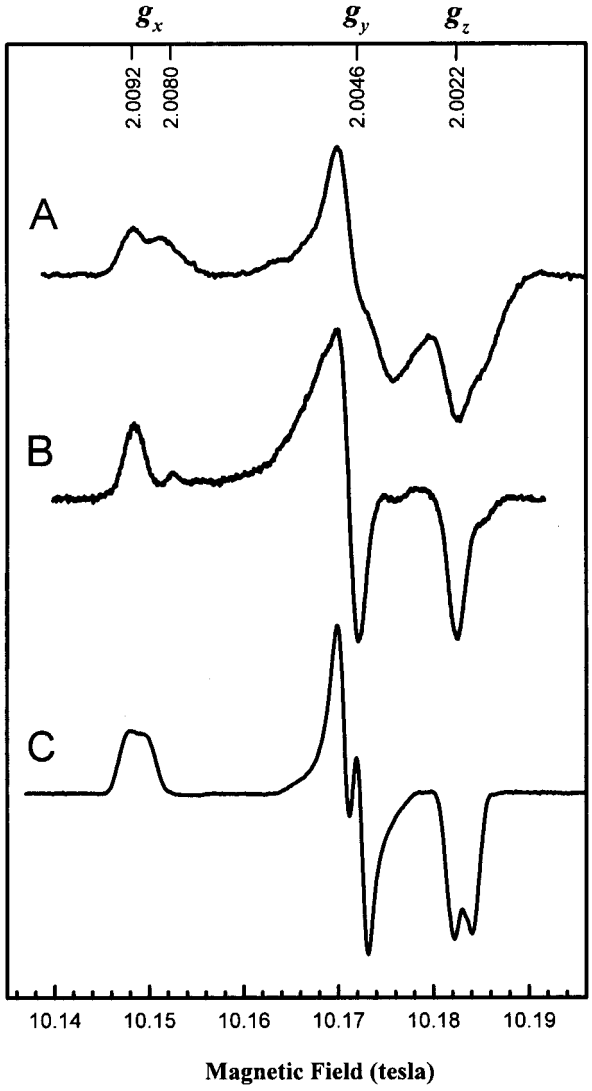

Figure 1. (A) 5 K, 285 GHz EPR spectrum of the tyrosyl radical in *M. tuberculosis* protein R2-2 of the class Ib ribonucleotide reductase. The tyrosyl radical in R2-2 was freshly formed by the reconstitution reaction of apoprotein with Fe^{2+} in the presence of O_2. The reaction was quenched by liquid nitrogen after 60 s reconstitution reaction. (B) 5 K, 285 GHz EPR spectrum of native protein R2-2 after purification procedures at 4 °C for less than a week. (C) Partly resolved 5 K, 285 GHz EPR spectrum of the tyrosyl radical in native protein R2 of the class Ia ribonucleotide reductase from *E. coli*. All EPR spectra were recorded with a maximum of 3 G modulation amplitude. A linear baseline correction has been applied to the spectra. Three principal *g*-value components (g_x, g_y, and g_z) are indicated. Traces A and B show two resolved g_x components, whereas trace C shows a partly resolved hyperfine coupling.

other. In an optimal preparation of native R2-2 we found an approximate ratio of 3:1 in height of the two peaks (Figure 2A). In another sample after repeated cycles of freezing−thawing and storage at −80 °C, the $g_x = 2.0080$ peak instead had become a broad slope (Figure 2B, see also ref 6). The broad g_x component of Figure 2B suggests a distribution of g_x values. There was clearly decreased activity in the enzyme assay for the R2-2 preparations after storage, and particularly after repeated freeze−thawing between 77 K and room temperature,[27] even though the tyrosyl radical content quantitated by 9.6 GHz EPR remained at a similar level.

The two observed g_x peaks correspond to *g* anisotropies of 0.0070 and 0.0058, respectively. As will be discussed in more detail below, different degrees of *g*-value anisotropy, $\Delta\mathbf{g}$, of

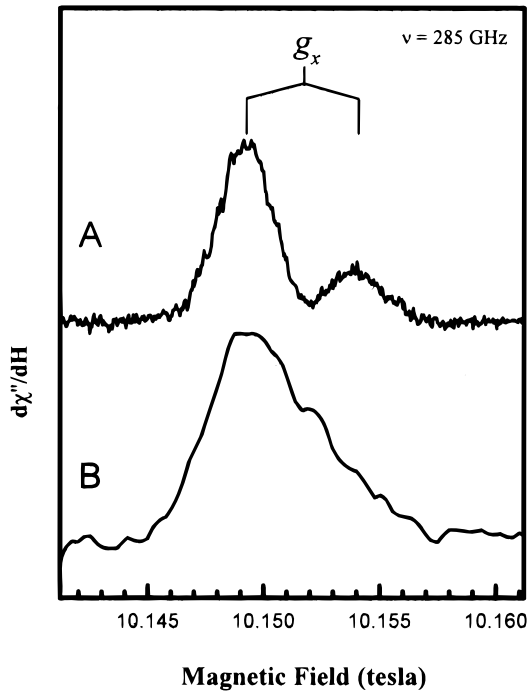

Figure 2. The g_x component of the tyrosyl radical of native *M. tuberculosis* protein R2-2: (A) freshly prepared sample that had never been thawed after initial freezing, showing two peaks of the g_x component with an approximate ratio of 3:1 in height; (B) a sample that underwent repeated freezing−thawing and 3 month storage at −80 °C, revealing a broad distribution of g_x values with a slope in the spectrum. The spectra were recorded at 5 K, 285 GHz.

tyrosyl radicals have been shown to indicate the absence (large anisotropy, ca. 0.0070) or the presence (smaller anisotropy, ca. 0.0050−0.0060) of an H-bond to the radical.[17−24,28] For comparison, Figure 1C shows a 285 GHz EPR spectrum of *E. coli* R2 under identical conditions, which displays a large $\Delta\mathbf{g}$ (0.0069, cf. ref 17). For *E. coli* R2 a partially resolved hyperfine doublet splitting is visible in all three *g* components. This large hyperfine coupling (about 1.8 mT) originates from one of the methylene protons in class Ia R2 proteins, but is much smaller and generally unresolved in class Ib R2 proteins, as is the case for *M. tuberculosis*.

In addition, all *M. tuberculosis* R2-2 spectra recorded at temperatures above 5 K contained a significant 6-line manganese contribution at the region of $g = 1.9924-2.0043$ (spectra not shown). This manganese signal was suppressed, more or less completely at 5 K or lower temperature, by depopulation of observable states, as in the present spectra. The manganese signal has no contribution at the magnetic field where g_x of the tyrosyl radical appears. The question of the manganese/iron dependence of class Ib RNR is however unclear and will not be dealt with further in this paper.

Structural Model Studies Suggest That a Water Molecule Located between the Tyrosyl Radical and the Diiron Center May Contribute to the H-Bond. It was found in our previous work on the *M. tuberculosis* R2-2 protein that the tyrosyl radical was relatively isolated from the dinuclear iron center and all other amino acid residues, which were located at least 4 Å away from the phenolic oxygen of the radical.[6] A molecular structural

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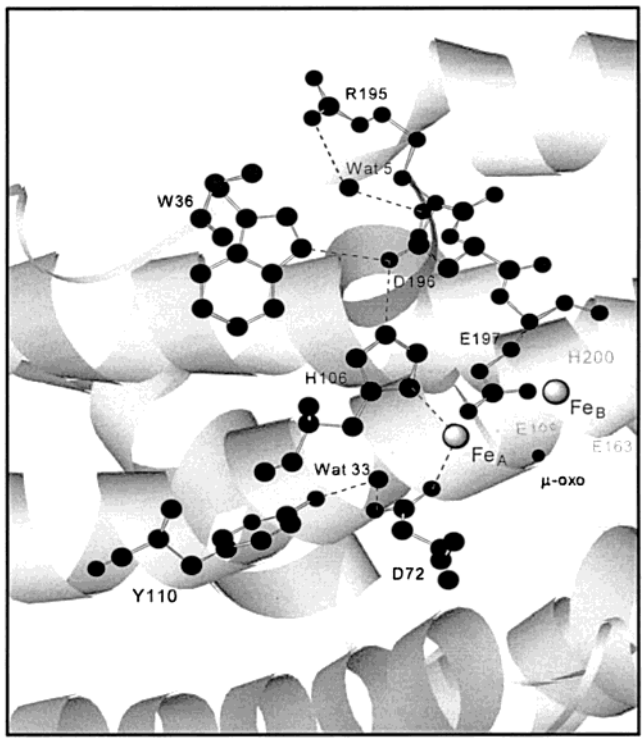

Figure 3. Hydrogen-bonding connection from the buried tyrosyl free radical to the R2-2 surface residues W36 and R195, which are proposed to interact with protein R1 in the holoenzyme. This H-bonded chain is believed to be part of the radical transfer pathway in *M. tuberculosis* ribonucleotide reductase. The water molecules 5 (atom No. 4584) and 33 (atom No. 4612) were adopted and modeled from the crystal structure of *S. typhimurium* $R2_F$.[29] The distances along the dashed connection starting from the phenolic oxygen of the tyrosyl radical to the surface residues are as follows: 2.5 (Y110−Wat33), 2.6 (Wat33−D72), 2.2 (D72−Fe_A), 2.2 (Fe_A−H106), 2.8 (H106−D196), 3.0 (D196−W36), 2.7 (D196−Wat5), and 3.1 (Wat5−R195) Å, respectively. The side chains involved (including part of the iron ligands) are shown in black ball-and-stick representation. The hydrogen bonds are depicted with dashed lines. The ribbon representation of the protein is shown transparently with labeled side residues for clarity.

model of the protein (details are given in the Supporting Information) was built from the crystal structure coordinates[29] of the highly related $R2_F$ protein from *S. typhimurium*. It was further improved by including all the detected water molecules inside the protein $R2_F$. The entire diiron-radical sites for the two class Ib proteins are physically and biochemically similar. Part of the radical transfer chain from the buried tyrosine to the surface of *M. tuberculosis* R2-2 through a hydrogen-bonded connection is depicted in Figure 3. The radical forming residue Y110 is located within a rigid solvent-inaccessible hydrophobic pocket. Only this single water molecule (No. 33) was found in the big hydrophobic pocket in the crystal structure of *S. typhimurium* $R2_F$ and in the model of protein R2-2. This water is about 2.5 Å distant from the tyrosyl radical and 2.6 Å from the iron ligand D72, and may approach the phenolic oxygen in a plane with a lone-pair orbital of the oxygen.

Discussion

The new g_x peak is not due to hyperfine interactions of the radical. In earlier EPR work at 9.6 GHz, we showed that the class Ib RNR tyrosyl radicals have only one large, partly resolved, methylene proton hyperfine coupling of ca. 1.0 mT both in the protein solution state (room temperature) and in frozen glass. This hyperfine coupling is too small to be resolved in the very high-frequency spectra. In contrast, in the EPR spectra from class Ia RNR radicals the larger methylene proton hyperfine coupling is about 1.8 mT[7,30] and is visible even in the 285 GHz frequency EPR spectrum, as seen for the radical in *E. coli* R2 (Figure 1C). Therefore, the new component of g_x (Figure 1A) indicates that a second local conformation is present in the iron-radical site of freshly prepared iron−oxygen-reconstituted radical-containing *M. tuberculosis* R2-2. This was further demonstrated by similar observations in freshly prepared native R2-2 samples (Figure 1B, 2A).

Recently, the **g**-tensor anisotropy, particularly the g_x parameter, has become accepted as a general indicator of H-bonding to the phenolic oxygen of tyrosyl radicals,[17−24,28] although other factors, such as electrostatic interactions with the iron center, ionized amino acids, or other charged centers could also be involved.

Previous results from the high-frequency EPR spectra of the tyrosyl radicals in RNR from HSV and mouse ($g_x = 2.0076$, i.e., a low *g*-anisotropy)[20] are in good agreement with Q-band ENDOR studies on similar samples, which after D_2O exchange showed weakly coupled H-bonded deuterons exchangeable with a water ligand in both radicals.[14] In contrast, ENDOR spectra did not show any evidence of an H-bonded proton in *E. coli* protein R2 alone,[7] and high-field EPR showed a g_x peak at 2.0091.[17] A very strong H-bond, e.g., that of photoionized tyrosine in aqueous glass, leads to a g_x of about 2.0067.[31]

From a theoretical point of view, the *g*-anisotropy of a tyrosyl radical is dependent on the spin density in the p_z-orbital of the oxygen and the excitation energy to the lone-pair oxygen π orbital.[26] An H-bond to the tyrosyl radical reduces the spin density (ca. 10−30%)[17,27] on the phenolic oxygen. A ^{17}O-labeling EPR study on $Y_D^{\bullet}$ of PSII verified that the spin density was weakly dependent on H-bonding.[32] Semiempirical analyses of **g**-tensors by molecular orbital methods[18,24] as well as recent *ab initio* calculations[33] have shown that H-bonding results in a smaller g_x value.[17] The small difference of g_x is practically invisible in low-field hyperfine-dominated EPR spectra (<30 GHz frequency). The presence in the high-field spectrum of two peaks of the g_x component may be interpreted as direct evidence of conformational heterogeneity, most likely the presence of both non-H-bonded and weakly H-bonded populations of tyrosyl radicals in the sample.

In the modeled structure of the *M. tuberculosis* R2-2 protein, the tyrosyl radical is ca. 7 Å away from the nearest iron ion of the binuclear iron center.[6,29] All other charged or noncharged species except a water molecule are at a distance beyond 4 Å. As shown in Figure 3, the H-bond donor in R2-2 is probably a water molecule, like in mouse R2.[14] However, in the present case the water should be H-bonded to the iron ligand D72. There is no reason to exclude the possibility that this water molecule carries the lost proton from the tyrosine residue and an oxygen atom from the dioxygen reacting during the cofactor assembly. It seems to be a common feature that one or more water molecules play a role in the radical transfer in RNR.

The two g_x peaks are present in native as well as reconstituted proteins. A similar heterogeneity with a distribution in distances

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and/or angles between H-bond partners has been described for the tyrosyl radicals in PSII.[22,34] However, the H-bonding of the tyrosyl radicals in PSII is complex.[2,22,23,32,34−40] The question here whether the observed decay of the resolved g_x component at 2.0080 is reversible requires further studies. Nevertheless, the fact that the g_x component has two peaks is significant for its use as a probe for the local electrostatic environment of different fractions of the tyrosyl radical and its potential neighbors in the long-range radical transfer.

In conclusion, we interpret the high-field g_x component as originating from a population of protein R2-2 where the tyrosyl radical is weakly H-bonded to a water molecule. The results presented here resolve at least partly one puzzle related to the postulated radical transfer pathway in RNR, i.e., the lack of evidence for H-bond(s) to the tyrosyl radical in some aerobic RNR R2 proteins. The observation of heterogeneity of protein conformations with different degrees of H-bonding of the tyrosyl radical may be important in understanding the catalytic reaction of the enzyme. The general hypothesis is that radical transfer should be activated (through allosteric effects) when the substrate and relevant allosteric regulators bind to protein R1. The activation may involve the formation of a complete H-bonded chain between tyrosine in protein R2 and cysteine in protein R1 through a conformational change involving also water-mediated H bonds, and possibly controlled by the dynamics of the protein.[41]

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Supporting Information Available: Tables of atomic coordinates of a 3D molecular model for *M. tuberculosis* protein R2-2, based on the crystal structure of *S. typhimurium* $R2_F$ at 2.0 Å resolution (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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