Supplementary information for:

Metal-free ribonucleotide reduction powered by a DOPA radical in Mycoplasma pathogens

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Supplementary information

The data presented in the manuscript allows the location of the radical species to be definitively assigned. The UV/vis absorption spectrum of the activated protein exhibits a moderately intense, narrow band in the 300-400 nm region indicative of an aromatic radical (Fig. 3a, Extended data Fig. 6a). This limits the identity of the radical to be, or at least derived from, an amino acid residue with an aromatic side-chain i.e. histidine, tryptophan, tyrosine and phenylalanine. Of this set of four aromatic amino acid residues histidine and tryptophan can be immediately excluded. The side-chain of both of these residues contains a nitrogen atom as part of the conjugated system, and as a consequence, the radical forms of these side-chains always resolve a nitrogen hyperfine coupling that can be detected using EPR/ENDOR (Extended data Fig. 7a). No such nitrogen hyperfine coupling is observed for the radical of *Mf*R2, leaving only tyrosine and phenylalanine residues as potential candidates for the location of the radical. Incorporation of deuterium-labeled amino acids into *Mf*R2 demonstrates that the stable radical is located at a tyrosine-derived residue (Extended data Fig. 6b).
Concomitant pulse EPR/ENDOR measurements conclusively shows that the radical is harbored by an asymmetric molecule. As such, an unmodified tyrosine residue cannot be the site of the radical species in *Mf*R2, as its phenoxyl side-chain is symmetric. This leads to the conclusion that the radical signal must instead be ascribed to a non-native (modified) tyrosine residue.
The inclusion of mass spectrometry and X-ray crystallography data then allows a definitive assignment as to the location of the radical species. Mass spectrometry shows the protein contains a single chemical modification corresponding to an additional oxygen atom at Tyr126 and X-ray crystallography confirms the presence of a modification at this tyrosine residue. Thus the site of the stable radical can only be attributed to the aromatic side-chain of this residue (Tyr126).
The detailed UV/vis and EPR/ENDOR analysis presented below corroborates this finding. These data show that the radical signal is consistent with a modified tyrosine residue, which includes an additional oxygen substituent. The oxygen substituent has the effect of breaking the symmetry of the phenoxyl side-chain, leading to its unique spectroscopic properties.

The UV/vis spectrum identifies an aromatic free radical. The position (300-400 nm) and intensity ($\varepsilon \approx 3000$ $M^{-1}cm^{-1}$) of the UV/vis bands associated with the radical species of *Mf*R2 are characteristic of an aromatic free radical.[1-3] Carbon centered radicals, which form the majority of amino acid radicals (glycyl, etc.) do not have strong absorption features in this region.[1] Similarly, while thiyl radicals do exhibit absorption features in the 300-400 nm region, these are broad and weaker.[4] We also note that all thiyl radicals characterized thus far are short-lived.[5] As the radical is derived from a protein residue this reduces the possible candidates for the radical to four: i) phenyl radical (phenylalanine), imidizol radical (histidine), phenoxyl radical (tyrosine) or an indole radical (tryptophan).
The lowest energy transitions of aromatic amino acid radicals that are easily observed are typically assigned as π to π^* transitions, with their position broadly corresponding to the size of the conjugated system e.g. the smaller phenyl radical appears in the blue (300 nm)[6] of the larger indole radical (460-600 nm).[7] Of the four candidate radicals, the phenoxyl (tyrosine) radical and its derivatives best match the UV/vis spectrum of the activated *Mf*R2. The phenoxyl radical has a $^2B_1$ ground state (C_{2v} symmetry). Its two lowest optical transitions ($^2B_1 \rightarrow {}^2A_2$, $^2B_1 \rightarrow {}^2B_1$) are both π to π^* transitions, appearing at 16,000 cm^{-1} (625 nm) and 25,000 cm^{-1} (400 nm).[8] It is the higher energy transition that is more readily observed, giving rise to a relatively sharp absorption with extinction coefficient of ≈ 3000 $M^{-1}cm^{-1}$, a marker band for tyrosyl radicals.[2-3, 9] As stated in the main text, OH substitution does not dramatically change the energy of this transition as it should only lead to a small perturbation of the ground

state ($^2B_1$, SOMO) and the relevant excited state (also $^2B_1$). For the set of hydroxyl substituted phenoxyl radicals in viscous paraffin solution, OH substitution (adjacent to oxyl group) leads to a downshift of the phenoxyl π to π* marker band from ≈402 to ≈383 nm.[3]

EPR/ENDOR data is also consistent with an aromatic free radical. The lineshape of an EPR signal of a radical comes about due to the interplay of two properties: i) the interaction of the unpaired electron spin with the applied magnetic field (g-value or chemical shift); ii) and the interaction of the unpaired electron spin with local magnetic nuclei via the hyperfine interaction. The g-value defines where the EPR line is observed while the hyperfine interaction leads to a splitting of the EPR line. Hyperfine splittings are additive, with all contributing to the observed spectral width. When measured in the solid state (frozen solution) the g-value and hyperfine splitting for each orientation of the molecule relative to the magnetic field axis can be different. This leads to a broadening of the EPR spectrum and a loss of resolution owing to the overlay of multiple splitting patterns. The problem can be overcome by measuring the EPR spectrum at multiple microwave frequencies. In addition, double resonance techniques, such as ENDOR allow each hyperfine interaction to be viewed in isolation (Extended data Fig. 7b).
The magnitude and number of hyperfine splittings allows aromatic and non-aromatic radicals to be distinguished. The unpaired electron spin density of non-aromatic radicals is more localized, leading to 1-2 large hyperfine interactions (couplings) with magnetic nuclei (e.g. ^1H) in the immediate vicinity of the radical bearing atom, and smaller couplings for more distant, second sphere nuclei. For example, the glycyl radical,[10-12] primarily located on the C_α carbon (72-76%)[13] resolves only three hyperfine couplings: one large ^1H coupling (proton attached to C_α, 33 MHz) one smaller ^1H coupling (proton attached to C_β 17 MHz) and a nitrogen coupling from the amine group (16 MHz).[14] Similarly alanyl radical, now primarily located on the C_β carbon, also resolves only three hyperfine couplings, two large ^1H couplings (C_α 62 MHz, C_β 73 MHz) and a nitrogen coupling from the amine group (9 MHz).[14] Valine and leucine give similar results, although the nitrogen coupling is not resolved.[14] In contrast, the unpaired electron spin density of an aromatic radical is distributed over the entire conjugated system leading to a larger set of similar hyperfine couplings of smaller magnitude. For example, tyrosyl radicals resolves at least five hyperfine couplings spanning 5 to 35 MHz.[15-17]

Quantitative description of the EPR/ENDOR spectra of *Mf*R2. The EPR spectrum of the radical seen in *Mf*R2 is centered at approximately g = 2.0044 (Fig. 3b, Extended data Fig. 7b). Its spectral width at X- and Q-band is 2.4 and 5.0 mT respectively. The increase in width is due to the anisotropy of the g-tensor. This also gives rise to the asymmetric hyperfine pattern at both X- and Q-band. At X-band at least two hyperfine splittings are observed of the order of 1 mT (28 MHz) and 3.6 mT (10 MHz). By way of comparison a typical tyrosine radical always displays at least three hyperfine splittings/couplings in excess of 15 MHz,[15-17] explaining why the *Mf*R2 radical signal is significantly narrower (Fig. 3b). Corresponding Q-band ENDOR data was collected at six magnetic field positions. These data resolve at least seven hyperfine couplings (Extended data Fig. 7b). These data constrain the magnitude and tensor symmetry of the three largest hyperfine couplings, although it is noted that the smallest component of hyperfine tensors A2 and A3 (i.e. y-component) is unresolved due to spectral congestion.
The entire dataset can be simulated using the spin Hamiltonian formalism. A basis set that describes the radical spin manifold can be built from the product of the eigenstates of the interacting electron (S = ½) and nuclear (^1H, I = ½) spins:

$$|S\ M\ \rangle \otimes |I_1\ m_1\ \rangle \otimes |I_2\ m_2\ \rangle \otimes |I_3\ m_3\ \rangle \otimes |I_4\ m_4\ \rangle \otimes |I_5\ m_5\ \rangle \otimes |I_6\ m_6\ \rangle \otimes |I_7\ m_7\ \rangle \qquad \text{Eq. 1}$$

Here, *S* refers to the electronic spin state, *M* refers to the electronic magnetic sublevel, I_i refers to the nuclear spin state of ^{1}H, and m_i refers to the nuclear magnetic sublevels of each ^{1}H. *S* and I_i take the value of ½ and *M* and m_i take the values ±½. The spin Hamiltonian that describes the spin manifold is:

$$\hat{H} = \beta_e \vec{B}_0 \cdot \hat{g} \cdot \vec{S} + \sum_{i=1}^{7} \left(-g_n \beta_n \vec{B}_0 \cdot \vec{I}_i + \vec{S} \cdot \hat{A} \cdot \vec{I}_i \right) \qquad \text{Eq. 2}$$

It contains: (i) an electronic Zeeman term describing the unpaired electrons interaction with the applied magnetic field (g_i); (ii) a nuclear Zeeman term describing the interaction of each ^{1}H nucleus with the applied magnetic field; and (iii) an electron-nuclear hyperfine term describing the magnetic interaction between the unpaired electron and each ^{1}H nucleus. Spectral simulations were performed numerically using the EasySpin package[18-19] in MATLAB.

EPR/ENDOR data excludes an aromatic free radical which contains a nitrogen as part of the conjugated ring system. Of this set of four candidate aromatic amino acid residues histidine and tryptophan can be immediately excluded. The side-chain of both of these residues contains a nitrogen atom as part of the conjugated system, and as a consequence, the radical forms of these side-chains always exhibit resolved nitrogen (^{14}N/^{15}N) hyperfine couplings. These couplings are characteristically highly anisotropic, with the $A_\perp$ (A_x, A_y) approximately zero and $A_\parallel$ (A_z) of the order of 44 MHz (histidine/imidazole)[20] and 24-29 MHz (tryptophan/indole).[21] The extensive EPR/ENDOR data reported shows that no nitrogen hyperfine coupling is observed for the radical of *Mf*R2 (Extended data Fig. 7a).

The EPR spectrum of the phenyl and phenoxyl radicals. As indicated above the EPR spectra of aromatic free radicals, such as a phenyl radical are complicated owing to the delocalization of the unpaired electron spin across the whole molecule. The unpaired spin resides in the p_z orbitals of the carbon atoms which make up the ring, aligned perpendicular to the ring plane. While these orbitals have no direct overlap with the protons of the ring, the exchange interaction between the unpaired spin in the p_z orbitals with the paired electron spins of the C-H sigma bonds leads to polarization of these bond and thus a hyperfine coupling between ^{1}H ring nuclei and the unpaired electron spin.[22] In addition to the ring protons, the unpaired electron of the conjugated ring also couple to protons of substituent groups. These coupling are typically larger than those of ring protons, as the unpaired electron directly interacts with the protons of the substituent via hyperpolarization.[22]
For the phenyl radical the p_z orbital of each carbon shares the unpaired spin equally (17%). For a substituted ring this is not the case. For example, for the phenoxyl radical it is estimated that the oxyl group and the carbon which it is attached to (C4) carries slightly higher unpaired spin density of 20-25%, with consequently lower spin densities at the C3 and C5 and particularly the C2 and C6 carbon positions of the conjugated ring. Interestingly, it is the para position (C1 on the opposite side of the ring to the oxyl group) that carries the highest spin density of 40%.

The magnitude of the ring and substituent ^{1}H couplings correlates with the unpaired spin density of the p_z orbital of the carbon it is attached to. The approximate magnitude of the coupling is given by McConnell's relation[22]

$$A_i^H = Q_{CH}^H \rho_i^\pi \qquad A_{\beta i}^H = Q_{CCH}^H \rho_i^\pi \qquad \text{Eq. 3}$$

Where Q_{CH} and Q_{CCH} are constant with assigned values of -2.37 mT (66.4 MHz) and 2.72 mT (63.6 MHz), respectively.

In solution, free rotation about the C-C bond axis of alkyl substituent groups, results in the proton couplings of the substituent being equivalent. This equivalence is lost in the solid state e.g. a tyrosyl radical embedded in a protein, in which the alkyl protons have a fixed position relative to the ring plane (Extended data Fig. 7c).

In this instance the hyperfine coupling is

$$A_{\beta i}^{H} = \rho_i^{\pi}\left(B' + B'' \cos^2\theta\right) \qquad \text{Eq. 4}$$

Where B' and B'' are constants and theta is the angle between the proton and a plane normal to the ring plane. B' is small and usually ignored. B'' is 5.8 mT (162.4 MHz).

Supplementary Table 1: **Fitted spin Hamiltonian Parameters**

	g-tensor					
	x	y	z	iso	aniso	
g	2.0065	2.0047	2.0019	2.0044	-0.0012	
	^{1}H hyperfine tensors /MHz					Assignment
A1	**31.7**	26.7	27.6	28.7	1.5	$C_{\beta 1}$
A2	14.6	**2.8***	11.9	9.8	-3.5	H5
A3	9.9	**2.1***	7.5	6.5	-2.2	H6
A4	**6.8**	4.0	2.5	4.4	1.1	H2
A5	**4.9**	-2.1	-2.1	0.2	2.3	Dipolar
A6	**3.8**	-1.4	-1.4	0.3	1.7	Dipolar
A7	-1.1	-1.1	**4.1**	0.6	1.7	Dipolar

*A_y component for A2 and A3 is not well defined

Supplementary Table 2: **EPR data for the ring protons of RNR tyrosyl radicals.[23]**

		^{1}H hyperfine tensors /MHz					
		x	y	z	iso	aniso	angle
S. typhimurium RNR[24]	H3,H5	32.1	**7.0**	19.8	19.6	-6.3	±23
	H2,H6	-	-	-	-	-	-
E. coli RNR[16]	H3,H5	26.7	**8.4**	19.6	18.2	-4.9	±25
	H2,H6	5.0	7.6	**2.1**	4.9	-1.4	±44
E. coli RNR[17]	H3,H5	26.8	**7.8**	19.6	18.1	-5.1	±27
	H2,H6	4.8	7.5	**1.1**	4.5	-1.7	±10
E. coli RNR[15]	H3,H5	26.9	**7.8**	19.7	18.1	-5.2	-
	H2,H6	4.8	7.6	**1.4**	4.6	-1.6	-
Mouse RNR[17]	H3,H5	25.1	**7.0**	19.0	17.0	-4.7	±25
	H2,H6	5.0	7.5	**1.1**	4.5	-1.7	-

Assignment of the fitted hyperfine tensors. As stated above, seven hyperfine tensors were needed to simulate the set of EPR and ENDOR spectra collected across the signal. These can be readily interpreted by comparison to phenoxyl/tyrosyl radicals. The largest hyperfine tensor (A1) is virtually isotropic (axial) and of the order of 30 MHz. It is assigned to a proton of a substituent group (non-ring proton) i.e. one of the protons attached to C_β. It gives rise to the broadest ENDOR lines. It is not clear why this is the case - it may reflect local heterogeneity. The second C_β proton is not observed and is assumed to be small.
The next three hyperfine tensors (A2, A3 and A4) are rhombic (see supplementary table 1). For the first two, A2 and A3, A_y is the unique tensor component. Their tensor structure is very

similar to that of ring protons located at the 3,5 position of a tyrosyl radical (see supplementary table 2). As such they are assigned to two, non-equivalent ring protons. Importantly, these couplings are systematically smaller (35%) than that seen for tyrosyl radicals. The third coupling (A4) is less well defined. Its unique tensor component can be assigned to either A_x or A_z. It bears some similarity to that of ring proton located at the 2,6 position of a tyrosyl radical and is approximately the same magnitude i.e. 4.5 MHz. The remaining fitted couplings are all dipolar, representing more distant protons in the vicinity of the radical (Extended data Fig. 7b).

Assignment of the radical signal seen in the *Mf*R2 protein (Extended data Fig. 7d). While the radical seen in *Mf*R2 protein bears some similarity to a tyrosyl radical its spectral width and overall structure is significantly different. An extended discussion is given below that explains why this signal cannot be assigned to an unmodifed tyrosyl radical or a tyrosyl radical with an additional alkyl substitution.[25]

Reasons to exclude an unmodified tyrosyl (phenoxyl) radical. There are two observations that rule out assigning the radical seen in *Mf*R2 to an unmodified tyrosyl radical: i) the magnitude of the beta proton coupling; and ii) the absence of two large, equivalent ring proton couplings. Both of these properties lead to the radical of *Mf*R2 being significantly narrower than all reported tyrosyl radicals.

i) Magnitude of the beta proton coupling. The crystal structure allows the position of the two beta protons to be inferred. Their positions are constrained by the dihedral angle (69-72.5) between the CH_2 side chain and the ring plane. The range of possible theta angles for the two beta protons are:

$$\theta_1 = \phi - 30^\circ = 39.0^\circ - 42.5^\circ$$
$$\theta_2 = 120^\circ - \theta_1 = 77.5^\circ - 81.0^\circ \qquad \text{Eq. 5}$$

Using Eq. 4 a theoretical estimate for the hyperfine coupling for the two beta protons can be obtained. To do this we need to assume the spin density at C1 position. This is fixed to the range measured for all tyrosines (0.34 - 0.43) with the spread arising due to the presence/absence of an H-bond to the phenolic oxygen[4]. Solution data for para substituted phenoxyl radicals also fall in this range (~0.4). The predicted values are:

$$A_{\beta 1} = 30.0 - 42.2 \quad \text{MHz}$$
$$A_{\beta 2} = 1.4 - 3.3 \quad \text{MHz}$$

Clearly both of these values (i.e. 30-42.2 MHz) are significantly larger than the largest fitted hyperfine coupling i.e. $A1_{iso}$ = 28.5 MHz. To reproduce this value the spin density at C1 must be significantly lower 0.29-0.32, outside the range of an unmodified tyrosine.

ii) Absence of two large, equivalent ring proton couplings. For all tyrosine radicals reported, two ring couplings of the same magnitude are observed (16-18.6 MHz). This is also seen for all phenoxyl radicals, including phenoxyl radicals with additional alkyl substituents. (Strictly speaking the alkyl substitution removes a ring coupling but introduces additional couplings of the same magnitude from the substituent itself). This means that the spin densities within the conjugated ring are not strongly affected by alkyl addition.

The two ring couplings observed for *Mf*R2 fall outside this range (A2 = 9.8, A3 = 6.5 MHz), further demonstrating that the observed radical is not an unmodified tyrosine or a tyrosine with an alkyl substitution.[23, 25]

The measured proton couplings also rule out its assignment to a symmetric semiquinone. o-benzosemiquinone is structurally similar to a tyrosine with an oxygen substituent at C3 (Extended data Fig. 7d). Both its oxygens have approximately the same radical character, and as such the C_2 symmetry axis of the molecule shifts to bisecting the C1-C6, and C3 and C4 bonds. The molecule however displays lower symmetry as compared to a phenoxyl/tyrosine upon substitution at the C1 position (i.e. protons of C2 and C5 are similar, but not equivalent). The addition of the second oxygen as part of the conjugated system acts to remove unpaired spin density from the ring, reducing all ^{1}H hyperfine couplings. This effect is dramatic with the C1 spin density dropping to 0.15-0.18.[26] This value is well outside the range for the spin density at C1 inferred for the *Mf*R2 radical i.e. 0.29-0.32. The largest ring coupling (10 MHz) of o-benzosemiquinone comes from the proton attached to C6 i.e. not derived from the protons on the carbons adjacent to those carrying the oxygens. The remaining ring protons typically display a coupling half this value i.e. below 4 MHz. While the 10 MHz coupling matches the large ring coupling of *Mf*R2, the absence of a second large ring coupling disfavors this assignment.

A phenoxyl radical with an oxygen substituent at position 3 is the most likely candidate. The radical observed in *Mf*R2 is therefore best described as something in between these two canonical radical types. In this respect, literature results on phenoxyl radicals with a methoxy substitution at C3 serve as a guide (Extended data Fig. 7d).[5] As with o-benzosemiquinone, the additional oxygen substituent decreases the unpaired spin density shared across the carbon atoms of the ring, leading to lower proton hyperfine couplings – but the magnitude of this effect is far smaller. The spin density at the C1 position for the o-methoxyphenoxyl radical, as compared to the unsubstituted form, decreases by approximately 20%, as inferred by the C1 proton coupling. We can use this value to scale the C1 spin density range seen for unmodified tyrosines (0.34-0.43), to make a rough estimate for the C1 spin density range for the o-methoxyphenoxyl radical.[25] This yields 0.28 to 0.35, matching the range estimated for the radical in *Mf*R2.
The o-methoxyphenoxyl radical also yields two large, non-equivalent ring couplings. As with an unmodified phenoxyl radical, the proton attached to C5 displays the largest coupling, which is reduced to 60% that of the unsubstituted form (18 vs. 11 MHz, see supplementary table 3). The proton attached to C2, which is adjacent to the methoxy group and the proton attached to C6, which is opposite to the methoxy group, both have smaller couplings, with only one resolved i.e. 5 MHz. These ^{1}H hyperfine couplings (11 and 5 MHz) broadly match those measured for the *Mf*R2 radical (9.8 and 6.5 MHz).
It is noted that a methoxy group introduces three additional ^{1}H couplings of the order of 5 MHz. This additional set of couplings are not observed for the *Mf*R2 radical species, suggesting that a methoxy is not the substitute, but instead the simpler OH unit. The precise nature of the additional oxygen substituent cannot be resolved solely based on the EPR/ENDOR data. It must: i) reduce the unpaired spin density of the ring; ii) break the symmetry of the ring protons; iii) have limited, but not negligible radical character. The exact chemical nature of this species, including its hydrogen bonding environment will require further study.

Supplementary Table 3:
Solution EPR data for p-substituted phenoxyl and o-semiquinone radicals.[25-26]

Substituent	Coupling Constant /MHz

3	1	5	a(3,5)	a(1)	a(2,6)
H	H	H	18.6	28.3	5.0
H	Me	H	16.8	(33.5)	4.1
H	Et	H	16.8	(28.4)	4.2
H	n-Pr	H	17.1	(24.4)	4.1
H	CH_2Ph	H	17.1	(24.4)	4.1
H	i-Pr	H	16.8	(16.8)	≤3.9
H	s-Bu	H	16.8	(12.0)	≤3.9
H	OMe	H	16.0	(4.8)	2.2
Me	H	Me	(18.2)	28.3	4.6
Et	H	Et	(16.0)	26.6	4.6
Me	Me	H	18.2 (18.2)	(31.4)	4.8
t-Bu	Me	H	18.5	(31.9)	4.8
t-Bu	OMe	H	17.4	(4.8)	2.5
OMe	H	H	11.8	23.2	5.0, -
OMe	H	OMe	-	23.2	4.2, -
OMe	CHO	H	12.0	-	6.2
O	H	H	-, 2.7	10.2	2.7,10.2
O	Me^1	H	3.9	12.3	2.0, 10.2
O	Me^2	H	2.8	14.3	0.7, 11.2

Substituent positions labeled using the same convention as supplementary table 2.

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