Statistical analysis of ENDOR spectra

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Electron–nuclear double resonance (ENDOR) measures the hyperfine interaction of magnetic nuclei with paramagnetic centers and is hence a powerful tool for spectroscopic investigations extending from biophysics to material science. Progress in microwave technology and the recent availability of commercial electron paramagnetic resonance (EPR) spectrometers up to an electron Larmor frequency of 263 GHz now open the opportunity for a more quantitative spectral analysis. Using representative spectra of a prototype amino acid radical in a biologically relevant enzyme, the Y42 in Escherichia coli ribonucleotide reductase, we developed a statistical model for ENDOR data and conducted statistical inference on the spectra including uncertainty estimation and hypothesis testing. Our approach in conjunction with 1H/2H isotopic labeling of Y42 in the protein unambiguously established new unexpected spectral contributions. Density functional theory (DFT) calculations and ENDOR spectral simulations indicated that these features result from the beta-methylene hyperfine coupling and are caused by a distribution of molecular conformations, likely important for the biological function of this essential radical. The results demonstrate that model-based statistical analysis in combination with state-of-the-art spectroscopy accesses information hitherto beyond standard approaches.

ENDOR | error model | bootstrap | statistical tests | tyrosyl radical

Structural information at atomic resolution is essential for many areas of physics, chemistry, and biology. Electron spin resonance (ESR) or electron paramagnetic resonance (EPR) (1) belongs to a pool of methods, including nuclear magnetic resonance, optical spectroscopy, X-ray diffraction, and cryo-electron microscopy, which can deliver this information. For biophysical applications, the EPR-based electron–nuclear double resonance (ENDOR) technique is particularly suited for mechanistic studies involving endogenous paramagnetic centers, such as redox-active amino acid radical intermediates or metal ions and clusters, as it measures the hyperfine (hf) interaction between these centers and their surrounding magnetic nuclei (2), providing information on the protein architecture and establishing structural to functional relationships. The method has identified and characterized a wealth of paramagnetic intermediates in enzymes; for recent examples see refs. 3–7. Moreover, ENDOR spectroscopy has recently been proposed as a method of choice to measure molecular distances in the angstrom to nanometer range by 19F spin labeling (8), bridging a critical gap in pulse EPR-based distance measurements in biomolecules and complementing 19F-based NMR spectroscopy (9, 10).

ENDOR spectra of magnetic nuclei are usually recorded in the solid state (a frozen solution of a biological sample) at very low temperatures (T < 80 K) and thus display the full anisotropy of magnetic interactions, resulting in very broad lines. The signals are recorded as a change of the EPR electron-spin echo (11), which is sensitive to drifts in experimental conditions, particularly at low temperatures. Therefore, a model-free analysis of the observed signals constitutes one of the main challenges of ENDOR spectroscopy. In the last decade, progress in EPR instrumentation enabled the implementation of pulsed EPR spectrometers operating in the quasi-optical regime (12). We have recently reported a 1H ENDOR study of the essential Y42 in the wild-type (WT) β2 subunit of Escherichia coli ribonucleotide reductase (RNR) (Fig. 1A), using a commercial instrument (13) operating at 9.4 T/263 GHz. The strong orientation selection (hole burning) under high field/frequency conditions leads to sharpening of the spectra, strongly facilitating their analysis. We demonstrated the effect with the 1H spectrum of Y42, illustrated in Fig. 1B, where the sharp peaks were attributed to internal 1Hs of the radical. Additional broad resonances became visible, whose attribution presents additional challenges. From visual inspection alone, the significance of these signals could not be inferred given their shallow line shapes and the difficulty to separate them from unknown baseline distortions.

The structure and function of amino acid radicals, particularly Y42 in prototype biological machineries such as RNRs or photosystem II, have been the focus of several studies since the 1990s, due to their representative role in biological redox reactions and proton-coupled electron transfer (14, 15). In class Ia RNRs, the Y42/diiron cofactor is essential for enzyme activity and thus for cell survival (16). In contrast to nonprotein Y42 model systems (17, 18), the conformation of Y42 in proteins appeared much more constrained (19, 20). This finding was rationalized as due to the interaction with the protein environment that confers redox properties and thus governs biological function. Nevertheless, the mechanism of action of Y42 at the diiron cluster in

Significance

Statistical modeling of experimental data is gaining increasing importance in biological science due to the availability of large datasets. Here we present a statistical analysis of electron–nuclear double resonance, a technique that delivers information on the angstrom to nanometer scale around paramagnetic centers in proteins. The described method allows for recognizing experimental artifacts and provides the most probable signal as well as its uncertainty. Application to representative high-field electron–nuclear double resonance spectra of a prototype tyrosyl radical in a protein, the β2 subunit of Escherichia coli ribonucleotide reductase, demonstrates that subtle information can be uncovered, such as a distribution of molecular orientations relevant for the biological function of this essential radical.

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The topic of this paper is a statistical analysis of ENDOR spectra and its representative applications to assess the significance of broad spectral features observed in the 263-GHz $^1$H spectra of the *E. coli* RNR $Y_{\alpha}^{\ast}$. We present a statistical model for the experimental data, which is used to extract the “most likely signal.” This model takes advantage of the information hidden in each individual scan (or batch) of the spectrum that is usually lost in the process of signal averaging. The treatment presents estimation of the uncertainty in the ENDOR spectra and permits subsequent statistical tests. The mathematical approach combined with spectroscopy of various isotopically labeled mutants of $Y_{122}^{\ast}$ ultimately uncovered a distribution of conformations of $Y_{122}^{\ast}$, yielding insight into the mechanism of action of this essential radical.

### Statistical Modeling of ENDOR Data

#### The ENDOR Signal.

ENDOR spectra display resonances of magnetic nuclei, which are hf coupled to the unpaired electron spin of a paramagnetic center. The observed doublet associated with one or more equivalent nuclei directly reports the hf splitting (Fig. 1). Resonances are recorded by detecting the effect of a radio frequency (RF) pulse on the intensity of the electron spin resonance signal, here a spin echo. Here, we discuss spectra recorded with a pulse sequence called Davies ENDOR, displayed in Fig. 24 (27); however, our statistical approach is not in any way limited to this pulse sequence. If the RF pulse excites a nuclear spin (27); however, our statistical approach is not in any way limited to this pulse sequence. If the RF pulse excites a nuclear spin, the intensity of the echo is modified (for details refer to SI Appendix, Fig. S1). The ENDOR spectrum is recorded by integrating the transient of the total echo intensity as a function of the RF, which is usually stochastically varied after each sequence shot to attenuate heating (28) and nuclear saturation effects (29).

To analyze and distinguish possible contributions in the ENDOR spectra, we first recapitulate some general features of the EPR and ENDOR signal detection, which is illustrated in Fig. 2R. In the 263-GHz spectrometer, the ENDOR signal is down-converted to 9 GHz, where it is subsequently split into two channels and mixed with a reference signal to permit the standard quadrature detection scheme. When the relative phase of the reference with respect to the signal channel is properly adjusted, the electron spin primary echo (two-pulse echo) appears on the real channel (11). With the phase of the mw pulses defining the $x$ direction, the echo signal is proportional to the $y$ magnetization or to the expectation value of the spin operator ($\langle S_y \rangle$). Due to the effect of mw pulses with finite bandwidth,
a dispersive echo signal appears also on the imaginary channel; however, its integration over time results in a net zero signal.

In pulse ENDOR, discussed in detail by Gemperle and Schweiger (30) using the product operator formalism, only integration over the echo in the real channel gives a detectable ENDOR effect. The physical description does not predict a signal component proportional to \( \langle x \rangle \) or \( \langle y \rangle \). Quadrature detection (QD) scheme in the 263-GHz spectrometer, leading to a real (\( \Re \)) and an imaginary (\( \Im \)) part of the signal, detected as proportional to \( x \) and \( y \) magnetization, or the expectation values \( \Re \) and \( \Im \), respectively, as explained in the text. Symbols containing crosses denote \( \text{mw} \) mixers, and triangles denote video amplifiers. A circle containing an arrow indicates a phase shifter.

ENDOR Spectra via Signal Averaging. Measurements of an ENDOR signal are carried out covering the radio frequencies \( f_b \) indexed by \( \nu \in \{1, \ldots, N\} \). The measurements are repeated and we refer to these repetitions as batches and number them by \( b \in \{1, \ldots, B\} \). Further details are given in Acquisition of ENDOR Spectra. Thus the entire measurement data are presented as a matrix \( Y \) with complex-valued entries \( Y_{b,\nu} \). The batches are affected by experimental noise as we expect random confidence intervals and enabling testing of a range of specific hypotheses, e.g., as to the presence of a particular baseline distortion.

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Honoring the constraints, with the following statistical model:

\[
Z_{\nu} = \psi + \phi e^{i \lambda_{\text{opt}}} (I_\nu + i \omega_\nu), \quad \nu \in \{1, \ldots, N\}.
\]  

We point out that while \( \psi \) is independent of the RF, it does not simply correspond to the EPR signal in the absence of the RF pulse, but may account for an additional offset contribution from the detector. The physical interpretation of these parameters including the offset contribution is schematically illustrated in SI Appendix, section 1. Inspection of Eq. 1 reveals that, even before finding \( \lambda_{\text{opt}} \) is attempted (Shape of the Orthogonal Component \( \omega_\nu \)), there are as many observations (namely \( 2N \) real-valued observations) as there are parameters (namely 2 for \( \psi \in \mathbb{C} \), 1 for \( \phi \), \( N \) for \( I_\nu \), and \( N \) for \( \omega_\nu \)) reduced by 2 + 1 due to the constraints in Eq. 2. Therefore, the parameter estimates \( \hat{\psi}, \hat{\phi}, \text{and} \hat{\lambda}_{\text{opt}} \) (given in closed form in SI Appendix, section 2.1) yield identical zero residuals

\[
R_{\nu} = Z_{\nu} - \hat{\psi} - \hat{\phi} \hat{\lambda}_{\text{opt}}.
\]  

Here and throughout, we use the hat to denote a statistical estimate, i.e., a value computed from experimental data, of a parameter of interest, e.g., estimating \( \psi \) by \( \hat{\psi} \).

Drift Model. The averaging procedure according to Eq. 1 works best when all observations have the spectrum in the same phase (corresponding to constant \( \lambda \)) and magnitude (corresponding...
to constant $\phi$). However, an ENDOR experiment over many hours may exhibit signal drift due to thermal and mechanical instabilities. To investigate the possible drift behavior in detail we analyzed a $^3$H ENDOR data matrix $Y$ consisting of $B = 233$ batches of ENDOR signals of the perdeuterated 1,3-bis(diphenylene)-2-phenylallyl (BDPA) radical. Its $^3$H ENDOR spectrum consists of a single matrix line only and therefore facilitates straightforward decomposition of the ENDOR signal $Y_{\nu\nu}$ into the frequency range $V_0$ that contains an ENDOR effect and the frequency range $V_B$ that contains only baseline. We apply principal component analysis (PCA) separately for each batch and frequency range (see SI Appendix, section 3 for details). The results indicate that the amplitudes $|Y_{\nu\nu}|$ averaged over either $V_0$ or $V_B$, respectively, drift in a parallel fashion across batches. Furthermore, the phases of the leading principal components of $Y_{\nu\nu}$ for the two frequency ranges also drift across the batches. Additionally, the phases differ between the two frequency ranges by an offset of approximately 90°, which stays constant across batches (SI Appendix, Fig. S2F). Overall, it becomes evident that there are baseline distortions and drift across the whole measurement for the parameters $\psi$, $\phi$, and $\lambda$ in Eq. 3. Therefore, the standard averaging procedure is not suitable and can cause severe distortions. Moreover, from the PCA in the frequency region $V_B$ of the baseline we observe an additional signal orthogonal to the spectrum which is discussed in Shape of the Orthogonal Component $\psi_{\nu\nu}$.

Based on these observations, we propose a refined model in Eq. 5 for extracting the ENDOR spectrum $I_\nu$ directly from the complex data matrix $Y$:

$$Y_{\nu\nu} = \psi_0 + \phi_0 \kappa_\nu + \epsilon_{\nu\nu}. \tag{5}$$

Here, $\epsilon$ is a Gaussian noise model with real and imaginary components with standard deviation $\sigma_r$ and $\sigma_i$, respectively. Since the real and imaginary components of $Y_{\nu\nu}$ result from mixing a single measurement signal with a reference in a quadrature detector, we expect correlation between the noise in the two channels and explicitly allow for (partial) correlation expressed by the correlation coefficient $\rho \in (-1, 1)$:

$$\epsilon_{\nu\nu} \sim N(0, \Sigma) \quad \Sigma = \begin{pmatrix} \sigma_r^2 & \rho \sigma_r \sigma_i \\ \rho \sigma_r \sigma_i & \sigma_i^2 \end{pmatrix}. \tag{6}$$

As evident from Eq. 5, there is a close connection to the averaging model: The scalar parameters $\psi$ and $\phi$ are replaced by complex vectors with components $\psi_0$ and $\phi_0$ ($\nu \in \{1, \ldots, B\}$), respectively, to capture variation of the average signal and the amplitude and phase of RF effects across batches enumerated by $b$. To ensure identifiability of the parameters $\psi, \phi, \kappa, \lambda$, and $\Sigma$, we retain the normalization conditions of Eq. 2.

We employ maximum-likelihood estimation to obtain complex-valued estimates $\hat{\psi}_0, \hat{\phi}_0, \hat{\kappa}_\nu$, our iterative algorithm for finding the maximum-likelihood estimator (MLE) is described in Shape of the Orthogonal Component $\psi_{\nu\nu}$. The estimation is minimal. Here, the residual imaginary part refers to what is left of the imaginary part after subtraction of a cosine function whose amplitude, phase, and frequency are chosen to minimize the $l^2$ norm of the residual imaginary part. Following the optimization we obtain the estimated ENDOR spectrum $\hat{I}_\nu$ and the estimated wave distortion $\hat{\omega}_\nu$ as real and imaginary parts of the rotated $\hat{\kappa}_\nu$, respectively. See SI Appendix, section 2.2 for more details.

Comparison of Model Fit. An algorithm for carrying out the estimation given the complex data matrix $Y$ has been implemented in Python. This applies either the averaging procedure in Eqs. 1–3 or the drift model from Eqs. 5 and 6 subject to the constraints in Eq. 2 and follows up by solving the optimization problem described in Shape of the Orthogonal Component $\psi_{\nu\nu}$. The estimation is performed on an example dataset ($^3$H ENDOR of $Y_{122}$ in E. coli RNR WT-$\beta_2$) and summarized in Fig. 3. Fig. 3A presents the EPR spectrum. Fig. 3B provides the resulting ENDOR spectrum $I_\nu$, and the resulting wave $\omega_\nu$ is shown in Fig. 3C. Fig. 3D–F is used to critically assess the averaging model’s goodness of fit and is referred to in greater detail below. Indeed, as the complex data matrix $Y_{\nu\nu}$ is available, we can calculate and inspect the residuals $R_{\nu\nu} = Y_{\nu\nu} - \hat{\psi} - \hat{\phi} \hat{\kappa}_\nu$, which, contrary to those of Eq. 4, are not identically zero and are plotted in Fig. 3D. It is apparent from Fig. 3D that the residuals do not follow any of the well-known distributions and a plot of the frequency-averaged residuals $\bar{R}_\nu = \frac{1}{N} \sum_{\nu=1}^N R_{\nu\nu}$ in Fig. 3E and $F$ reveals the origin of this problem. The residuals are not identically distributed across batches as their mean value changes over batches. This behavior is consistent with the experimental observation that phase and intensity of the echo signal drift with time.

Drift model fit. Results of fitting the drift model to the example dataset ($Y_{122}$ in E. coli RNR WT-$\beta_2$) are summarized in Fig. 4. Analyses of drift model fit for a representative subset of all datasets in the study are available in SI Appendix, Figs. S3 and S4 and Table S1. Comparison with the results from the averaging procedure in Fig. 3 shows that both the
The previously reported broad features in the spectrum (Fig. 1B, asterisks) are reproduced using the drift model (Fig. 4A) and visual comparison suggests that they are indeed part of the ENDOR spectrum and not a result of either random variations due to noise or the wave. Such an assessment is crucially supported by the availability of confidence intervals (displayed in Fig. 4A) from the drift model, which are absent in the averaging procedure. Formal statistical hypothesis tests to further support our observation of broad features are carried out in Statistical Analysis of the \( Y_{122} \) Spectra. The eponymous drift of \( \phi_0 \) and \( \phi_b \) is clearly observed in Fig. 4C and D, respectively. Detailed analysis of the residuals is provided in Fig. 4E–I. Correlation of the real and imaginary components of the residuals \( \epsilon_0, \epsilon_b \) is clearly visible in Fig. 4E and persists after averaging over batches as is demonstrated in Fig. 4G.

Both components of the residuals are remarkably well modeled by normal distributions with mean zero. We visualize this using a normal quantile–quantile plot that plots the sorted sample values of the residuals versus the corresponding quantiles under the normal distribution: Deviations from the diagonal indicate differences between empirical and theoretical distributions. As Fig. 4H and I shows, these deviations appear very small. Indeed, Kolmogorov–Smirnov tests for goodness of fit of the variance-standardized residuals for normality yield \( P \) values of \( P_{KS} = 0.91 \) and \( P_{KS} = 0.69 \) for the real and imaginary components, respectively, each of a sample size of 108,000.

Therefore, the null hypothesis that the residuals follow a normal distribution cannot be rejected. This is a remarkable finding given the sample size and demonstrates the superior fit of the drift model both compared to the averaging model and judged against commonly encountered goodness of fit in statistical practice.

**Application: Testing for Broad Resonances Reveals Conformational Distributions**

The drift model enables extraction of spectra with an estimate of their associated uncertainty. These estimated uncertainties can, in turn, be used to decide whether broad features in ENDOR ENDOR spectrum \( I_\nu \) (in Fig. 3A) and the wave \( \omega_\nu \) (in Fig. 3B) resulting from fitting the two models are similar except for improved signal-to-noise ratio (S/N). This is more clearly visible in SI Appendix, Fig. S5. To see how this improved S/N results from taking the drift into account, consider the following hypothetical example: Assume the phase of the detector drifts at a constant rate covering one full period (360\(^\circ\)) from taking the drift into account, consider the following hypothesis.

\[
C = B \text{ constant rate covering one full period (theoretical example: Assume the phase of the detector drifts at a constant rate covering one full period).}
\]

This would lead to the signal covering the entire range of directions in the complex plane so that contributions pointing in opposite directions will cancel. Mathematically, this is seen by plugging Eq. 5 into Eq. 1. Thus, such a drift would lead to complete cancellation and the spectrum obtained through the averaging procedure would be nothing but noise. In real data cases, phase drift is likely to lead to partial cancellation and thus accounts for varying degrees of improvement in signal-to-noise ratio (SI Appendix, section 8), depending on the amount of drift present in the data.

Fig. 3. Results of applying the averaging model to an example ENDOR measurement. (A) Echo-detected 263-GHz EPR spectrum of \( Y_{122} \) in E. coli RNR WT-\( \beta \). (B) The \(^1\)H ENDOR spectrum of \( Y_{122} \), obtained as \( I_\nu \) in Eq. 3. (C) The approximately cosine-shaped distortion \( \omega_\nu \) (the wave). (D) Kernel density estimate of residuals from Eq. 7. (E) Real component of frequency-averaged residuals \( R_\nu \) plotted versus batch number. (F) Imaginary component of frequency-averaged residuals \( R_\nu \) plotted versus batch number. Experimental settings for ENDOR are given in Materials and Methods. The data were recorded in \( B = 300 \) batches.

Both components of the residuals are remarkably well modeled by normal distributions with mean zero. We visualize this using a normal quantile–quantile plot that plots the sorted sample values of the residuals versus the corresponding quantiles under the normal distribution: Deviations from the diagonal indicate differences between empirical and theoretical distributions. As Fig. 4H and I shows, these deviations appear very small. Indeed, Kolmogorov–Smirnov tests for goodness of fit of the variance-standardized residuals for normality yield \( P \) values of \( P_{KS} = 0.91 \) and \( P_{KS} = 0.69 \) for the real and imaginary components, respectively, each of a sample size of 108,000. Therefore, the null hypothesis that the residuals follow a normal distribution cannot be rejected. This is a remarkable finding given the sample size and demonstrates the superior fit of the drift model both compared to the averaging model and judged against commonly encountered goodness of fit in statistical practice.

Fig. 4. Results of applying the drift model to an example ENDOR measurement. (A) The \(^1\)H ENDOR spectrum of \( Y_{122} \) from \( I_\nu \) in Eq. 3. (B) The distortion \( \omega_\nu \) (the wave). (C and D) Real (black) and imaginary (red) components of \( \phi_0 \) and \( \phi_b \), respectively. (E) Kernel density estimate of residuals \( R_\nu \). (F) Dependence of the real (black) and imaginary (red) components of the averaged residuals \( \bar{\epsilon}_0, \bar{\epsilon}_b \) on the radio frequency. (G) Standard deviation of the real (black) and imaginary (red) components of the residuals \( R_\nu \) over radio frequencies \( \nu \) versus batch number \( b \). (H and I) Empirical quantile—theoretical quantile plots for the real and imaginary components of \( R_\nu \), respectively. Shaded gray and red regions indicate pointwise 95% confidence intervals in A–D, very narrow in A and even more so in C.
spectra, as discussed in the Introduction in the case of Y_{122}.
are statistically significant. To this end, we propose two different
statistical tests.

**Flatness Testing.** The flatness test tests the null hypothesis that
the spectrum is flat over a specified frequency region of interest V_i,
mathematically speaking that it equals an unknown constant,
quantifying the evidence against flatness by the P value P_{flat}.
To accomplish this, we define a test statistic T_{flat} based on the
summed squares of the differences of J from its average value
over the region of interest V_i according to Eq. 8:

\[ T_{flat}(I) := \sum_{i \in V_i} \left( I - \frac{1}{|V_i|} \sum_{i' \in V_i} I_{i'} \right)^2, \]

where |V_i| denotes the number of radio frequency indexes in V_i.
We recall that whenever the P value P_{flat} = P( T_{flat} > \lambda_{bias})
is below the chosen significance level, the null hypothesis of flat-ness is rejected. Here, \( \lambda_{bias} = T_{flat}(\hat{I}) \) is the observed value of
the test statistic whereas T_{flat} denotes the random variation of the
test statistic under the null hypothesis, i.e., assuming that the
deviation from flatness arises only from the modeled noise.
We then use a bias-corrected bootstrap sample from the distribution of T_{flat} to estimate the P value P_{flat} resulting from the test as well as a \( \chi^2 \)-based asymptotic approximation \( P_{flat,X} \). We inspect the standard significance levels of 0.1\%, 1\%, and 5\% Benjamini–Hochberg corrected for multiple testing (a total of 56 flatness tests are conducted herein). Details are given in Section S1 and all \( P_{flat} \) values are reported in SI Appendix, Tables S2 and S3.

**Difference Testing.** To assess the effect of partial deuteration when comparing the ENDOR spectra \( I_{w_t} \) of the Y_{122} and \( \bar{I}_{D2} \) of the \( \beta, \beta'-[\text{H}]_2\text{-Y}_{122} \) samples, a test ascertaining whether an observed difference between \( I_{w_t} \) and \( \bar{I}_{D2} \) is statistically significant is desirable. To avoid comparison of \( P \) values from two individual flatness tests for the individual spectra, we propose a difference test considering both measurements and their uncertainties simultaneously.
The method’s null hypothesis is that a straight line least-squares fitted to the difference between \( I_{w_t} \) and \( \bar{I}_{D2} \) in the frequency region of interest V_i of the spectrum has slope zero; i.e., it is horizontal. We again employ bias-corrected bootstrap samples of both spectra. Details on how the test computes the resulting P value P_{diff} as well as its Gaussian-based asymptotic approximation \( P_{diff,X} \) are given in Section S1. Since we will perform a total of 16 difference tests, we employ a Benjamini–Hochberg correction for multiple testing.

We note explicitly that our slope-based approach has high power for detecting monotonous differences covering a broad spectral range and is therefore well suited to test for the linear component of the suspected broad features in the Y_{122} spectra. The region of interest has to be chosen with care, as the test inherently has low power if positive and negative slopes of the difference between the spectra are canceled out on average within V_i.

**Statistical Analysis of the Y_{122} Spectra.** To analyze the origin of the broad features in the Y_{122} spectrum (Fig. 1B) with the aid of statistical methods, three \( \text{H} \)-isotope–labeled \( \beta_2 \) protein samples were prepared and investigated. All protein mutants were catalytically active and contained about 1 Y_{122} per \( \beta_2 \) (Incorporation of D_{2}-Y, D_{3}-Y, and D_{7}-Y in the E. coli RNR \( \beta_2 \) Protein). Echo-detected EPR spectra at 263 GHz of the four samples, i.e., Y_{122} (WT), \( \beta, \beta'-[\text{H}]_2\text{-Y}_{122} \) (D2), 2, 3, 5, 6-[\text{H}]_2\text{-Y}_{122} (D4), and 2, 3, 5, 6, \( \beta, \beta', \alpha-[\text{H}]_2\text{-Y}_{122} \) (D7), are reported in SI Appendix, Fig. S6A. The spectra are clearly dominated by \( g \)-factor anisotropy and their comparison nicely demonstrates the expected effect of partial deuteration: The large hf interaction with one of the \( \beta \)-methylenes protons, which produced the doublet structure visible in Fig. 3A, in the EPR spectrum of Y_{122}, is not observed in the \( \beta, \beta'-\text{deuterated analogs} \) (D2 and D7).
The \( ^1 \text{H} \) Davies ENDOR spectra of the four samples were typically measured at five different resonance positions in the 263-GHz EPR line, of which only the position denoted g_z is shown in Fig. 5—all other positions for WT and D2 data are provided in SI Appendix, Fig. S5. Note that the spectra in Fig. 5 and in SI Appendix, Fig. S5 are obtained from the drift model as opposed to the spectrum in Fig. 1, where the averaging model was employed. The five resonance positions, at which the ENDOR spectra were typically recorded, are denoted by g_x, g_y, g_z = 5 mT, and g_z and they are illustrated in SI Appendix, Fig. S6B. All displayed spectra were analyzed with the drift model and frequency-wise 95\% confidence intervals were computed using J = 1,000 bootstrap samples to obtain error bars.

![Fig. 5. The 263-GHz \( ^1 \text{H} \) Davies ENDOR spectra (recorded at EPR resonance position g_z) of the four protein samples with different \( \text{H} \)-content of Y_{122} and results of flatness tests—the deuteration scheme is given in Left Insets. (A) Wild-type data, (B) D2 data, (C) D4 data, (D) D7 data. The \( \chi^2 \)-approximated \( P \) values obtained from the flatness test are included and significance (after Benjamini–Hochberg BH correction across all flatness tests in the paper) is indicated by * for \( P \) values achieving the 0.001 BH-corrected significance level and by *** for \( P \) values achieving the 0.05 BH-corrected significance level. The orange center line indicates the \( ^1 \text{H} \) Larmor frequency.](https://doi.org/10.1073/pnas.2023615118)
The Davies ENDOR spectra show the expected disappearance of the ring \(^1\)H\(^{3,5}\) resonances in the D4 and D7 samples (Fig. 5 C and D at ±10 MHz). The broad features (Fig. 1B) seem absent in spectra in Fig. 5 B and D; however, ascertaining the presence of the broad features in the spectrum in Fig. 5C by simple inspection is difficult and requires statistical analysis. Additionally, we note that surprisingly little variation is observed among isotopologues in the central area of the spectra (white, central areas in Fig. 5). Thus, the lines attributed to ring \(^1\)H\(^{2,6}\) resonances (±1 MHz) are only reduced in intensity. The large number of resonance lines in the spectrum of the D7 sample is the first clear indication of multiple contributions due to the protein environment.

We apply the flatness test to all ENDOR spectra in those spectral ranges lacking resonances due to known hf couplings of the ring protons. The results are depicted in Fig. 5. For all spectra shown, two or four regions (I to IV) of interest were examined: the left and right marginal regions (regions I and IV in Fig. 5) as well as the intermediate regions, i.e., the parts where broad features are suspected (regions II and III for spectra in Fig. 5 A–C). For all spectra in Fig. 5 A–D, these regions were defined using the same offsets relative to the proton Larmor frequency. Regions III and IV were chosen to be mirror images of regions II and I, respectively, following the approximate mirror symmetry of the ENDOR spectra with respect to the proton Larmor frequency. The resulting \(P\) values and an indication of Benjamini–Hochberg-corrected significance (by asterisks) are also included in Fig. 5. We observe that in all marginal regions, the \(P\) values are above the standard 0.05 significance level (even before correction for multiple testing), consistent with our null hypothesis of flatness. Only in the intermediate regions of spectra in Fig. 5A and C do we observe \(P\) values that are surprisingly small. Since we have already confirmed goodness of fit of the drift model (Drift model fit), we can rely on the \(P\) values and cannot attribute the surprisingly small \(P\) values to model misfit. Therefore, either an extremely low probability event yields the surprisingly small \(P\) values or the null hypothesis (flatness) is wrong in the regions concerned.

We thus conclude that statistically significant deviations of the spectrum from flatness are found in the intermediate regions for the WT and the D4 isotopologue. Similar results are obtained in an analogous fashion for the remaining spectra at other orientations as summarized in SI Appendix, Tables S2 and S3.

We apply the difference test to the WT-D2 pair of ENDOR spectra and the small \(P\) values indicate statistically significant differences in all intermediate regions (Fig. 6). In contrast to this, the difference test does not reject the null hypothesis (i.e., no difference in the spectra) in any marginal regions. Since the test has sufficient statistical power to detect differences (as seen in the intermediate regions), it is reasonable to assume that the spectra are in fact not different in the marginal regions.

Thus, while the flatness test confirms that the WT spectrum is not flat in the intermediate regions, the difference test confirms that the WT and the D2 spectra are different from one another in these regions. In the marginal regions, on the other hand, D2 and WT spectra seem to both be flat and show no differences from one another. This supports the attribution of the differences in the intermediate regions to deuteration because other potential variations between measurements (e.g., in detector offsets, RF heating, etc.) would likely have caused statistically significant differences or nonflatness in other regions, too.

Together, both statistical tests therefore unequivocally confirm the presence of the broad features in the WT and D4 spectra suspected upon visual inspection and support their attribution to the \(\beta\)-methylene protons.

**Evidence for a Distribution of Hyperfine Couplings.** Given the association of the newly discovered broad features in the ENDOR spectra of \(Y_{122}^\star\) with the \(\beta\)-methylene protons (\(H^{\beta_1}\) and \(H^{\beta_2}\)), the question arises: What is the origin of their peculiar line shape? Considering that one of these two protons (\(H^{\beta_2}\)) produces the dominant, almost isotropic hf splitting (ca. 60 MHz) observed in the EPR spectrum (Fig. 3A), the smaller interaction to \(H^{\beta_2}\) is likely responsible for the broad features. In our previous analyses (13, 33) we attributed the \(H^{\beta_2}\) coupling to the sharp inner peaks at \(g_\beta\) (±0.5 MHz; Fig. 5A). However, from the D7 spectrum in Fig. 5D it becomes evident that most sharp peaks in the central part of the \(^3\)H ENDOR spectrum (within ±2 MHz) are conserved upon deuteration and must arise from the protein environment. This finding together with the establishment of the new broad features caused us to reexamine the \(^3\)H assignments.

Previous density functional theory (DFT) studies (18, 34) have predicted that the isotropic part of the \(H^{\beta_1}\) and \(H^{\beta_2}\) couplings strongly depends on the ring dihedral angle (defined by the
Cα, Cβ, Cγ, and Cε atoms; Fig. 7, Inset) of the tyrosyl radical. To illustrate this dependence, we have plotted the hf tensor components of Hβ1 and Hβ2 as a function of this dihedral angle (Fig. 7A) as calculated by DFT on a small tyrosyl radical model system (Materials and Methods). As expected, the hf couplings are nearly isotropic. We note that for orientations of Hβ2 close to the ring plane (corresponding to a dihedral of 120°) its hf coupling is nearly zero. Nevertheless, very subtle reorientation of the radical can strongly affect the coupling. Thus, we presume that the broad features in the ENDOR spectrum arise from a distribution of ring dihedrals in Y_{122}. A distribution of Hβ couplings due to structural heterogeneities has been previously reported from 2H-electron spin echo envelope modulation studies of tyrosyl radicals in frozen solutions produced by light irradiation (17). However, to our knowledge, distributions of Hβ2 couplings specifically have not been reported for protein Y_{122}.

To validate the assignment of the broad features to Hβ2, we performed spectral simulations combined with DFT predictions for the Hβ couplings. For simplicity, we describe the distribution of ring dihedral angles using a normal distribution with only two parameters (mean μ and half-width σ) (Fig. 7B). These two parameters were adjusted to reproduce the new broad features in the ENDOR spectra as well as the resonances of Hβ1. All ring 1H couplings were adopted from the previous reports in the literature (13, 20, 33, 35). Representative ENDOR simulations for values of μ = 144° and σ = 5° and including only the internal Yβ1-H hf couplings are depicted along with the experimental spectra in Fig. 7 C–E. The relatively narrow distribution (width of 2·σ ≈ 10°) is sufficient to spread the hf values of Hβ2 over a range up to 22 MHz and to qualitatively reproduce the line shape of the broad absorptions. Interestingly, the Hβ1 coupling covers a range of ca. 10 MHz and is less sensitive to the conformational distribution. This is due to the cos² δ dependence of the hf coupling on the dihedral angle δ combined with the particular mean value of the dihedral angle in Y_{122} (Fig. 7A and B). Nevertheless, the distribution significantly improves the simulation of the Hβ1 ENDOR resonances (SI Appendix, Fig. S7). A more elaborate mathematical procedure to analyze the distribution in terms of possible radical conformations, as for instance proposed in ref. 17, is in progress but goes beyond the scope of this work. It will require a global statistical analysis of the ENDOR spectra including the wider range of the Hβ1 resonances.

All simulation parameters and comparison with previous assignments are reported in Table 1. We note that the distribution of dihedral angles also affects the coupling of Hα, albeit to a lesser extent in comparison with Hβ1 and Hβ2. This coupling is included in the simulation and gives rise to signals close to the matrix line. Simulations were also performed assuming only one conformation of the Y_{122}, i.e., the most probable one with dihedral angle μ = 144°. This yielded significantly sharper and more structured features not consistent with the spectra (SI Appendix, Fig. S8). Notably, the EPR spectrum is also consistent with the observed distribution, which produces a line broadening of less than 0.3 mT. The broadening is within the intrinsic EPR line width at 263 GHz (13) and therefore not visible in the EPR spectrum, as confirmed by the observable Hβ1 splitting (Fig. 3A) as well as by the EPR simulation performed with the same parameters as the ENDOR simulations (SI Appendix, Fig. S6b).

Inspection of various structures of the E. coli RNR β2 determined by X-ray diffraction (PDBs 1rib, 1pfr, 1mxr, 5ci4) (22, 36–38), in which Y_{122} is protonated and reduced, reveals that the crystal structure shows the ring dihedral angle Y_{122} ranges from 127° to 142°. The Y_{122} g tensor was found to be reoriented by 5° to 10° compared to the side-chain orientation in the reduced form (22, 39). From initial ENDOR studies at low EPR frequencies, the ring dihedral in frozen solution was estimated to be around 120° (20, 35). All these values are in principle close to or within the range of the distribution identified here, but taking them individually, they would not be consistent with the spectra. We thus conclude that the dihedral angle approximately follows a normal distribution with mean value μ ≈ 144° and strictly positive standard deviation σ ≈ 5°. Our results give evidence that Y_{122} in its protein pocket has some conformational flexibility that has never been observed before, despite substantial spectroscopic efforts since the 1980s.

**Conclusion and Outlook**

We have demonstrated that statistical analysis is able to model ENDOR spectra, taking into account the information hidden in multiple individual experiments (batches) that is lost in the usual process of signal averaging. This permits us to reconstruct the ENDOR signal with confidence intervals, valuable for statistical hypothesis testing, without having to rely on assumptions on the unknown spectrum. This approach can distinguish experimental baseline distortions from subtle absorption features, such as less populated conformations or distributions that are important to understand biological function. Moreover, the approach is general as it can in principle not only be adapted to ENDOR spectra at any microwave frequency but also expanded for other types of spectroscopic methods. Specifically, for ENDOR the availability of a statistical model that fits the measured data well allows uncertainty quantification and opens the way for more sophisticated data modeling and interpretation, for instance to systematically achieve better agreement of simulated with measured spectra.
Table 1. Hyperfine coupling parameters used for the EPR and ENDOR spectral simulations

| Nucleus | $A_x$/MHz | $A_y$/MHz | $A_z$/MHz | $\alpha^x/\gamma$ | $\beta^x/\gamma$ | $\gamma^x/\gamma$ |
|---------|------------|------------|------------|-----------------|----------------|----------------|
| H$^3$   | −27.2      | −7.9       | −19.8      | 20              | 0              | 0              |
| H$^5$   | −27.2      | −7.9       | −19.8      | 155             | 0              | 0              |
| H$^2$   | 4.9        | 7.7        | 25         | 0               | 0              | 0              |
| H$^6$   | 4.9        | 7.7        | −155       | 0               | 0              | 0              |
| H$^\alpha$ | 1.4 (1.2 to 1.6) | −2.1 (−2.9 to −1.8) | −1.8 (−2.2 to 1.6) | 83 (73 to 88) | 106 (95 to 82) | −40 (−35 to −43) |
| H$^{\beta_1}$ | 60.2 (54.7 to 61.6) | 53.7 (48.3 to 54.9) | 55.5 (49.9 to 56.9) | 3 (43 to 19) | 33 (38 to 36) | −1 (−35 to 18) |
| H$^{\beta_2}$ | 59        | 55         | 56         | 7               | 0              | 0              |
| H$^{\beta_4}$ | 12.8 (6.9 to 21.9) | 7.0 (1.3 to 15.9) | 7.5 (1.8 to 16.5) | −81 (−94 to −77) | 135 (172 to 99) | 68 (60 to 82) |

As values for H$^\alpha$, H$^{\beta_1}$, and H$^{\beta_2}$ depend on the ring dihedral angle, values for the highest-probability conformation (144°) are given along with the maximum and minimum values (in parentheses). For comparison, values for H$^3$, H$^{\beta_5}$, and H$^{\beta_3}$ from our previous report (13) are included in italic font. For the ring protons previously reported values (13) were used. Euler angles follow the $x'y'z'$ convention and describe the rotation of the A tensor to the g tensor. Positive values correspond to clockwise rotation when viewed along the rotation axis. The g values were $g_{x'y'} = 2.00915, 2.00460, 2.00225 (13)$.

Applying this approach to data collected for the essential tyrosyl radical Y$^{T22}$ in the wild-type $\beta_2$-subunit of E. coli RNR as well as three additional mutants, in which the radical was partially deuterated, allowed the unambiguous distinction of broad spectral features from the baseline. We verify the presence of previously unrecognized hf couplings using two statistical tests, a flatness test and a difference test. The result establishes that the coupling is contributed by the H$^{\beta_2}$ and is consistent with a distribution of conformations. Such a distribution has not been observed before due to the difficulties of analyzing broad ENDOR resonances in the powder pattern of multiple contributing nuclei.

A wealth of studies have been reported to characterize the detailed electronic structure and the role of tyrosyl radicals in proteins, with the Y$^{T22}$ of E. coli RNR being a prototype. The observation of a conformational distribution of Y$^{T22}$ strongly suggests that very small conformational changes might be sufficient to couple and uncouple this radical from the adjacent diiron cofactor and from its hydrogen bond donor, likely a water molecule at this cluster. Thus, our finding draws attention to the role of less populated states in such a highly regulated mechanism. Further, since the diiron cofactor and from its hydrogen bond donor, likely a water molecule at this cluster. Thus, our finding draws attention to the role of less populated states in such a highly regulated mechanism. Thus, our finding draws attention to the role of less populated states in such a highly regulated mechanism. Such a distribution has not been observed before due to the difficulties of analyzing broad ENDOR resonances in the powder pattern of multiple contributing nuclei.

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a linear sweep of the RF range. Prior to each ENDOR measurement, an echo-detected EPR spectrum was recorded to determine the excitation position for ENDOR. Comparison of echo-detected EPR spectra before and after the ENDOR measurements indicated negligible field drifts of about 0.2 mT during the measurement time (ca. 22 h). Davies ENDOR spectra of the protein samples were measured at 5 K using microwave pulses of equal amplitude. If not otherwise indicated in the figure legends, the preparation pulse length was set to 200 ns, while for spin echo detection a corresponding π/2 − π − π sequence with a × value of 550 ns was used. RF pulse length was 60 μs, and the repetition time of the sequence was 12 ms at one shot per point. Acquisition times were typically between 10 and 20 h.

**Acquisition of ENDOR Spectra.** For statistical purposes, the availability of every single measurement is desirable but this exceeds the on-board storage capacity of the detection unit in the case of several thousand repetitions. As a compromise, several experiments are accumulated (i.e., averaged) in what we herein term a batch to balance averaging time per batch and memory requirements. In all experiments described herein, 50 individual experiments were accumulated in less than 5 min per batch and we typically used 8 = 300 batches. A data matrix X was recorded at five representative excitation positions in the EPR lines to cover most molecular orientations according to their g values (SI Appendix, Fig. S5).

**DFT Calculations.** The dipeptide Ac-Tyr-Me was chosen as a model system as it allows meaningful use of the dihedrals Φ (defined by the peptide backbone atoms C−1, N, Cα, and C) and Ψ (defined by N, Cα, C, and N−1) to constrain the overall amino acid orientation to the values observed in the solid-state structure determined by X-ray diffraction (PDB 1mrx) (22). Following the results of a single-crystal EPR study of the system, the backbone dihedral (defined by atoms C, Cα, Cβ, and Cγ) was constrained to −169.4° and the ring dihedral was varied in steps of 0° to 180°. The radical structure was then optimized (Orca 4.0.1.2) (44) without any further constraints using the B3LYP functional in conjunction with the def2-TZVP basis set (45). Normal self consistent field convergence criteria and a dispersion correction were used (46). Subsequently, hf couplings were calculated using the DFT method, the hf coupling size depends strongly on the degree of spin delocalization. The corresponding spectra were then averaged over all ring dihedral values, weighted by their corresponding probability. The simulated EPR spectra were convolved with a Gaussian line shape of 10−4 mT width, and the ENDOR spectra are simulated with Lorentzian line shapes with a width of approximately 20 kHz for a single transition.

**Data Availability.** Python code and measured data have been deposited in the University College London data repository (47).

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