

Direct Detection Of A Globin-Derived Radical In Leghaemoglobin Treated With Peroxides

Posted on March 25, 2025

Introduction

Introduction to Leghaemoglobin

Leghaemoglobins (Lb) are a homogeneous group of monomeric proteins with a molecular mass of ~16,000. They are mainly distributed in nitrogen-fixing root nodules of leguminous plants. The contribution of leghaemoglobin in these nodules is to aid the transport of oxygen into the bacteroids (nitrogen-fixing symbiotic bacteria). Nitrogen fixation is essential to plant growth because it is the process of taking nitrogen out of the atmosphere and converting that into a form available for plants, which helps create healthier soil [1]. Leghaemoglobins are structurally similar to other oxygen-transporting heme proteins in this respect, such as myoglobin and hemoglobin. The haem group is a prosthetic found in all globins, and it performs the function of oxygen binding. Fe^{2+} represents the majority of iron in protohaem when it is reduced, i.e. active. This leghaemoglobin is a form with very high oxygen affinity, which guarantees efficient supply of molecular oxygen to the bacteria even in a hypoxic environment, as it occurs within root nodules. Although the oxygen affinity is high, oxygen concentration in the nodule must be tightly regulated to avoid inhibiting nitrogenase; otherwise, it would not function properly. Consequently, even in a state of steady-state leghaemoglobin, only about 20% is oxygenated, thereby rendering oxyleghaemoglobin (OxyLb). That which is left stays as a deoxygenated leghaemoglobin in case some oxygen needs to be bound later. This equilibrium is vital for the balance necessary to fix nitrogen efficiently [2].

Link with Globin-Derived Radicals

Leghaemoglobins play a critical role in the transportation of oxygen within root nodules, but their interaction with O_2 and other reactive species can generate globin-derived radicals (GDRs), which are very active and could also act as toxic molecules. This process commences when oxyleghaemoglobin (OxyLb) autoxidises, a reaction in which the iron within its haem group is oxidised from ferrous (Fe^{2+}) to ferric ion state (Fe^{3+}), converting OxyLb into Metleghaemoglobin (MetHlb). This is followed by the release of superoxide radicals (O_2^-), one form of Reactive Oxygen Species (ROS) [3].

Superoxide radicals are short-lived and may readily dismutate to form hydrogen peroxide (H_2O_2) in a reaction known as disproportionation. Although less reactive than superoxide, hydrogen peroxide can interact with methaemoglobin to generate the overall more harmful radicals. One of them is the

Fenton type interaction, in which hydrogen peroxide reacts with MetLb iron that giving rise to hydroxyl radicals ($\bullet\text{OH}$), one of the most reactive and deleterious ROS. These hydroxyl radicals can react with the leghaemoglobin protein directly, causing oxidative damage of, especially Tyr residues in that protein. For example, leghaemoglobin has an oxidation-sensitive tyrosine residue Tyr133 that forms a globin-derived tyrosyl radical. These radicals may lead to structural changes in the leghaemoglobin molecule, compromising its functionality and subsequently triggering numerous processes of oxidative damage within the cell. The formation of radicals from globin is not merely a biochemical oddity; the radical has important physiological consequences for the plant. When not properly regulated by the antioxidant defenses of the plant, these radicals can wreak havoc on cellular machinery causing huge damage to lipids, proteins and DNA with eventual cell death. This should disrupt nitrogen fixation inside the nodules that are rootsome, additionally putative to extend growth of the plant and improve soil with nutrition like elements [4]. Therefore, elucidating the relationship between leghaemoglobins and globin-derived radicals is essential to understanding how leguminous plants control oxygen availability within their root nodules. Similarly, it reveals general principles of how plants balance their metabolic requirements for oxygen against preventing oxidative damage, optimizing health and productivity not only at the level of a plant but also with respect to its soil-associated ecosystem [5].

Importance of Tyrosine Residue at Position 133 (Tyr133)

Tyrosine residues are frequently found in active sites of proteins where they catalyze different kinds of chemical reactions. Of these, the tyrosine residue Tyr133 is particularly noteworthy in the case of leghaemoglobin. This residue, situated within the protein structure of leghemoglobin acts as a strategically important amino acid and plays therefore an essential role, especially under oxidative circumstances, for structural and functional stability.

One of the most physiologically relevant targets is Tyr133, which is an attractive site for formation of globin-derived radicals because this residue is susceptible to oxidation. Under conditions of [oxidative stress](#), auto-oxidation of oxyhemoglobin generates hydrogen peroxide (H_2O_2), which has been postulated to react with methemoglobin, resulting in the generation of highly reactive hydroxyl radical ($\bullet\text{OH}$). The tyrosyl radical is formed after the radicals abstract a hydrogen atom from Tyr133 [6]. This radical can make substantial changes to the structure and function of the leghaemoglobin molecule. Several can follow from the radical formation at Tyr133.

Firstly, it can disrupt the ability of the protein to effectively engage in oxygen binding and transport. This is especially problematic in the case of nitrogen-fixing root nodules, because oxygen supply needs to be highly efficient throughout a petiole and into bacteroids. Presumably, N_2 -fixation-mediated growth promotion and the ability of a plant to increase soil fertility can fail when oxygen transport is impaired. In addition, the radical generated in Tyr133 could trigger an oxidative chain reaction within CALB, indirectly leading to considerable damage to both the protein and surrounding cellular structures. Leading to additional degradation of the leghaemoglobin molecule and other

essential cell components such as lipids, nutrition acids, proteins, etc. This kind of oxidative damage can weaken the general health and degradation in nitrogen fixation efficiency at the root nodules level, ultimately leading to death. In addition, the oxidative modification of Tyr133 may also be an indicator for intracellular oxidative stress. Thus, detecting tyrosyl radicals or other oxidative modifications at Tyr133 may be developed as an index of the degree of oxidation within the root nodules, representing a criterion to assess in vivo stress physiological status of the plant [7].

Overall, Tyr133 in leghaemoglobin is an important residue that facilitates the structural integrity and overall function of the protein. As it is a hotspot of oxidative modification, this study focused on the changes that occurred in leg-haemoglobin during oxidative stress and whether such changes can affect its function within nitrogen-fixing root nodules. Understanding Tyr133 and its interaction provides a vital understanding of the potential mechanisms behind oxidative damage in plants, which can help scientists work on strategies to improve plant resistance against stress caused by oxidation, thus improving agricultural productivity as well as sustainability [8].

Soybean: An Ideal Model Organism for Our Study

Soybean (*Glycine max*), one of the most widely cultivated leguminous crops in our agricultural systems, serves as an excellent model species for studies on nitrogen fixation and related biochemical processes. This was especially interesting since soybean is crucial in this context and hence an ideal candidate for probing leghaemoglobins under induced oxidative stress responses including the generation of globin-based radicals [9].

1. **Economic and Agricultural Significance:** Soybean is a crop, which occupies an important place in world economics (provides about 70% of all protein and oil content for human consumption and animals). It has the ability to fix atmospheric nitrogen through symbiosis with rhizobia bacteria in root nodules, and this also significantly reduces the need for chemical nitrogen fertilizers, which is important since its production releases large quantities of carbon dioxide into industrial waste streams. Knowledge of the molecular mechanisms driving this beneficial relationship, notably concerning leghaemoglobins (Lb), is key to further improving soybean yields and stress resistance, with direct relevance for global food security [10].

2. **Extensive Research Foundation:** Soybean has been studied in-depth from the aspects of genetics, physiology and biochemistry over many years setting a solid base for research. Well-characterized soybean cultivars, genetic tools and comprehensive genomic data availability make it possible to carry out detailed molecular oriented studies (Severin et al. This was made possible because of a vast amount of pre-existing knowledge that we could leverage to investigate something new about soybean biology, the contribution and role in leghaemoglobin function defined by individual amino acids, such as Tyr133 [11].

3. **Symbiotic Relationship and Nitrogen Fixation:** Since soybean establishes a symbiotic relationship with N_2 -fixing bacteria and has the most substantial nitrogen fixation amount in terrestrial

ecosystems, it became an ideal model for analysis of biochemical pathways responsible for this process. In rhizobia-infected cells, leghaemoglobins are essential to supply oxygen for the bacteroids and at the same time protect nitrogenase from oxygen inactivation. The well-described model of nodule formation and function in soybean provides an excellent opportunity to undertake comprehensive studies on their impact on nitrogen fixation efficiency, oxidative stress a special focus on sites such as Tyr133 [12].

4. Genetic and Environmental Variability: Due to the wide range of genetic diversity and environmental adaptability present in soybean, it would serve well as a model for understanding how various leghaemoglobin function-related factors are influenced by oxidative stress. This natural diversity can be utilized by researchers to identify soybean lines that are less susceptible to oxidative damage, which could also translate into legume crops. Additionally, this will facilitate identifying conditions under which the formation of globin-derived radicals in soybean is affected by environmental factors and hence offer leads for designing strategies to alleviate oxidative stress under agricultural settings [13].

5. Practical Relevance to Crop Improvement: Studying soybean, as a model organism has direct implications in crop improvement programs. Knowledge of the role that leghaemoglobins play in nitrogen fixation and upon Tyr133 possibly oxidative stress could be utilized to develop a precise sort staining soybean which will fix additional N-demand from the literature fixing regions under hostile environmental conditions. That can, in turn, increase yields and make for a more ecologically friendly food production system that includes sustainable agriculture practices to support farmers [14]

Electron Paramagnetic Resonance (EPR) Spectroscopy

Electron Paramagnetic Resonance (EPR) spectroscopy, also known as Electron Spin Resonance (ESR), is the most important method to determine materials with unpaired electrons, and it has been successfully applied in different areas from physical chemistry to biology. This method is based on the sensing of interaction between these unpaired electrons and an external magnetic field. Simply put, in practice, the sample is placed under a static magnetic field and irradiated with microwaves. The unpaired electrons in your sample will absorb microwave energy at certain frequencies to cause transitions between these different spin states [15].

The main data obtained from EPR spectroscopy are the g-value, which tells us about the electronic structure and magnetic surroundings of unpaired electrons, and hyperfine splitting, which reveals interactions between the electrons and nearby nuclear spins. By analyzing these EPR spectra, researchers can gain a detailed understanding of the electronic properties and chemical environment of the radicals or paramagnetic centers within the sample [16].

UN Scan It

UN Scan It is a powerful converting tool that allows digitalization of visual (spectroscopic) data, i.e. from scanned images with EPR spectra. The digitization process consists of capturing the graphical image of the spectrum and converting it into a numerical dataset to be analysed computationally. This tool is a vital part of our research as it permits the accurate extraction of spectral datasets from visual formats. Correct digitization guarantees the consistency of subsequent analyses to actual properties of EPR spectrum (like peak positions and intensities) that are necessary for interpretation of radicals in leghaemoglobin [17].

MATLAB

MATLAB (Matrix Laboratory) is a high-level programming environment used for numerical computation, data analysis and visualization. For our work, MATLAB is used for various purposes such as calibration and data processing of EPR spectra. It is capable of taking in the digitized data and performing complex mathematical operations for manipulation of quantities such as Angle Theta (the angle between magnetic field and sample) or ROC1 parameter controlling signal alignment. The broad spectrum of functions and toolboxes available in MATLAB allows for studying the EPR data at a much more detailed level, as the comparison with theoretical models can reliably govern experiments by fine-tuning experimental conditions [18]. This initial processing is crucial to guarantee that you get robust and trustworthy results in your study.

EasySpin

EasySpin is a MATLAB toolbox that was designed specifically for the simulation and analysis of EPR spectra. A group of tools to perform simulations on the behaviour of radicals and other paramagnetic species in a sample. EasySpin simulations perform the EPR spectra on a theoretical level in order to compare these with experimentally collected data of leghaemoglobin. With an associated toolbox, one can accurately fit the simulated spectra to these experimental results in great detail, essentially providing a blueprint of electronic and magnetic properties for those radicals. EasySpin helps in elucidating the interactions and mechanisms of these radical centers within the protein using more advanced models and spectral data fitting [19].

TRASSA

TRASSA (Transient Spectroscopy and Simulations Assistant) is Specialized software for the in-depth simulation and fitting of EPR spectra This consists of several parts: TRASSA Stimulation Y — allowing to simulate EPR spectra; Trassa, y Fitting (for the fine tuning fitting of simulations with experiment data) and TRASSA-Y File for incorporation optimization results. TRASSA, in our study, aids in adjusting the spectral simulations such that they mimic experimental observations more faithfully. The program

features allow for iterative refinements in addition to offering clear specific instances of the drastic changes occurring throughout leghaemoglobin, improving confidence and accuracy within EPR data interpretations [20].

PDB Swiss Reviewer

PDB Swiss Reviewer is a sophisticated tool with which you can visualize and validate three-dimensional protein structures. This helps researchers load protein models and visualize their 3D arrangement. In your study, you use PDB Swiss Reviewer to examine the 3D structure of leghaemoglobin and focus particularly on important residues like Tyr133. The software yields a detailed landscape of the conformational intricacies in relation to this protein, which is important as it can be used for validating the structural accuracy of computation models and verifying their consistency with experimental data. This is a key analysis in clarifying how the spatial distribution, namely Tyr133 position, affects protein function and interaction, as well as confirming the structural error of computational models [21].

Objective of the study

Detection and Characterization of Tyrosine Residue at Position 133 (Tyr133)

The study aims to identify and characterize the tyrosine residue at position 133 (Tyr133) within the soybean leghaemoglobin protein structure. This involves determining the precise location and spatial arrangement of Tyr133 to understand its role in the protein's stability and functionality. The focus will be on assessing how this residue contributes to the overall structure of leghaemoglobin and its susceptibility to oxidative modifications. This characterization will provide insights into the potential impact of Tyr133 on the protein's ability to function under oxidative stress conditions.

Analysis of EPR Spectra

The analysis of Electron Paramagnetic Resonance (EPR) spectra will involve digitizing and examining the spectra using advanced computational tools, including UN Scan It, MATLAB, EasySpin, and TRASSA. Initial steps include calibrating and processing the EPR data in MATLAB to ensure accurate signal alignment and normalization. Subsequently, the spectra will be simulated and fitted using EasySpin and TRASSA to elucidate the nature of radical species and their interactions within the leghaemoglobin protein. Finally, the EPR data will be integrated with 3D structural models of the protein, using PDB Swiss Reviewer, to validate and interpret how the positioning of Tyr133 affects radical formation and the overall stability of leghaemoglobin.

Significance of the Study

This study holds significant importance for several reasons. Firstly, by focusing on the tyrosine residue at position 133 (Tyr133) within soybean leghaemoglobin, the research provides critical insights into the structural and functional roles of specific amino acids in the protein. Understanding the exact location and characteristics of Tyr133 can reveal how this residue influences the protein's stability and its interaction with oxidative agents. Additionally, the use of advanced computational tools for EPR spectra analysis enables a detailed examination of radical species and their behavior, contributing to a deeper understanding of the oxidative mechanisms affecting leghaemoglobin. This knowledge is vital for applications in agriculture and biotechnology, as it can help in engineering more resilient proteins or optimizing leghaemoglobin-based systems for industrial uses. Moreover, this study contributes to the broader field of protein science by demonstrating the application of sophisticated computational techniques and EPR analysis in structural biology. It serves as a model for similar investigations into other proteins, potentially leading to advancements in protein design, functional analysis, and the development of novel strategies for mitigating oxidative damage in biological systems.

References

- [1] Becana, M., Moran, J. F., & Iturbe-Ormaetxe, I. (1995). Structure and function of leghemoglobins. *INSTITUCIÓN «FERNANDO ELCATÓLICO»*, 203.
- [2] Singh, S., & Varma, A. (2017). Structure, function, and estimation of leghemoglobin. *Rhizobium biology and biotechnology*, 309-330.
- [3] Kosmachevskaya, O. V., Nasybullina, E. I., Shumaev, K. B., & Topunov, A. F. (2021). Expressed soybean leghemoglobin: Effect on *Escherichia coli* at oxidative and nitrosative stress. *Molecules*, 26(23), 7207.
- [4] Moreau, S., Davies, M. J., Mathieu, C., Hérouart, D., & Puppo, A. (1996). Leghemoglobin-derived radicals: evidence for multiple protein-derived radicals and the initiation of peribacteroid membrane damage. *Journal of Biological Chemistry*, 271(51), 32557-32562.
- [5] Davies, M. J., & Puppo, A. (1993). Identification of the site of the globin-derived radical in leghaemoglobins. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*, 1202(2), 182-188.
- [6] Ordentlich, A., Barak, D., Kronman, C., Ariel, N., Segall, Y., Velan, B., & Shafferman, A. (1995). Contribution of aromatic moieties of tyrosine 133 and of the anionic subsite tryptophan 86 to catalytic efficiency and allosteric modulation of acetylcholinesterase. *Journal of Biological Chemistry*, 270(5), 2082-2091.

- [7] Palomino-Hernandez, O., Buratti, F. A., Sacco, P. S., Rossetti, G., Carloni, P., & Fernandez, C. O. (2020). Role of Tyr-39 for the Structural Features of α -Synuclein and for the Interaction with a Strong Modulator of Its Amyloid Assembly. *International journal of molecular sciences*, 21(14), 5061.
- [8] Sanjeev, A., & Satish Kumar Mattaparthi, V. (2017). Computational investigation on tyrosine to alanine mutations delaying the early stage of α -synuclein aggregation. *Current Proteomics*, 14(1), 31-41.
- [9] Modgil, R., Tanwar, B., Goyal, A., & Kumar, V. (2021). Soybean (glycine max). *Oilseeds: health attributes and food applications*, 1-46.
- [10] Kaur, J., Ram, H., Gill, B. S., & Kaur, J. (2015). Agronomic performance and economic analysis of soybean (Glycine max) in relation to growth regulating substances in Punjab, India. *Legume Research- An International Journal*, 38(5), 603-608.
- [11] Anderson, E. J., Ali, M. L., Beavis, W. D., Chen, P., Clemente, T. E., Diers, B. W., ... & Tilmon, K. J. (2019). Soybean [Glycine max (L.) Merr.] breeding: History, improvement, production and future opportunities. *Advances in plant breeding strategies: legumes: Volume 7*, 431-516.
- [12] Hungria, M., & Mendes, I. C. (2015). Nitrogen fixation with soybean: the perfect symbiosis?. *Biological nitrogen fixation*, 1009-1024.
- [13] Jain, R. K., Joshi, A., Chaudhary, H. R., Dashora, A., & Khatik, C. L. (2018). Study on genetic variability, heritability and genetic advance in soybean [Glycine max (L.) Merrill]. *Legume Research- An International Journal*, 41(4), 532-536.
- [14] Kofsky, J., Zhang, H., & Song, B. H. (2018). The untapped genetic reservoir: the past, current, and future applications of the wild soybean (Glycine soja). *Frontiers in Plant Science*, 9, 949.
- [15] Eaton, S. S., & Eaton, G. R. (2004). Electron paramagnetic resonance. In *Analytical instrumentation handbook* (pp. 375-424). CRC Press.
- [16] Bertrand, P. (2020). *Electron Paramagnetic Resonance Spectroscopy*. Springer.
- [17] Edwards, P. M. (2002). UN-SCAN-IT version 5.0 for Windows. *Journal of chemical information and computer sciences*, 42(5), 1272-1272.
- [18] Lopez, J., & Hueso, J. (2015). Introducción a MATLAB. *Valencia: Universidad Politécnica de Valencia*.
- [19] Stoll, S., & Schweiger, A. (2006). EasySpin, a comprehensive software package for spectral simulation and analysis in EPR. *Journal of magnetic resonance*, 178(1), 42-55.
- [20] Gavrilov, R. V., Kislov, A. M., Romanenko, V. G., & Fenchenko, V. N. (2004). The software TRASSA

for the analysis of spacecraft thermal conditions. *Kosmichna Nauka i Tekhnologiya*, 10(4), 3-16.

[21] Rodrigues, J. P., Teixeira, J. M., Trellet, M., & Bonvin, A. M. (2018). Pdb-tools: a Swiss army knife for molecular structures. *F1000Research*, 7.

Methodology

Introduction to methodology

This methodology outlines the steps taken to analyze the soybean (Glycine max) leghaemoglobin protein structure, with a focus on the tyrosine residue Tyr133. The process commenced with the retrieval and preprocessing of the protein's 3D structure from the Protein Data Bank (PDB), ensuring its readiness for computational simulations. Key steps were the digitisation of the EPR spectrum with UN Scan It and subsequent analysis in MATLAB; where ROC1, Theta, Cam & Microwave Frequency etc. essential parameters are fine-tune to allow for a robust calibration and signal alignment EPR data interpretation and Tyr133 structural model building. The EPR data was analysed through advanced spectral simulation and fitting by using EasySpin, in collaboration with TRASSA software for the refinement of the structure model built. The last part of the process was visualizing a 3D protein model in PDB Swiss Reviewer to ensure that our folded-state models were indeed accurate and also to confirm where Tyr133 is located from a spatial perspective. The properties and importance of Tyr133 could instead be uncovered for the individual functions or folded forms separately, using this broad variety of proteins.

Data Preparation and Initial Modeling

The research began by extracting the 3D structure of soybean (Glycine max) leghaemoglobin from the Protein Data Bank [PDB]. The protein structure was initially pre-processed by deletion of redundant hetero-atoms and inclusion of missing hydrogen atoms through Swiss PDB Viewer to ensure accurate modeling. Computational simulations that followed required preparation. The space structure of the protein, in particular Tyr133, was examined and analyzed for its spatial arrangement.

Digitizing and Computational Analysis

The EPR spectrum was then digitally transformed using the software UN Scan It, which entails downloading the spectral data to produce a digital image for an EPR signal. To work with those spectrum images, first, they are opened in Un-Scan-it, where the EPR Spectrum files have already been uploaded, which makes it really easy. The axes are then set up, with the X-axis indicating magnetic field strength (usually measured in gauss or mT), and the Y-axis corresponding to EPR response signal intensity. The software from there allows the fetching of numerical values along the spectrum curve pertaining to changes in signal amplitude related to magnetic field strength. Each

step in the workflow is designed to represent both peak positions and line widths of all detected peak features, given by [1].

The digitized spectrum is illustrated in Figure 1, showing the EPR spectrum converted into a usable data format for further analysis.



Figure 1 Digitized EPR spectrum

The digitized EPR spectrum data were imported into MATLAB for preliminary analysis, where several key parameters were utilized to ensure accurate calibration and reliable results.

Initially, a MATLAB with toolboxes including Signal Processing Toolbox was set up for EPR data analysis and processing. This was then extended by arming MATLAB and coupling it with EasySpin, built in house utilizing MATLAB. Installation of EasySpin (download followed by adding paths to directories for use as functions related to EPR spectral simulation) [2]. These files were imported into MATLAB using specialized scripts and commands to read the data structures, when TRYSSA files are integrated along with defining specific simulation characteristics and then further dependable behavior of radicals. The TRYSSA documents also provided experimental conditions such as the strength of the applied magnetic field, spin Hamiltonian parameters. Required for development and investigation in EPR spectrum simulation [3].

According to our measurements, the ROC1 parameter was tuned between 0.35 and 0.37 in order for accurate EPR signal alignment with theoretical models after resonance offset calibration optimization was performed. The Angle Theta was set in the range of 35 to 38 degrees, which is the angle between the magnetic field and sample orientation with respect to the calculation simulation, which is important for determining how the EPR signal interacts with the Magnetic Field. The parameter Cam, as adjusted with a value of 1.3,5, was responsible for tuning the detector sensitivity (signal detection & measurement).

The Experiment Factor was a scaling factor set at 0.5 used during the normalization process of experimental data such that appropriate control could be maintained between runs for comparative purposes. Also, the Position for Experimental Setup, which was defined quite exactly in terms of 20 setups to avoid errors due to a bad positioning of EPR measurements. The Microwave Frequency as set at 9.47291 GHz, was essential to excite the electrons and record a precise EPR spectrum. The peaks corresponding to the g-tensor and splittings associated with nuclear interaction are easily resolved in an EPR experiment. Together, these parameters enabled the fine-tuning of EPR data.

Advanced Spectral Simulation and Fitting

After the data was collected from MATLAB, it then underwent further analysis in EasySpin. The software assists in the elaborated EPR simulation and fitting procedures to provide a better

understanding of its spectrum. EasySpin was used to analyze the spectra in detail and thus also to obtain additional information about the radical generation mechanism. After experimental data were collected, analysis in the form of detailed simulation and fitting to EPR data was carried out with TRASSA software. This involved:

TRASSA Stimulation Y: Used for simulating the EPR spectra.

TRASSA Y Fitting: Used for fitting the simulated spectra to the experimental data.

TRASSA Y File: The TRASSA Y File was used for integrating simulation results and optimizing the fit.

Protein Structure Analysis

With the EPR data processed and analyzed, our attention turned to examination of protein structure with a specific focus on residue Tyr 133. These experiments have provided key details both on the spatial properties and the electronic characteristics of the protein, which were supported by data from MATLAB as well as EasySpin and TRASSA. MATLAB performed computational modeling on the EPR data and preliminary models of the protein structure, while EasySpin simulated spectra that further defined those. TRASSA fitting processes were utilized to refine the model towards experimental EPR data. This step analyzed the 3D structure of the protein to know about Tyr 133 in which direction & configuration it is, along with the whole structure. This included examining the spatial relationship between Tyr 133 and other residues and possible interactions with neighboring amino acids or molecular entities. Analysis of these samples was critical for understanding the function and participation in biochemical or biophysical mechanisms by Tyr 133. This was to map the precise placement of Tyr133 and determine in what ways its positioning could influence enzyme function or stability.

Final Visualization with PDB Swiss Reviewer

The model was further validated and visualized by detailed analysis using PDB Swiss Viewer in 3D. The first step was opening the Swiss Viewer (Swiss-PDBViewer) and importing a theoretical protein model obtained from some previous computational analyses that used MATLAB, EasySpin to predict its distance restraints; Those models were generated as PDB files. This structure was then used as the basis for a detailed structural analysis. After loading the model, a more detailed image of all 3D protein structure is demonstrated by highly powerful rendering tools available in software [4] was finally developed. We particularly focused on the Tyr 133 residue because it is an important functional region of this protein.

The spatial position and conformation of Tyr 133 in the protein were further investigated by zoom, rotation, and focus functions of this software. It included taking into account its distance and orientation with respect to other important residues, identifying any molecular interactions that may

meddle in their function as hydrogen bonds or van der Waals forces. After processing this visualization (hit), we started the validation with a real professional Swiss-Viewer, calculating almost all folds and structural quality for our protein. This part of the project examined bond angles and inter-residue distances as well as performing minor refinement to bring it into better agreement with protein primary structural principles. Further analysis of the Tyr 133 environment illustrated how its protein conformation might influence stability and function via neighboring amino acids. To further validate the model, these structural analyses were corroborated with EPR spectroscopy experiments. This comparison was important to ensure that the computational model correctly reflected experimental observations, which related especially to the conformation of Tyr 133. At last, the visualizations of the 3D model were exported, emphasizing on Tyr133 region. While this might be true for a few useful visualizations, it was necessary to capture at least some of the images on paper or electronically in order to illustrate structurally the appropriateness and correctness associated with how well protein folding-based EPR data is being replicated by the model. In theory, this rigorous validation and visualization confirmed the accuracy of our model with respect to the location of Tyr 133 relative to its function within prostate-specific antigen.

Results Visualization

Results from MATLAB, EasySpin and TRASSA are shown combined visually. Album feedback, including spectrum analyses and protein structure representations of results, was examined. These have involved computerized representation of the EPR spectrum (Fig. 1) as well as differences between images showing the predicted structure of proteins or location (or not) of Tyr133 in experiments. Computational results were cross-validated with available experimental data to improve credibility. Mixed Use of Theoretical and Experimental Results for Calibration Parameters and Models in Simulation. In addition, sensitivity analysis was performed which evaluated the robustness of these findings through investigating how changes in computational input parameters influenced simulation results. PDB Swiss Reviewer was used to accomplish a final analysis of protein structure. This step consisted of viewing and assessing the structure of the protein as a whole (overall quality), but also, for what concerns only Tyr 133 that where this residue was clearly visible.

References

- [1] Steen, A. D., Arnosti, C., Ness, L., & Blough, N. V. (2006). Electron paramagnetic resonance spectroscopy as a novel approach to measure macromolecule-surface interactions and activities of extracellular enzymes. *Marine chemistry*, 101(3-4), 266-276.
- [2] Pribitzer, S., Doll, A., & Jeschke, G. (2016). SPIDYAN, a MATLAB library for simulating pulse EPR experiments with arbitrary waveform excitation. *Journal of Magnetic Resonance*, 263, 45-54.
- [3] Stoll, S., & Schweiger, A. (2006). EasySpin, a comprehensive software package for spectral simulation and analysis in EPR. *Journal of magnetic resonance*, 178(1), 42-55.

[4] Johansson, M. U., Zoete, V., Michielin, O., & Guex, N. (2012). Defining and searching for structural motifs using DeepView/Swiss-PdbViewer. *BMC bioinformatics*, 13, 1-11.

Results And Discussion

Advanced Spectral Simulation and Fitting

For detailed spectral simulations and advanced fitting procedures of EPR data for tyrosyl radicals in soybean leghaemoglobin, EasySpin69 and TRASSA76 software⁵⁹ were applied. EasySpin was used to make broad simulations that were further processed using TRASSA to fit the simulation in great detail with experimental data. Important parameters were changed, including the spin density and theta (θ) angle, in order to achieve a better match between experimental and simulated spectra. This value corresponds to the distribution of those electrons within the radical, and it was readjusted after each calculation in order to be an accurate intensity as well as resolution for simulated spectra. The theta angle, which defines the orientation of Tyr133 within the magnetic field, was set between 35° to 38°. This particular orientation was pivotal in matching the computer-generated spectra to experimental results and for accurately adjusting the hyperfine interactions that gave a detailed description of the electronic environment around Tyr133. The blue curves were experimental spectra, and the orange curves are simulated ones for different θ angles. The striking agreement between the experimental and simulated spectra, with nearly identical peak positions and line shapes, confirmed that these computational models are accurate. Minor discrepancies in signal intensity and line width highlighted areas for further refinement. This thorough comparative analysis confirmed the reliability of the simulations and enhanced the understanding of the radical's electronic environment and its role within the protein structure.



Figure 3: Results from MATLAB, EasySpin, and TRASSA Tyr133 Spatial Analysis

The detailed analysis of the protein structure revealed the specific location and orientation of Tyr133. The data obtained from MATLAB, EasySpin, and TRASSA highlighted the interactions between Tyr133 and surrounding residues. This analysis was critical in understanding the role of Tyr133 in the protein's function, particularly in relation to radical stabilization and its potential influence on the protein's overall stability. In the UnScan it, software, the X-axis maximum was set at -500, and the Y-axis maximum at 100, further refining the spectra to achieve accurate peak positions and intensities. Using the Swiss PDB Viewer, in our analysis of the soybean (Glycine max) leghaemoglobin protein, Psi values play a significant role in understanding the spatial arrangement of Tyr133. Specifically, the Psi 6 value of -96.86 and the Psi 2 value of -79.62 were critical in accurately modeling the secondary structure and spatial orientation of Tyr133. The Psi values supplied us with invaluable insights into the dihedral angles of amino acids surrounding Tyr133 in the polypeptide chain backbone, and thus its

direct impact on spatial coordination within the 3D protein framework. With these Psi values included in the computational models, it provides the correct structure of Tyr133 well-mimicking its experimental one. The correct alignment was necessary to perform a simulation, interpret the EPR spectra accurately. The Psi angles were also useful for determining the role of Tyr133 in stabilizing the radical and maintaining overall protein stability. Therefore, Psi values were fundamental in validating the structural model of Tyr133 and assessing whether our simulations are representing its biological role in leghaemoglobin.

The angle theta in Matlab was turned out to be 38 degrees precise, and the theta of the Swiss PDF was 51.4, which was found by this formula

$$\theta = ((180^\circ - \phi_6) - \phi_2)/2 - 30^\circ \text{ (note that } \phi_2 \text{ and } \phi_6 \text{ have opposite signs). [0]}$$

This value was relative to a previous study.



Figure 4: Spatial Location and Orientation of Tyr133

Comparative Visualization and Validation

The protein structure validation was done through PDB Swiss Reviewer. Validation of our computational models based on this step was essential. By validating the various computational predictions against this structure, an accurate 3D model was created that provided insights to verify if any given conformation predicted through calculations resembles its actual structural form. A critical component of this validation was the correct positioning of Tyr133 within the protein structure. Swiss Reviewer of PDB gave detailed orientation relations amongst them and molecular structures to position Tyr133. The agreement of all this alignment with both theoretical models and experimental data confirmed that the 3-dimensional structure used in the calculation correctly reflects protein conformation, location and function. Experimental results are in remarkable agreement with the theoretical predictions, which gives strong evidence of the high reliability of the employed computational models. Using data from multiple resources and visualization tools allowed us to ensure that the computational approach reflected both the location, 3D orientation of Tyr133 as well as its functional importance. The high stringency of that validation process shows the reliability of our data and reinforces the trustworthiness regarding Tyr133 located in soybean leghaemoglobin protein as well.



Figure 5: Comparative Visualization of Protein Structure

Protein Structure Modeling

The 3D structure of soybean (*Glycine max*) leghaemoglobin was successfully retrieved and preprocessed. Swiss PDB Viewer was employed to eliminate redundant heteroatoms and add missing hydrogen atoms for correct modeling. In terms of spatial orientation, the direction in which Tyr133 was positioned within the protein structure was determined explicitly. This pre-processing step set the stage for later computational simulations.



Figure 1: Protein Structure of Soybean Leghaemoglobin

Final Protein Structure Visualization

The final protein structure, visualized using PDB Swiss Reviewer, provided a clear and detailed representation of the entire leghaemoglobin molecule, with particular emphasis on the positioning of Tyr133. The visualization confirmed that Tyr133 is strategically located within the protein, potentially influencing its biochemical activity and stability.



Figure 6: Final Protein Structure with Tyr133 Highlighted

Discussion

We have investigated the tyrosine residue at position 133 (Tyr133) in soybean leghaemoglobin as a phenoxyl radical, since it has been proposed to be an important initiator site for enzymic O₂ reduction by peroxidases. An important feature observed from the EPR analysis was that we established an angle θ with respect to Tyr133 at 51°. This angle is pivotal as it influences the orientation and interaction of the phenoxyl radical within the protein structure. The observed EPR parameters for Tyr133, including a_1 at 1.99 mT, a_{2H} at 0.70 mT, and g at 2.0044, were consistent with those reported for tyrosine phenoxyl radicals in previous studies [1]. In particular, the appreciable doublet-splitting that is observed from these experiments accords with the spectral splitting resulting from interaction of the unpaired electron on a Tyr133 side chain -CH₂- proton reported for systems such as ribonucleotide reductase and Photosystem II. The smaller triplet splitting is expected to be the result of interactions with two ortho hydrogen atoms on the aromatic ring in Tyr133.

The value of 51° for the θ angle is found to be particularly important and allows us to change in orientation, facilitating interactions with its protein environment. This sizeable angle suggests that Tyr133 is oriented so as to correspond with the EPR parameters viewed, hence providing strong evidence for anisotropy in the measured EPR signal. The constraints on the phenolic ring of Tyr133 by its protein environment and to slow rotational motion of the radical about the phenoxyl axis, in

conjunction with tumbling times long compared to the haem protein contributing to this anisotropic signal. Our findings are compared to previous studies [2]. It is worth noting that the angle θ has varied in other research contexts. In previous studies, the θ angle was observed to be different, usually in a range from 30 to 45 degrees. This change in θ we see within our study (51 degrees) could be due to variations of the protein environment, such as spatial constraints imposed by neighbouring residues or conformational differences in the dynamic states. These factors may influence how Tyr133 is positioned relative to the haem ring and other structural elements, thereby affecting the angle of the phenoxyl radical. In the case of Tyr133, as it is close to the haem ring (or more specifically, there are hydrogens in addition to a proximal atom such that domino effects could lead ultimately to decarboxylation), θ of 51° supports this being one of the sites for radical formation. This alignment supports the part of tyrosine 133 on radical creation. Other investigations, especially with other legumes like lupin, which retains Tyr133 but lacks Tyr30, may provide further insight into the relevance of this angle in radical generation and stabilization within species. Comparative studies such as these may reveal whether the angle θ is a consistent predictor of radical formation across different legume species or if it varies because of interspecific variation in structural configuration.

References

- [0] Svistunenko, D. A. (2004). Tyrosine residues in different proteins. Phenol ring rotation angle database.
- [1] Davies, M. J., & Puppo, A. (1992). Direct detection of a globin-derived radical in leghaemoglobin treated with peroxides. *Biochemical Journal*, 281(1), 197-201.
- [2] Davies, M. J., & Puppo, A. (1993). Identification of the site of the globin-derived radical in leghaemoglobins. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*, 1202(2), 182-188.