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Babeș-Bolyai University

Faculty of Chemistry and Chemical Engineering

In extenso abstract of the Ph.D. Thesis

Insights into the reactivity of heme and non-heme iron in biologically relevant contexts

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Cluj-Napoca
2025

Keywords: iron, iron reactivity, heme, myoglobin, non-symbiotic phytoglobin, prooxidant, antioxidant, gallic acid, liposomes, oxygen, phytoglobin, *Arabidopsis thaliana*, nitrite, nitric oxide, cyanamide, UV-vis, EPR spectroscopy, DFT.

Table of contents of the Thesis

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General considerations

All chapters of this thesis have already been published as scientific papers, or have been submitted for publication, or are in preparation as follows:

Chapter 2: submitted to *Talanta*, 2025;

Chapter 3: published as Zăgrean-Tuza, C., Matei, A., Silaghi-Dumitrescu, R. 2024. A biomimetic assay for antioxidant reactivity, based on liposomes and myoglobin. *Journal of Inorganic Biochemistry*, 258, 112613;

Both Chapters 2 and 3 refer to Zagrean-Tuza, C., Igescu, I., Lupan, A., Silaghi-Dumitrescu, R. 2024. A study of the molecular interactions of hemoglobin with diverse classes of therapeutic agents. *Inorganica Chimica Acta*, 567, 122053;

Chapter 4: submitted to *Physical Chemistry Chemical Physics*, 2025;

Chapter 5: published as Zagrean-Tuza, C., Pato, G., Damian, G., Silaghi-Dumitrescu, R., Mot, A. C. 2024. Redox Reactivity of Nonsymbiotic Phytoglobins towards Nitrite. *Molecules*, 29(6), 1200;

Chapter 6: submitted to *Nature Communications*, 2024;

Chapter 7: published as Zagrean-Tuza, C., Padurean, L., Lehene, M., Branzanic, A. M., Silaghi-Dumitrescu, R. 2024. Globin ferryl species: what is the nature of the protonation event at pH< 5? *Journal of Biological Inorganic Chemistry*, 1-10.

Chapter 8: submitted to *Inorganic Chemistry*, 2025.

1. Introduction on a Fe-nomenal element

The Great Oxygenation Event has significantly changed the chemistry on the Earth's surface – with particular relevance to the biosphere. While the soluble Fe^{2+} was oxidized to the insoluble Fe^{3+}, the oxidation of Cu^{+} to Cu^{2+} increased its solubility and hence its bioavailability[1]. The ferric cation could still be used, but only if trapped by ligands. This aspect could be the reason why aquatic organisms rely on a much more diverse spectrum of cations (e.g., vanadium, nickel, cadmium) to carry biocatalytic functions. GOE unlocked a wealth of reactions which would normally occur to a lesser extent in a reductive atmosphere: radical-based processes – and especially those involving reactive oxygen species. One-electron oxidation of oxygen produces the O_2^{-}•, which could further lose another electron and produce H_2O_2; Fe^{3+} can be reduced to $Fe^{2+}$by O_2^{-}• or other molecules. Fe^{2+} and H_2O_2 could react in two ways, depending on the iron's coordination sphere, by either generating the hydroxyl radical, or by producing a high-valent intermediate. Both HO• and Fe(IV)=O can further engage in a variety of chemical processes; it can be argued that such processes greatly contributed to building the living world as we now know it.

Probably the greatest effect of GOE on life on Earth was kicking the aerobic metabolism. When oxygen is used as a final electron acceptor, far more energy is produced compared to anaerobic metabolism. For example, glycolysis produces roughly 5 ATP equivalents, while the Krebs cycle produces 20 ATP equivalents. This increase in available energy allowed prehistoric organisms to further evolve and diversify instead of just merely surviving. Eukaryotes are highly dependent on oxygen to produce energy, yet oxygen in itself is potentially a threat to their survival; this paradigm is known as the "oxygen paradox". Two processes revolving around the production of free radicals in the presence of iron are discussed in Chapters 2 and 3 of this thesis.

To adapt to and unlock the full chemical potential under the emerging aerobic conditions, organisms had to expand the structure and the scope of their biochemistry, especially with respect to biocatalysts. It is important to note that (metallo)proteins had long existed before the Great Oxygenation Event. It would have been a shame not to use iron in biotransformations. Iron exists over a wide span of oxidation states; the Fe^{2+}/Fe^{3+} duo operates in electron transfer or acid-base reactions. Their redox potential can be fine-tuned based on the ligand's identity, thus diversifying the chemical portofolio. Moreover, iron can bind to both hard (oxygen-based) and soft (nitrogen- and sulfur-based) ligands; Fe^{3+} is a hard center, while Fe^{2+} has an intermediate character.

Heme proteins are great to illustrate the versatility of iron chemistry. The oxy form contains a ferrous heme that binds and, in globins, transports oxygen. The binding of oxygen alters the electronic structure of the heme, stabilizing the iron in a low-spin state and enabling the reversible release of oxygen in tissues that require it. The deoxy form, in contrast, lacks bound oxygen, leaving the Fe^{2+} in a high-spin, five-coordinate state (in some globins). We explore the reactivity of oxy and deoxy forms of phytoglobins in Chapters 5 and 6.

The met form, also known ferric (Fe^{3+}) heme, results from oxidation of the ferrous iron. In this state, heme iron is unable to bind oxygen; however, ferric heme is able to bind a wide array of other small ligands, albeit at lower affinities. We discuss about and explore this ability

in Chapter 4 through EPR spectroscopy and DFT. Moreover, the ferric form is particularly important for the functioning and high turnover of catalase, which is discussed in Chapter 8.

The interplay between the ferrous and ferric heme forms is important in cellular processes. The Fe^{2+}/Fe^{3+}redox cycling in cytochrome *c* allows the transfer of electrons between Complex III (cytochrome bc_1 complex) and Complex IV (cytochrome c oxidase, one of the oldest enzymes). Subsequently, the $Fe^{2+}/Fe^{3+}/Fe^{4+}$ triumvirate contributes along with a Cu center and a key tyrosine to proton and water production in cytochrome *c* oxidase. Both cytochromes are part of the Electron Transfer Chain, which is the last step of cellular respiration in aerobic organisms. Moreover, this ferrous/ferric interplay is essential for cytochrome P450 catalytic cycle: the resting ferric form is reduced to ferrous, which binds oxygen to generate Compound 0, which undergoes homolysis to Compound I that hydroxylates substrates.

Compounds I is one of the not so many high-valent iron-oxo species encountered in biochemistry. Fe^{4+} might be a rare redox state, but its involvement in the manipulation of strong chemical bonds is physiologically important and inspires alternative catalysts. Compound I (Fe(IV)=O coupled with a porphyrin-based free radical) is one of the most reactive heme species trapped and characterized so far. It is an integral part of the catalytic cycle of cytochromes P450 and peroxidases which allows the hydroxylation of C-H bonds or the decomposition of hydrogen peroxide. In Chapter 8 we hypothesize the involvement of a globin Compound I in the hydroxylation of cyanamide.

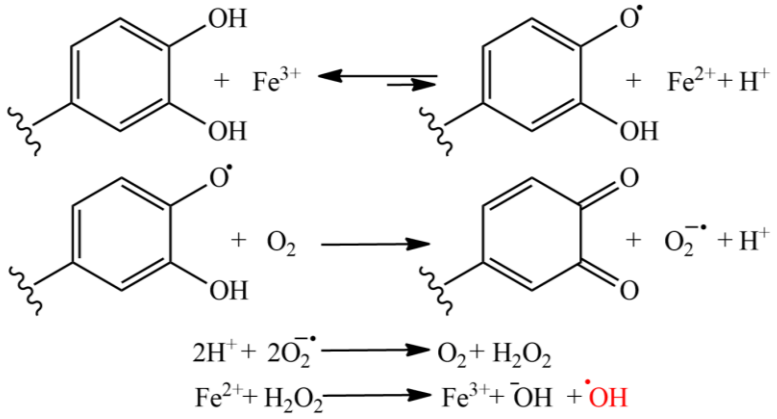
The factors that enable and fine-tune the reactivity of Compound I are still debated, but some theories gain traction, such as those based on the two-state reactivity concept or on the strength of the O-H bond D(OH) formed during the H-atom abstraction step, rooted in the Bell-Evans-Polanyi principle[2,3]. Compound I in turn generates Compound II, a clean Fe(IV)=O heme intermediate. In Chapter 7 we comment upon the protonation state of the ferryl form in globins. Normally, the potential of Compound II is too low to allow the hydroxylation or halogenation of substrates, but it can still take part into electron transfer reaction or generate free radicals, as described in Chapter 3.

In the following chapters we would like to indulge in understanding heme behaviour and reactivity in proteins, especially in phytoglobins. We use a complex array of tools that includes spectroscopy (especially UV-vis and electron paramagnetic resonance, EPR – though in work connected to the thesis the candidate also explores the use of fluorescence, nuclear magnetic resonance NMR, and Mössbauer), molecular biology with recombinant proteins, protein purification and characterization, kinetics (steady-state, stopped-flow and, in work connected to the thesis, rapid freeze-quench, RFQ) and computations (Density Functional Theory (DFT) and docking).

2. Iron-y reactions: when polyphenols generate fire (hydroxyl radicals) and how to make use of it[4]

In this chapter, we discuss the development and optimization of a new analytical method for the determination of the prooxidant activity. This prooxidant assay is rooted in physiologically relevant processes and revolves around the determination of the Midas curse extent of polyphenolics and ascorbic acid: their ability to generate free radicals. To serve the purpose of this PhD thesis, the underlying mechanism of the prooxidant effect is studied in detail, rather than describing the analytical performance of the method; the latter aspect is nevertheless treated in more detail in the article submitted for publication. Special focus is put on the reactivity of iron towards polyphenols and their ensuing Fenton-like reactivity.

The principle of the prooxidant assay is based on the ability of Fe^{3+} to oxidize polyphenols to semiquinone as outlined in Scheme 1[5]. The ferric compound is converted to its ferrous form and needs to ideally stay in this oxidation state, as Fe^{3+} is not able to generate the hydroxyl radical. Once formed, the semiquinone radical can further transfer one electron to molecular oxygen, rendering the superoxide anion radical. The latter undergoes disproportionation. The hydrogen peroxide thus formed reacts with Fe^{2+} in the Fenton reaction. The hydroxyl radical is the product.



Scheme 1

The hydroxyl radical is very reactive and only a handful of approaches allow its detection and determination. A very facile way to detect small reactive free radicals is by spin trapping. In such experiments, a nitrone-type molecule (e.g. DMPO-5,5-dimethyl-1-pyrroline N-oxide) traps the free radical.

More accessible methods of detection make use of substrates that undergo significant UV-vis absorbance or fluorescence profile changes when the hydroxyl radical is present. One such substrate is 2-methyl-4(5)-nitroimidazole, which is attacked by the hydroxyl radical to yield nitrite. While this reaction itself does not have a significant spectral shift, nitrite can be detected with the Griess reagent, a highly specific and sensitive analytical test.

Control experiments were performed to prove the working principle with Fe3+-EDTA and gallic acid at pH 7. The HO• production was assayed with 2-methyl-4(5)-nitroimidazole and the Griess reagent. No hydroxyl radical production is detected when one or more of the reactants (Fe3+-EDTA, gallic acid and/or 2-methyl-4(5)-nitroimidazole is(are) removed (1-6 in Figure 1). To investigate the role of oxygen in this Fenton-like system, the assay was performed

under normoxic, anaerobic and oxygen-enriched conditions (7-9 in Figure 1). The results indicate that oxygen availability is crucial for radical generation, as the absence of molecular oxygen leads to diminished hydroxyl radical formation (8 in Figure 1). Oxygen-enriched conditions lead to a three times higher yield of hydroxyl radical (9 in Figure 1). Based on these considerations, oxygen is indeed crucial in this Fenton-like reaction sequence.

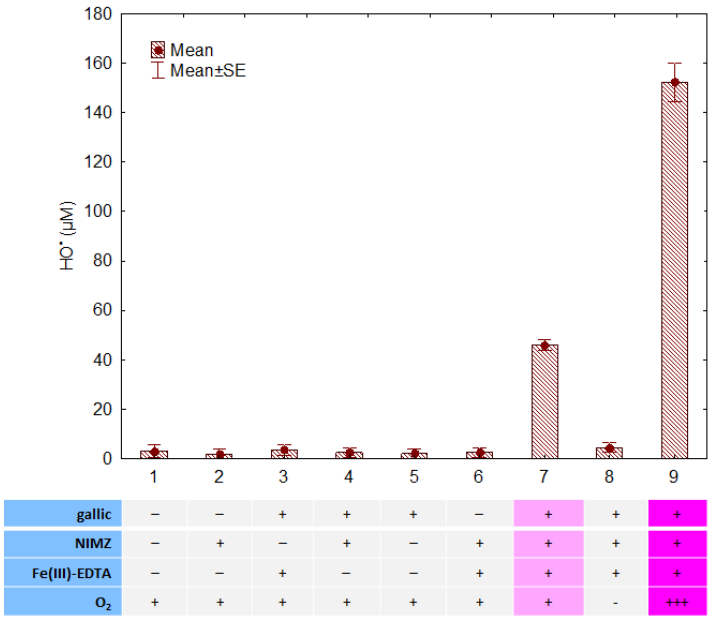


Figure 1. *Control experiments with gallic acid and Fe(III)-EDTA. NIMZ stands for 2-methyl-4(5)-nitroimidazole.*

We probe the ability of gallic acid to produce hydroxyl radicals in the presence of a number of Fe^{3+} complexes, over a pH range and in the presence of organic solvents. Additionally, we discuss our observation from the structural and mechanistic perspective. We used spin trapping and X-band CW EPR spectroscopy to conform the Griess-based UV-vis detection. Polyphenols in the presence of Fe^{3+}-EDTA at pH 10 produce the most hydroxyl radical and the system can be used as a prooxidant assay.

3. Lipids in danger: myoglobin's oxidative wrath vs. antioxidant shields. What inhibits the myoglobin-induced liposome peroxidation?[6,7]

A methodology based on the inhibition of liposome peroxidation was developed by our group (ILA-protein: inhibition of liposome autooxidation by protein)[8–11]. Such principle can be used to assess the antioxidant capacity of a wide range of compounds, regardless of their polarity. Liposomal peroxidation can be accelerated by a range of heme proteins, and even by free heme[8,12–20]. As illustrated in Figure 2 A, heme proteins can act as radical initiators due to heme iron chemistry through pseudoperoxidase-like (or Fenton-like) catalytic decomposition of traces of peroxides and hydroperoxides already present in the lecithin samples. When cytochrome *c* is replaced with myoglobin as catalyst, the liposomal autooxidation requires much shorter times to reach completion, with a clear dependence on myoglobin concentration (Figure 2 B).

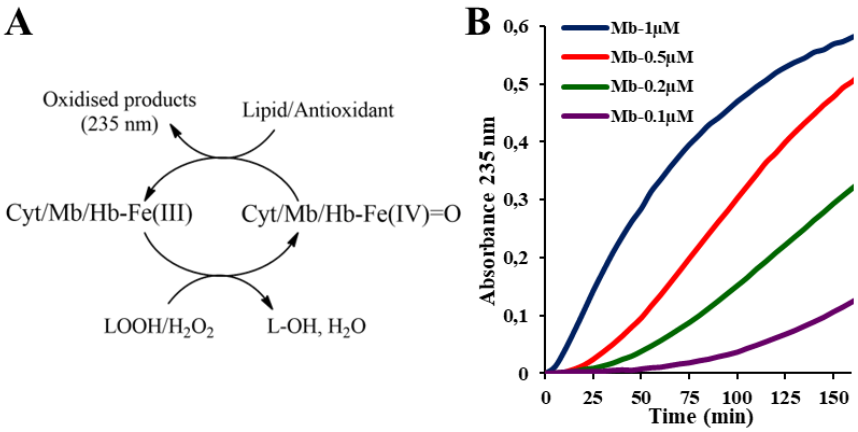


Figure 2. A. *Proposed reaction scheme highlighting the heme-based players. Liposome (0.5 mg/mL) peroxidation in the absence of hydrogen peroxide as dependent on* ***B.*** *myoglobin.*

As previously found in IC_{50} or K_M analyses using other antioxidant assays, the redox potentials of the antioxidants do not correlate with the $IC_{50}^{ILA\text{-}Mb}$ [21]. It may then be assumed that the antioxidant mechanism evaluated in ILA-Mb assay is not solely dependent on outer-sphere reaction electron transfers or hydrogen atom transfers between small molecules (e.g., antioxidant and lipid-based radicals). Specific binding, interaction and molecular recognition of the antioxidant by either liposomes or myoglobin are most probably at play.

To explore the binding of small molecules to myoglobin at a theoretical level, we performed blind docking calculations using the five selected antioxidants (ascorbate, catechin, gallate, ferulate, and rutin) as ligands. The binding site of catechin (highest affinities) is shown in Figure 3; catechin forms a hydrogen bond with the propionate group. The propionate may facilitate a more efficient PCET by both hydrogen bonds and electrostatic interactions with substrates (in this case, with the small antioxidants). This observation may help explain ascorbate and catechin behavior in ILA-Mb: both of them form at least one hydrogen bond with the propionate (antioxidants are the H donors).

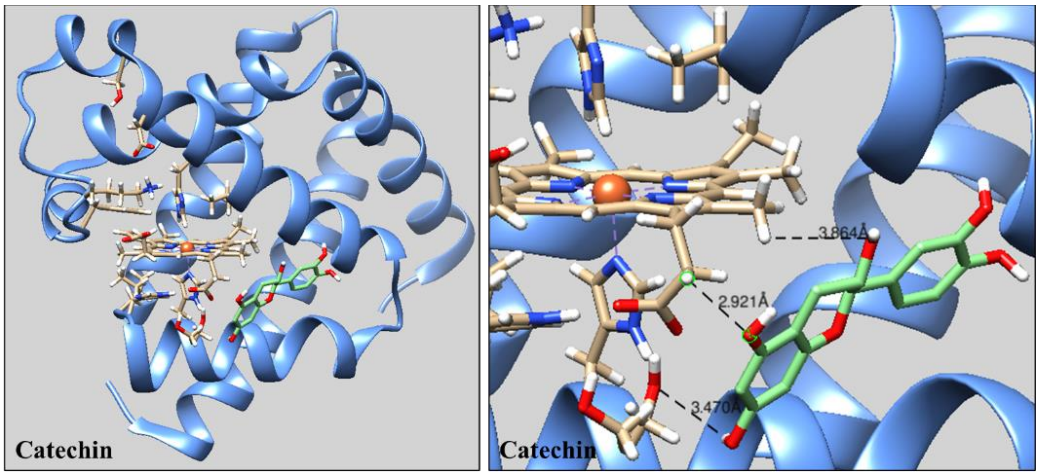


Figure 3. *The predicted most favorable binding site of catechin to myoglobin.*

In terms of interactions with lipids, all five antioxidants and the hydrophobic control, α-tocopherol, induced changes in the EPR signal of 16-MeDSAE as a result of them interacting with the liposomes (Figure 4). The treatment with ascorbate, catechin, ferulate and gallate yield identical spectral profiles: single component, with a pronounced linewidth broadening, with a correlation time around 1,5 x 10^{-9} s for all four spectra, which indicates a slow-motion regime. On the other hand, treatment with α-tocopherol and, surprisingly, rutin, drastically changed the shape of 16-MeDSAE spectrum, with the sharp decrease in the linewidth. The sharper spectral component in both antioxidants is characterized by a correlation time of 1 x 10^{-10} s, indicating a fast motion regime, which can be correlated with an increase in membrane fluidity.

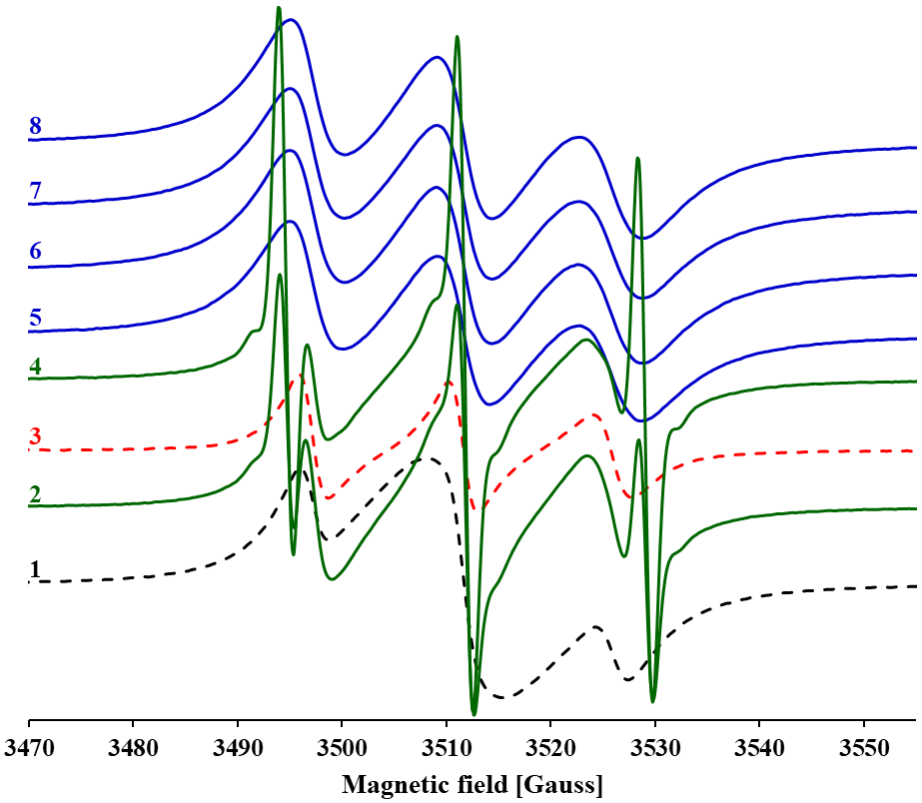


Figure 4. *EPR spectra as follows: 1- 16-doxyl stearic acid methyl ester (16-MeDSAE) in buffer; 3- Liposomes labeled with 16-MeDSAE in buffer (control) treated with rutin (2), α-tocopherol (4), ascorbate (5), catechin (6), ferulate (7) and gallate (8).*

4. Beauty is in the eye of the resonator: small-molecule probing of an atypical hemoglobin site using electron paramagnetic resonance spectroscopy combined with DFT simulations[22]

Heme systems in the ferric state are optimal candidates for EPR spectroscopy. Fe^{3+} has five d electrons which can be arranged in either a high-spin configuration (S=5/2), or a low-spin configuration (S=1/2), depending on the ligands. Ferric heme EPR signals tend to have either axial geometry (in high-spin configuration), or rhombic (in low-spin configuration). Due to high spin-orbit coupling values, such EPR signals can be usually observed at very low temperatures[23].

AtHb3 (truncated hemoglobin 3 from *Arabidopsis thaliana*) is a very interesting nonsymbiotic phytoglobin. It lacks the distal histidine, or any other distal amino acid for that matter[24]. The cavity above the heme is larger than the one in regular globins and has a more hydrophilic character, with amino acids that would easily engage in hydrogen bonding to stabilize ligands: Tyr44, Gln71 and Trp111.

In this chapter we explore several low-spin ferric AtHb3 adducts through EPR spectroscopy using a range of small-molecule probe ligands that bind to the iron and display a range of electronic structure differences, spin state preferences, ligation states / linkage isomerism (Figure 5). One of these AtHb3 complexes especially caught our eye due to its relaxation time. As a consequence, as a first report on this set of complexes, we explore several properties determined from the CW EPR spectrum or calculated using density functional theory (DFT). We speculate and evaluate the influence of properties such as tetragonality, rhombicity, spin density, and hyperfine components.

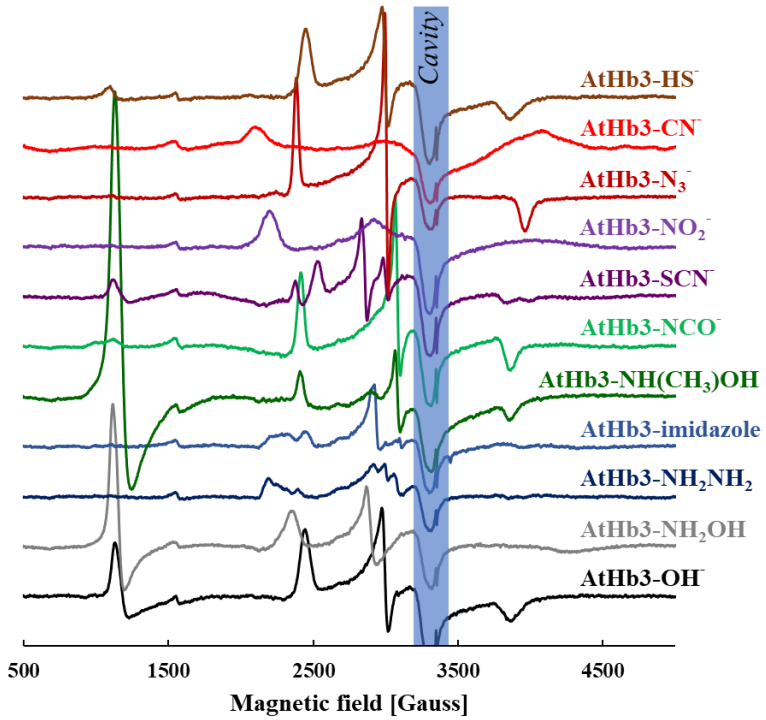


Figure 5. *Raw CW X-band EPR spectra of selected AtHb3 adducts (300 μM in 200 mM Tris pH 7 buffer) measured at 100 K. The signal of the cavity is marked. The spectra are shown without any mathematical processing (smoothing, subtraction, multiplication or otherwise).*

5. A nitrite/nitric oxide juggling primer for phytoglobins. The reactivity of plant hemoglobins towards nitrite[25]

A thorough kinetic exploration of *Arabidopsis thaliana* nonsymbiotic phytoglobins' (met, oxy and deoxy) reactivity towards nitrite is reported in this chapter. Mammalian myoglobin is used for comparison. We try to link the mechanistic observations to known *A. thaliana* physiology (especially under abiotic or biotic stress) in our discussions.

Nonsymbiotic phytoglobins are frequently based on iron coordination in deoxygenated form,[26] especially in spectroscopic studies. As per Figure 6, the hexacoordinated phytoglobins, such as the nonsymbiotic AtHb1 (class 1) and AtHb2 (class 2), display a highly conserved so-called distal histidine bound to the heme iron, hence the ”hexacoordinated” designation. Truncated phytoglobins such as AtHb3 (class 3) exhibit a more compact 2/2 helical fold and lack the distal histidine, hence the ”pentacoordinated” designation.[27–29]

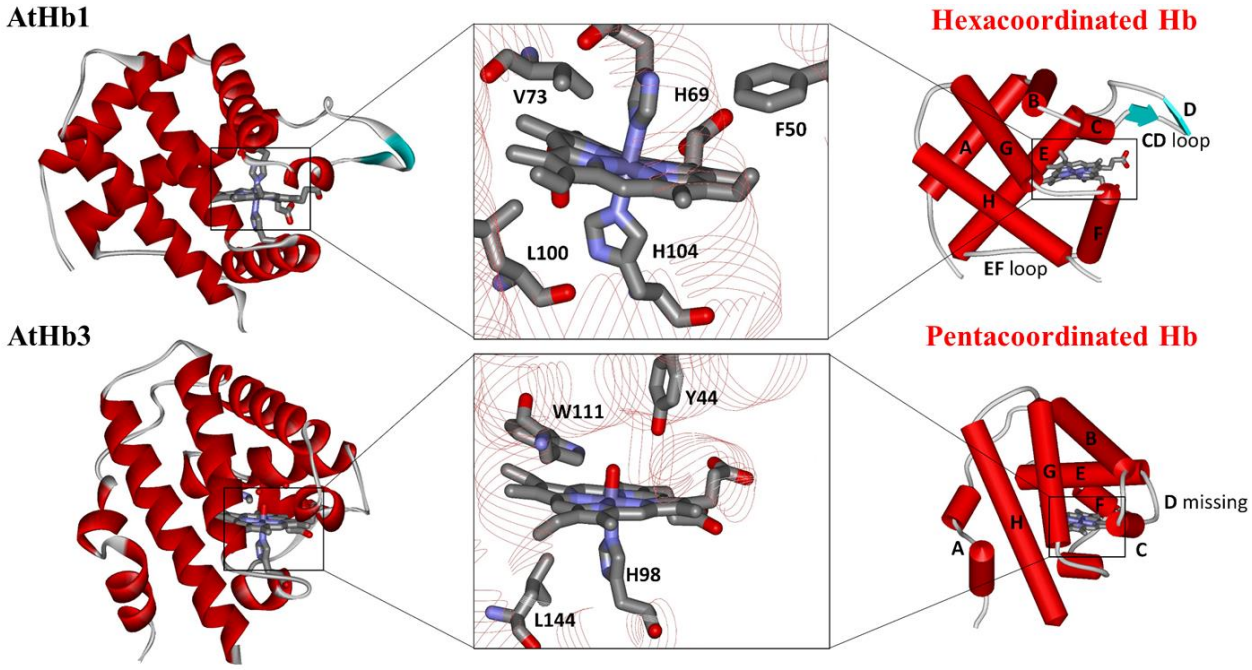


Figure 6. *The two representative folding structures of nonsymbiotic phytoglobin 1 (3ZHW[30]) and truncated phytoglobin 3 (4C0N[24]) from A. thaliana. Figure reproduced from[27].*

In anaerobic conditions, mammalian myoglobin and hemoglobin are known to act as nitrite reductases, converting nitrite to nitric oxide. Figure 7 presents detailed findings of this activity for the studied phytoglobins. The spectral shift of the Soret band and the formation of the nitrosyl feature in the 475 – 600 nm spectral region, with clear isosbestic points, is present in all four globins. These findings support the previous observations that nitrite reduction leads to nitric oxide, when ferric Hb is reduced by dithionite.[31]

We have shown in our previous works that in the presence of oxygen or hydrogen peroxide, mammalian globins and phytoglobins display conspicuous differences.[32–35] Oxy mammalian hemoglobin and myoglobin are known to react with nitrite; the mechanism is very complex: it involves an initial slow phase (lag phase) that accelerates into a rapid phase (propagation phase) of oxidation. The lag time in minutes for this slow phase and the maximum

decay rate in the fast phase were experimentally determined for all three phytoglobins and myoglobin (Figure 8 is illustrative for AtHb1). The reaction of oxyAtHb1 with nitrite shows a much longer lag time as compared to AtHb2. OxyAtHb3 exhibited a different kinetic behavior, with the presence of only the propagation phase, in any of the conditions investigated. Since high-valent ferryl globin was detected as intermediate before[17,36,37], it is reasonable to assume that oxyAtHb3 exhibits a totally different behavior in the presence of nitrite due to its involvement.

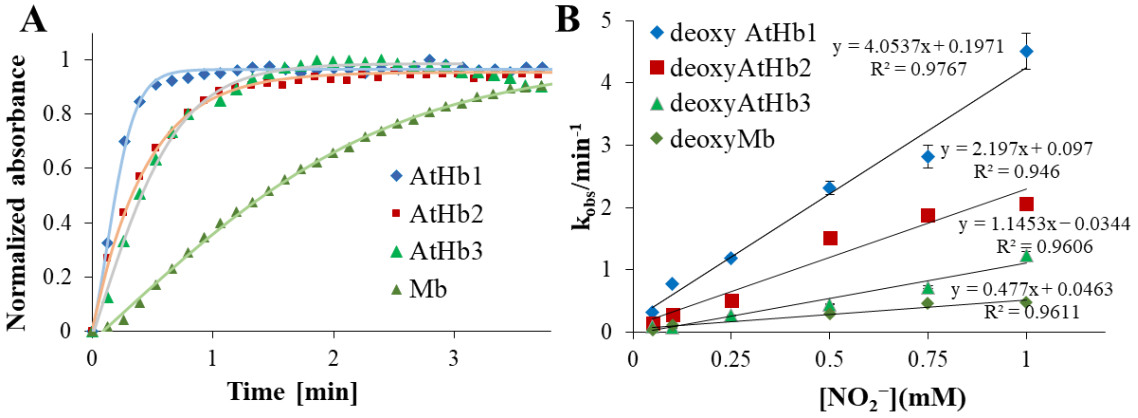


Figure 7. *Kinetics of nitrite reaction with A. thaliana nonsymbiotic deoxyhemoglobins and horse heart deoxymyoglobin.* ***A.*** *Kinetic profile of each Hb and Mb in the presence of 1 mM nitrite at 413 nm for AtHb1, 412 nm for AtHb2, 419 nm for AtHb3, and 422 nm for Mb.* ***B.*** *Plot of the observed rate constants (k_{obs}/min^{-1}) versus nitrite concentration in 50 mM phosphate buffer pH 7 and 25°C.*

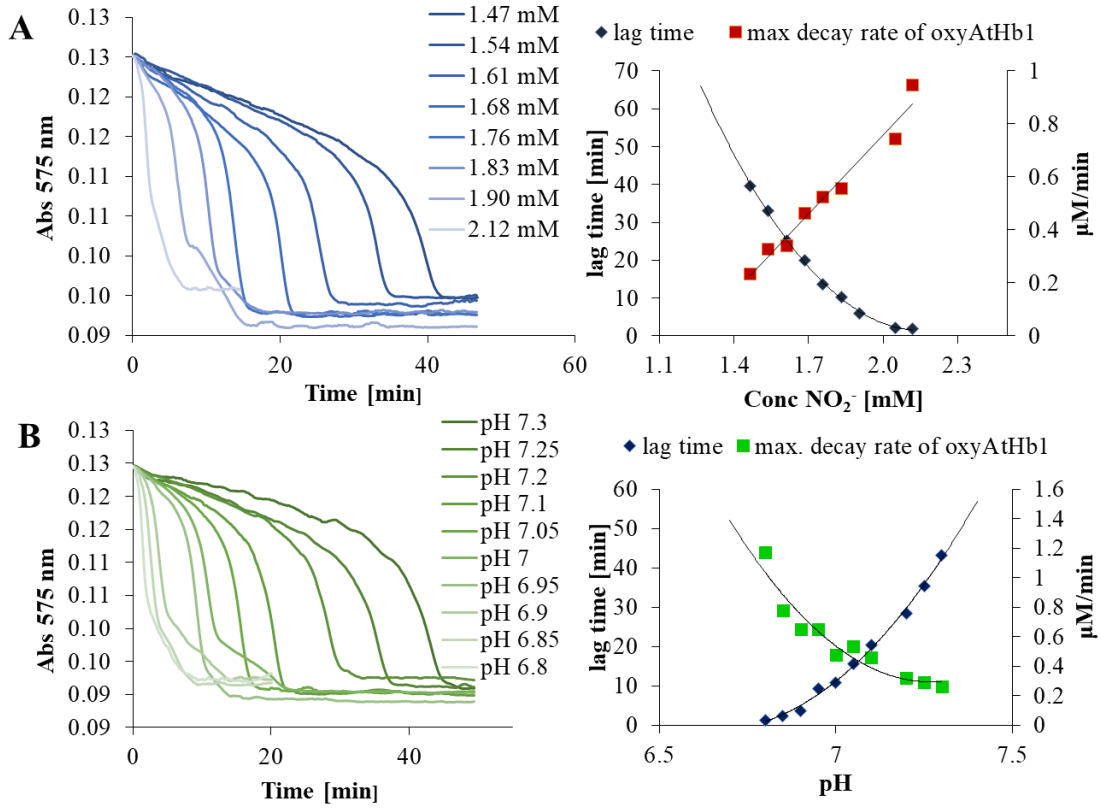


Figure 8. *Kinetic profiles and their corresponding lag times and maximum decay rates for the reaction between 8 µM oxyAtHb1and nitrite* ***A.*** *at different concentrations of nitrite (1.47-2.12 mM) in 50 mM phosphate buffer pH 7 and* ***B.*** *at different pH values (6.8-7.3) with 1.83 mM $NaNO_2$.*

This two-faced reactivity towards nitrite may account for more than just a response to hypoxia. The reactivity of oxyphytoglobins towards nitrate was previously explored *in vivo* and may form an alternative respiratory pathway during hypoxia, as NO_3^- plays the role of an intermediate electron acceptor.[38–41] Nitrite would be the product of nitrate reduction, but it would not accumulate in plants, as it will be subsequently reduced to nitric oxide in hypoxia. The reduction of nitrite can be coupled with proton translocation to maintain ATP biosynthesis.[42] What is even more interesting is that, to ensure a form of anaerobic respiration, other enzymes, presumably oxyphytoglobins, based on the mechanism discussed in this paper, can oxidize NO back to nitrate so that the cycle can continue.[43]

6. A Nitrosyl Affair under Hypoxya starring methionine aminopeptidases and non-symbiotic phytoglobin 1[44]

The removal of the N-terminal methionine is a pivotal post-translational modification in around 60-70% proteins and is carried out by methionine aminopeptidases (MAPs). Due to their fundamental function, such enzymes need to be tightly regulated mostly through transcriptional means or post-translational modifications[45]. In this chapter, we discuss how the reactivity of *A. thaliana* methionine aminopeptidases may be regulated through metal and S-nitrosylation. Building on the foundation established in the previous chapter, we explore a possible nitric oxide transfer from a nitrosylated methionine aminopeptidase to the non-symbiotic hemoglobin 1 from *A. thaliana*.

Upon the addition of $GS^{14}NO$ or $GS^{15}NO$ on MAPs, a high intensity well-defined EPR signal arises at g value 2.004. This is a clear indication of a metal-nitrosylated complex, highly likely with a non-heme iron center, with an important contribution from a cysteine in the vicinity[46,47]. The formation of a nitrosylated MAPx resulted in partial inhibition of the activity in both MAPs tested.

Experimental evidence from our collaborators at Universidad de Salamanca shows that *Arabidopsis* methionine aminopeptidases are involved in hypoxia responses and are regulated through nitrosylation[44]. We show how methionine aminopeptidases transfer the nitric oxide to AtHb1 through EPR spectroscopy, an interaction which could be relevant *in vivo*.

7. pKa and the globin ferryl identity crisis: what is the nature of the protonation event at pH< 5?[48]

Reported is this chapter are the UV-vis measurements (including stopped-flow) on ferryl myoglobin (Mb) over a wide pH range (2-10), revealing for the first time the Soret band of this species at pH < 5. Comparisons between the behavior of ferryl and that of both met and oxy Mb under the same conditions are provided. Also, to deconvolute the possible contributions of protein denaturation below pH = 5, control experiments are performed with an alternative denaturing agent – guanidine (Gu). We describe detailed efforts to rationalize the differences in spectra between ferryl Mb at acidic *vs.* neutral/basic pH.

Figure 9 reiterates the previously-reported UV-vis data on ferryl Mb obtained from the pH jump experiments, and now enhances them with newly-collected data in the Soret region. The pKa calculated based on the Soret region is 4.6 (by applying the Henderson-Hasselbach equation on the ratio between the 418 nm and 422 nm absorbances), which is in reasonable agreement with the pKa determined from the α and β region (4.2 in Figure 9, or 4.6 in another paper[49]). Overall, further control experiments suggest that a notable protonation event occurs upon acidification of ferryl, but neither upon acidification of met or of oxy Mb, nor upon the simple denaturation of the protein. Hence, this protonation event (and the spectral change thereafter) are intrinsic to the ferryl unit rather than to the protein in general.

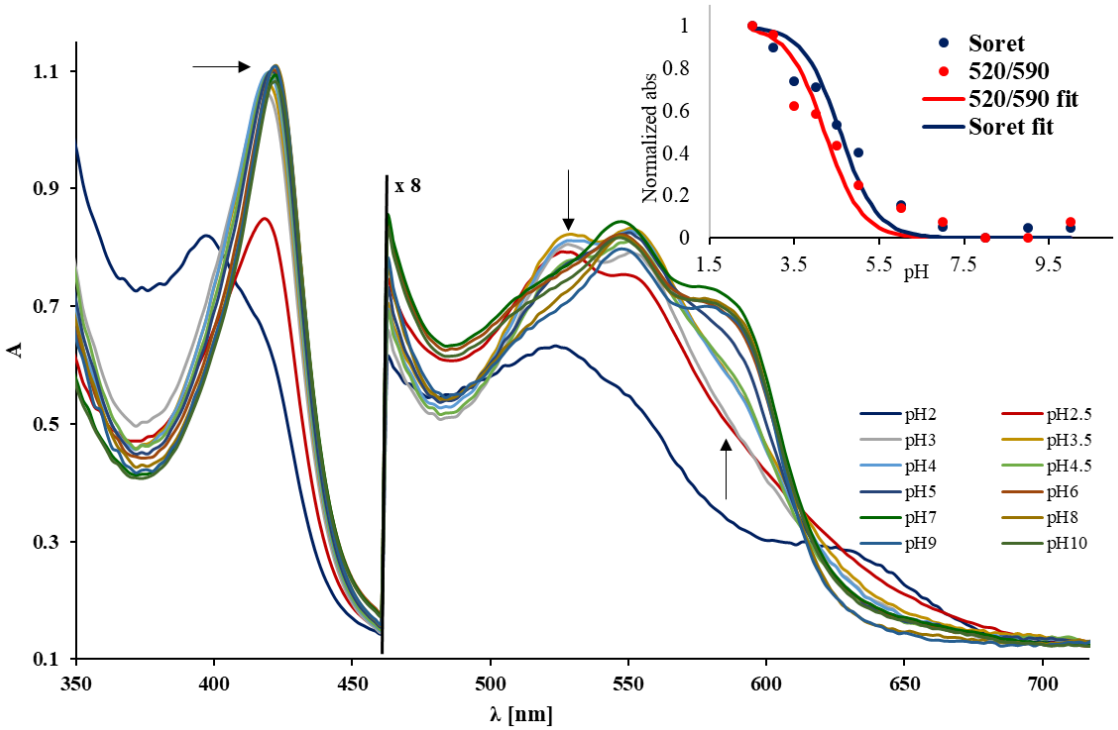


Figure 9. *UV-vis spectra collected in a stopped-flow experiment titrating 6.7 μM ferryl Mb against 120 mM universal buffer of pH values at room temperature. The insert shows the titration curves of the ferryl Mb determined from the pH-jump experiment. The pK_a calculated using the Soret-derived curve is 4.6 (4.58), while the pK_a determined based on the changes in the α and β is 4.2.*

8. Globi-Wan Kenobi the Third and the Cyanamide Menace: the catalase-like activity of a truncated phytoglobin towards cyanamide[50]

It was observed that cyanamide could interact with ferric heme proteins in a poisonous way[51,52], effect which was augmented by alcohol consumption. In turn, cyanamide has been used as an alcohol deterrent[53], as one of its metabolites inhibits aldehyde dehydrogenase[54–57]. The experimental evidence suggests that cyanamide is hydroxylated by the Compound I form of catalase to N-hydroxycyanamide, which in turn decomposes to cyanide and HNO[58–60]. The relatively spacious binding cavity of AtHb3[61] and its reactivity towards hydrogen peroxide may be taken as premises for a peroxidase/catalase-like activity. To explore the possibility of this activity, cyanamide was used as substrate.

Adding hydrogen peroxide to the cyanamide-AtHb3 complex triggers interesting spectral changes, as shown in Figure 10. The α and β bands shift in a hypsochromic fashion to 534 and 562 nm, showcasing characteristics of a low spin adduct.

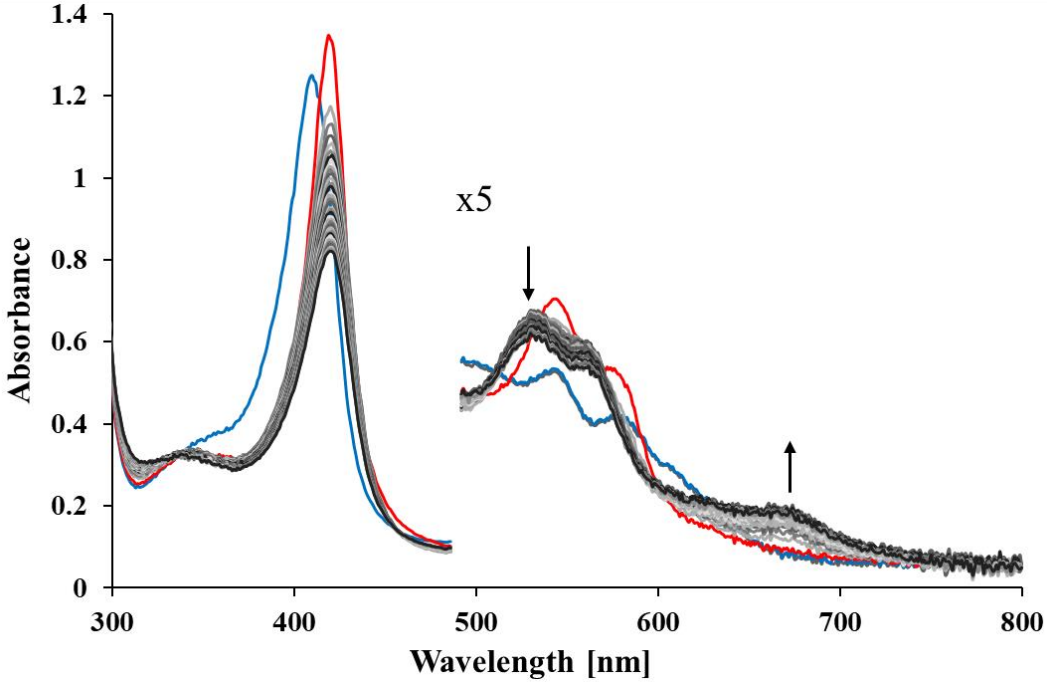


Figure 10. *UV-vis kinetics of the reaction between the metAtHb3-NH_2CN adduct and hydrogen peroxide. MetAtHb3 (blue spectrum) is converted to metAtHb3-NH_2CN (red spectrum), which in turn reacts with hydrogen peroxide to generate a new species (grey/black spectra).*

The ability of globins to convert ABTS to its free radical form is used to measure their pseudoperoxidase activity[62]. The addition of cyanamide inhibits the peroxidation of ABTS in a concentration-dependent manner with an inhibition constant equal to 1.6 mM (Figure 11). Taken together, all experimental observations seem to point to the hydroxylation of cyanamide in a similar way to that catalyzed by catalase.

The mechanism which describes the reaction between the metAtHb3 and cyanamide adduct, and hydrogen peroxide appears to comprise the following steps:

1. Cyanamide dissociates from metAtHb3 in the presence of hydrogen peroxide; metAtHb3 is converted to Compound I AtHb3.

2. A small fraction of Compound I AtHb3 hydroxylates the N-H bond in the amino group of cyanamide to generate N-hydroxycyanamide, which decomposes to cyanide and possibly N_2O.
3. Simultaneously, Compound I AtHb3 is rapidly converted to Compound II AtHb3 (ferrylAtHb3); the lack of distal histidine and the hydrophilic binding site, together with the lack of an efficient reducing agent most likely result in the degradation of the protein.

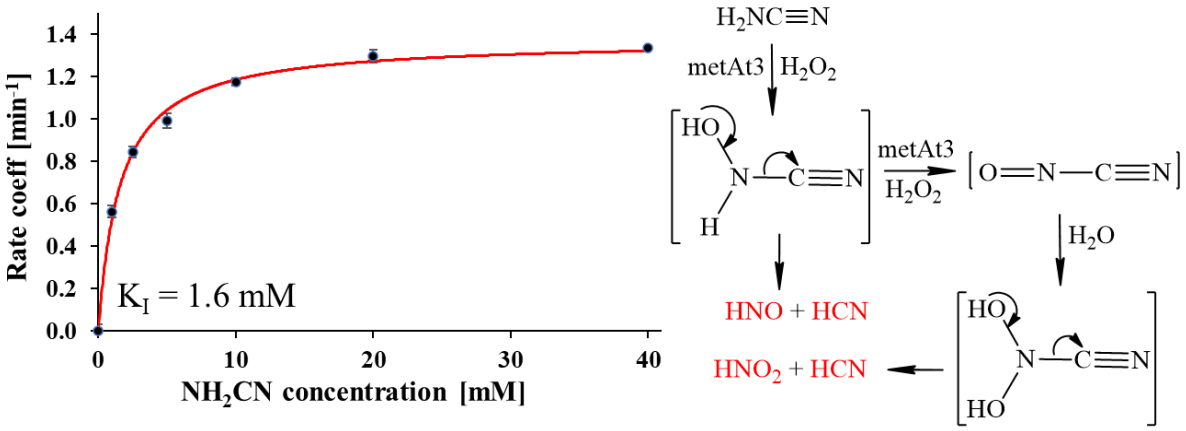


Figure 11. Left: *The Michaelis-Menten plot with varying concentration of cyanamide in the presence of 20 nM metAt3, 0.5 mM ABTS and 2 mM H_2O_2 at pH 7.4.* ***Right:*** *A proposed mechanism for cyanamide decomposition in the presence of catalase*[60] *adapted for metAtHb3.*

There is no clear proof in support of the reactivity of AtHb3 towards cyanamide *in vivo*, but it should be noted that no other enzyme (except for catalase) displays such a catalase-like reactivity towards this substrate. AtHb3 might be the first non-genetically tailored globin to perform hydroxylation reactions using Compound I and not a free radical-based pathway. The experimental trapping and characterization of Compound I AtHb3 could deepen our current understanding of high-valent heme intermediates, especially on how histidine-ligated hemes could stabilize high-valent intermediates to perform hydrogen abstractions.

9. General conclusions

In Chapter 2, the intricate balance between the antioxidant and prooxidant behavior of polyphenols was discussed, with an emphasis on their potential to generate hydroxyl radicals in the presence of iron complexes. By developing and optimizing a colorimetric assay to assess this prooxidant activity, we have provided a novel analytical tool to investigate the Fenton-like chemistry of plant phenolics. Our findings demonstrate that polyphenol structure, pH, and iron coordination significantly influence free radical generation.

The mechanism of liposomal peroxidation in the presence of myoglobin and its inhibition by polyphenols was explored in Chapter 3. The inhibitory activity has been explored in terms of mechanism, pointing out the importance of interactions of small molecules with both the liposomes and the globin.

In Chapter 4, we investigated the low-spin ferric AtHb3 adducts using EPR spectroscopy. Our enquiry has revealed significant variations in their anisotropy and relaxation behavior. Notably, certain adducts exhibit relaxation behaviors that deviate from conventional expectations, pointing to complex electronic effects beyond simple spin-orbit coupling consideration. The combination of experimental EPR data with DFT calculations has provided valuable insights into the fundamental factors governing the spin properties of heme systems, such as spin density and hyperfine components.

Despite their structural and possible functional differences, all three nonsymbiotic plant hemoglobins from *A. thaliana* display a nitrite reductase-like activity under anaerobic conditions. As discussed in Chapter 5, all three phytoglobins display better rate constants than horse heart myoglobin. All studied globins reacted with nitrite in aerobic conditions as well; among them, AtHb3 is by far the most reactive one with regard to this function. Ferryl involvement is also discussed based on spectroscopic and kinetic observations.

Chapter 6 provides compelling evidence that nitric oxide could modulate the activity of *Arabidopsis thaliana* methionine aminopeptidases (MAPs) through both metal nitrosylation and S-nitrosylation. Our findings demonstrate that MAPs could engage in trans-nitrosylation with non-symbiotic phytoglobin AtHb1, transferring NO and potentially contributing to hypoxia signaling in plants.

In Chapter 7, we show how ferryl Mb undergoes a protonation event below pH 5, as assessed using pH jump experiments with stopped-flow UV-vis spectroscopy. This event is characterized by hypsochromic shifts in the Soret band (~5 nm) as well as in the α and β bands (~20-40 nm) and a ~10-nm reduction in the energy difference between the α and β bands. Control experiments suggest that the protonation event in these latter experiments is localized on the iron-bound oxygen atom, as opposed to somewhere on a hydrogen-bonding partner.

Chapter 8 proposes a catalase-like reactivity of the truncated phytoglobin 3 from *A. thaliana* towards cyanamide. The observed spectral changes are consistent with the involvement of high-valent heme intermediates, akin to those found in catalase. Kinetic data reveal a pseudoperoxidase-like activity of AtHb3 which is significantly inhibited by cyanamide, suggesting an inner-shell reaction mechanism. Further investigations are consistent with the involvement of Compound I AtHb3. This catalase-like activity of AtHb3 towards cyanamide seems to be unique among heme-containing enzymes so far.

10. References

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