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Summary of the PhD Thesis

INVESTIGATION OF SOME OXYGEN-TRANSPORT PROTEINS BY EPR SPECTROSCOPY

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Cluj-Napoca
2013

ACKNOWLEDGEMENT

In order to elaborate a doctoral thesis, in addition to the scientific background and experience, it takes a professional leadership, persistence, the necessary infrastructure for the practice, the financial and the social support.

I would like to express my gratitude to Prof. Univ. Dr. Grigore Damian, as my scientific coordinator in elaborating my thesis, in addition to his knowledge and experience has provided me the necessary bibliography and support, and also helped me through the past few years.

Particular thanks to Conf. Dr. Radu Silaghi-Dumitrescu, Dr. Augustin Cătălin Moț and Dr. Cristina Bischin from the Faculty of Chemistry and Chemical Engineering from the University of Babeș-Bolyai, Cluj-Napoca, for the advices and hints provided in the field of biochemistry, which helped me complete the practical part of this thesis.

I am thanking my guidance commission, Prof. Dr. Viorica Șimon, Prof. Dr. Leontin David and Conf. Dr. Vasile Miclăuș, for giving practical advices and for providing quality guidance during the research. Also special thanks to Dr. Laura Bolojan for introducing me in the practice of writing a thesis, and Dr. Cristina Gruian who guided me during the time spent in Germany.

Prof. Dr. Heinz-Jürgen Steinhoff and his team from the University of Osnabrück, Germany, I owe thanks, because they helped in my professional development during my practical semester spent at their group.

Material security is a prequisite for a scientific work and I would like to thank for the financial support recieved from the Sectoral Operational Programme for Human Resources Development 2007-2013, co-financed by the European Social Fund, under the project number POSDRU/107/1.5/S/76841 with the title „Modern Doctoral Studies: Internationalization and Interdisciplinarity".

I also want to express my gratitude and thank for my family that they suported me over the past three years, in particular my parents, my sister and my wife, because they belived in me and in the idea that succes can be achieved through fulfillment in the scientific world. They accepted that most of the time I was devoted to my thesis and work, they encouraged me and assured their love.

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Keywords: site directed spin labeling (SDSL), continuous wave EPR spectroscopy, protein dynamics, protein denaturation, hemerythrin, hemoglobin, thermodynamic parameters, Gibbs free energy, enthalpy, entropy, heat capacity

INTRODUCTION

The evolution of biochemistry was explosive over the last century rivaling with other disciplines like physics and chemistry. Thanks to this evolution we know now that living systems are based on organic elements like carbon, oxygen, nitrogen and hydrogen mixed with the inorganic elements like iron, copper, sodium, potassium and magnesium and that the laws of physics apply in the same way in the living systems. By combining ideas from chemistry and physics the complexity of the living systems can be studied and understood more easily.

Proteins have unique features amongst the macromolecules by taking part in every reaction occurring in the biological systems and supporting a large amount of biological activities.

The investigation of the protein behavior represents a great importance in the modern scientific community because molecular genetics studies showed that many diseases are connected to protein defects and malfunctions, by studying and understanding a protein behavior the obtained information can be an influence in the pharmaceutical drug and health care products development.

This work presents the results obtained from a study of two oxygen-transport responsible protein, hemerythrin and hemoglobin, by using the site directed spin labeling (SDSL) technique in combination with electron paramagnetic resonance (EPR) spectroscopy [Altenbach et al 1989, Altenbach et al 1990]. Also this work can be considered the first spin labeling study on the hemerythrin protein [Takacs et al 2013a].

By using EPR spectroscopy in protein studies we have the advantage that the studied samples are live proteins and they can be investigated in a close to their native like environment.

The two proteins, hemerythrin and hemoglobin have a great importance due to their property to bind and release oxygen and also recently the hemerythrin protein was proposed as an alternative solution to hemoglobin based blood substitutes [Alayash 2004, Tsuchida et al 2009, Mot et al 2010, Fischer-Fodor et al 2011].

This work is structured into four main chapters.

In chapter 1 the proteins are presented, a general description of protein structural elements is given along with their function and specific properties. Besides the general introduction of protein structure and function, the two studied proteins are presented the hemerythrin and hemoglobin.

In chapter 2 a few techniques are described which are currently used in the protein study field (IR spectroscopy, Raman spectroscopy, circular dichroism and the NMR technique) they are presented as comparative information to the technique used in this work. The continuous wave EPR theory is presented further and a description is given of the continuous wave X-band EPR spectrometer.

The chapter 3 gives a short introduction to the nitrone derivatives. The nitrones can be classified into two subchapters the nitroxide radicals which are the spin labels and are used in protein studies where direct detection is required and possible, and spin traps which are used in cases where the indirect detection is possible only. Spin traps are used when the studied free radicals are short lived and

by using the spin trapping technique it is possible to form spin adducts which can be detected by EPR spectroscopy.

Further in this chapter the site directed spin labeling technique is presented along with different nitroxide radicals used in this field, the attachment to the protein (labeling) and the advantage of the various types of spin labels. The nitroxide radicals can be used in EPR oxymetry, which is considered an important field since allows the quantification of the molecular oxygen in biological samples. An EPR oxymetry study is presented of molecular oxygen upon nitroxide radicals; encapsulated TEMPO spin label in liposomes [Takacs et al 2011a, Takacs et al 2011b]. The spin trapping technique is presented along with the obtained results [Bolojan et al 2012] and the technique used in a study where the superoxide radical generated from potassium superoxide was monitored using the spin trap DEPMPO and EPR spectroscopy.

The chapter 4 brings focus on the main subject of this work, by using the background information presented in the previous chapters. This chapter presents the first spin labeling attempt and result of the hemerythrin along with the spin labeling of hemoglobin [Takacs et al 2013a]. The spin labeled proteins were subject of two types of study. First the dynamics of these two proteins was investigated by using various controlled environments in order to mimic their native environment [Takacs et al 2013b, Takacs et al 2013c]. The viscosity of the environment was controlled using two types of viscosity agents, glycerol and PEG-4000. The chosen two agents were used in order to investigate the dynamical behavior of the studied protein in a viscous environment with and without macromolecular collisions. The glycerol molecule has a small molecular weight compared to the studied proteins and was used in the dynamical study without macromolecular collisions. The PEG-4000 has a noticeable molecular weight so it is an ideal candidate for macromolecular collision studies.

Also the crowding effect [Ellis 2001] was studied by EPR spectroscopy in a biological environment by using Bovine Serum Albumin as a crowding agent. The BSA is a relatively large sized protein, and has the property to attach to other proteins or de-attach subunits from proteins in its environment.

The two spin labeled proteins were a subject of a denaturation study by using chemical and physical denaturation [Takacs et al 2013d]. Guanidinium chloride was used in order to induce an overall unfolding of the proteins. By introducing the samples in environment with various pH the stability of the proteins was monitored. Also the thermal stability of the proteins was investigated.

With the help of the denaturation studies important thermodynamic parameters were determined; the Gibbs free energy in the absence of denaturant representing the stability of the protein to a denaturation process, the enthalpy, entropy and the heat capacity was also obtained. By using two approaches, spectral area subtraction and average rotational correlation time, an important and alternative method to calorimetric studies on proteins was determined and described by using EPR spectroscopy.

1. CHARACTERISTICS OF PROTEINS

1.1. Proteins

This chapter will bring the focus to one of the major component of living systems, the proteins. They are to be found in all living systems, from bacteria to mammals. They make up over 50 percent of the dry weight of the cells and are present in the highest ratio compared to any other bio-molecule [Whitford 2005].

Proteins can have a large field of biological functions like transport, cell binding, storage, receptor and immune system proteins, but despite the large functional diversity all proteins have something in common, all have a linear arrangement of amino acid residues linked together in a polypeptide chain.

Although the large number of protein structure and function there are only 20 amino acids and they have similar composition. These amino acids can be considered also the building blocks of proteins and good way of understanding proteins is to understand the working mechanism of these amino acids.

Amino acids are compounds containing a carboxyl group at one end and an amino group next to it, bound to the carbon atom found in the carboxyl group. This carbon atom is called α-carbon. The carboxyl group donates a proton to the amino group, so that in the absence of additional acids or bases the amino acids carries a negative and a positive charge. In this way the molecule appears uncharged, this phenomenon is also called zwitterion.

All amino acids have common elements they all contain carbon, hydrogen, nitrogen, oxygen and two of the amino acids contain additionally sulfur. Amino acids can be found in white crystalline solid form, this is explained by their high melting points, having the most atypical properties of organic molecules. Their decomposition and melting point can be placed in the range of 200-300°C [Whitford 2005].

Compounds like amino acids with the possibility of acting both as acid and base have an important property, the isoelectric point *pI*. The isoelectric point is the pH value at which the number of positive charges on the molecule equals with the number of negative charges, and the zwitterion phenomenon exists. The isoelectric point is given by the average of the *pK*-s that involves the zwitterion or sets its boundaries [Buxbaum 2007]. The linear arrangement of the amino acids [Alberts 2002] in a protein sequence is called the protein primary structure. The primary structure is the two dimensional arrangement of a protein also referred to as the backbone of the protein.

The primary structure starts with the N terminal, amino group, of the first amino acid and ends with the C terminal, carboxyl group, of the last amino acid from the linear sequence.

Due to the different bonding angles between the forming chemical elements and peptide bonds the proteins have a three dimensional structure. This three dimensional structure is called the secondary structure, the spatial arrangement which describes the conformation of the amino acid residues that are next to each other in the primary sequence.

There are three basic units which are referred to as secondary structure and these are: α helices, β sheets or strands and γ turns. The rest of the structures are the combinations of these three basic elements.

By linking the elements of the secondary structure together would result in a final three-dimensional form. This three-dimensional structure of a protein [Alberts 2002], which is mostly a result of a large number of non-covalent interactions between amino acids is called the tertiary structure, represented on the Figure 1.1.1. It describes the global conformation of the protein or more precisely the spatial arrangement of the elements of the proteins secondary structure. Tertiary structure can be also called the global folding of a single polypeptide chain [Alberts 2002].

Tertiary structure is determined by various interactions occurring between sequences, like ionic interactions between charged amino acid R-groups or Van der Waals bonds. A dominating force in determining the tertiary structure of proteins is the hydrophobic effect. The polypeptide chains fold in a way that the side chains of the non polar amino acids are buried within the structure and the side chains of the polar residues are exposed on the outer surface, a great example for this are the globular proteins [Whitford 2005, Alberts 2002]. The stability of the tertiary structure of proteins is resolved by hydrogen bonding involving groups from the peptide backbone and side chains and disulfide bonds or bridges (S - S bonds) between cysteine residues [Buxbaum 2007].

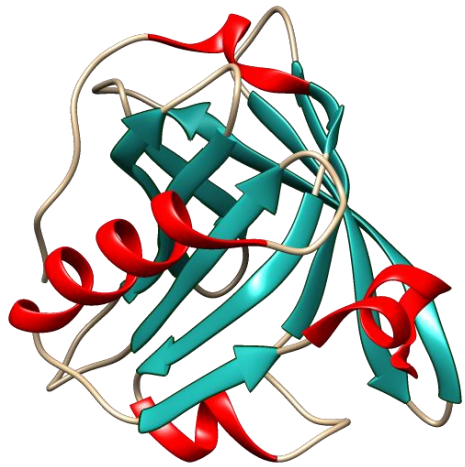

Figure 1.1.1. Representation of the tertiary structure of the beta-lactoglobulin protein. The drawing was generated with the visualization software UCSF Chimera [Pettersen et al 2004] using the coordinates from 2GJ5 [Yang et al 2008] entry of the Protein Data Bank.

Often many proteins contain more than one polypeptide chains, more polypeptide chains combined in order to form one functional protein would result in a quaternary structure [Whitford 2005, Alberts 2002]. The quaternary structure is very similar to the tertiary structure but with the difference that the interactions are present between polypeptide chains or subunits. It was shown that proteins with quaternary structure allow the formation of catalytic or binding sites at the interface between subunits [Whitford 2005], which is physically impossible in a monomeric protein. The advantage of oligomeric proteins is that the ligand binding allows

conformational changes for the whole structure and by this way the protein can take part in processes of regulating biological activities.

Some proteins are more sensitive to denaturants or heat some are not so much. A few proteins can be completely denatured by heat and denaturants and after cooling down and removing the denaturant by dialysis the protein will go back into its initial conformation and becomes functional again.

Proteins can be classified according to their function in three major categories: fibrous proteins, membrane proteins and globular proteins.

Fibrous proteins are structural and form long filaments, rod-like forms, which are insoluble in aqueous solutions. The second category the membrane proteins are usually receptors, provide channels for charged molecules to pass through the cell membrane or contribute in cell adhesion allowing cell identification and interaction. The globular proteins are proteins formed by compact amino acid chains, which are often folded into spherical shapes. The main difference between fibrous and globular proteins is that globular proteins are soluble in aqueous solutions.

1.2. Hemerythrin

Hemerythrin (Hr) is an oxygen binding [Kurtz 2003] oligomeric metalloprotein. It is a respiratory protein responsible for the oxygen transport and oxygen storage in the blood of marine invertebrates. Although the name of the protein contains the word heme, the hemerythrin lacks the heme group and has a non-heme di-iron site [Farmer et al. 2000; Jin et al. 2002; Kryatov et al. 2005].

The hemerythrin protein can be found in the marine invertebrate phyla like sipunculids, brachipods, priapulids and annelids. These are sedentary worms and small shelled invertebrates usually found in deep seas, with a length ranging from 4 - 10 cm. Most widely the octameric hemerythrin is studied and used which comes from the sipunculid *Phascolopsis gouldii* or on its common name the *Peanut worm*. The studies performed in this work are also based on this version of the protein

The hemerythrin is generally formed by 8 identical subunits giving its main octameric form, represented on the Figure 1.21, but it also can exist in other oligomeric structure formed like monomer [Langeman et al 1969], dimmer [Sieker et al 1981], trimmer [Smith et al 1983] and also a tetrameric [Sieker et al 1981] form is possible. The octameric hemerythrin has a total mass of 108 kDa.

According to [Stenkamp 2004] the subunits have the following structure they are nearly cylindrical shaped around 40 Å long and with a 20 Å width, they have a mass of 13.6 kDa and contain 114 amino acid residues. The subunits are formed by four α-helices folded into an anti parallel packing.

The α-helices are not exactly parallel with each other they form an inter helical angle giving an overall wedge shape to the subunit. The helices are closer at one end and they diverge at the other end, leaving space this way between them that is used for binding prosthetic groups like binuclear iron complexes, also the iron complex in hemerythrin is placed around at one-third of the full length of the subunit from the wider end [Stenkamp 2004].

In addition each of the subunits contains a native cysteine at the 51st position (51C), which can be used for site directed spin labeling studies by EPR spectroscopy without performing mutation on the proteins amino acid sequence.

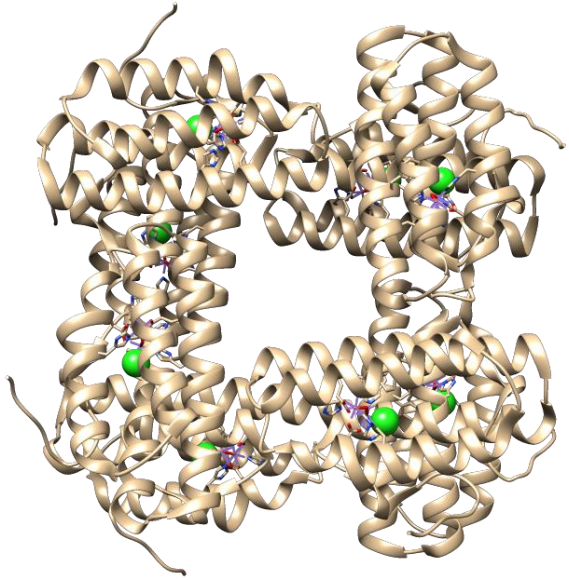

Figure 1.2.1. Representation of the octameric quaternary structure of Phascolopsis gouldii hemerythrin. The drawing was generated with the visualization software UCSF Chimera [Pettersen et al 2004] using the coordinates from 1I4Y [Farmer et al 2001] entry of the Protein Data Bank.

The *Phascolopsis gouldii* octamer hemerythrin structure described by [Stenkamp 2004] has 422 (D4) symmetry with four subunits packed around a four-fold rotation axis, allowing heterogonous interactions with the neighboring subunits in the same layer. Two layers are interacting via homogenous interactions forming the octamer structure. The two layers are coordinated by two kinds of two-fold rotation axes perpendicular to the four-fold axis. The size of an octamer is around 60 x 60 x 40 Å and has a 20 Å wide channel that passes through the center of the protein perpendicular to the two layers along the four-fold rotation axis. There has been no functional significance discovered and reported for the presence of this channel yet.

The di-iron metal complex [Farmer et al. 2000; Jin et al. 2002; Kryatov et al. 2005] in hemerythrin in addition to oxygen (O_2) can bind other small molecules like azide, chloride or nitric oxide [Garbett et al 1969, Nocek et al 1988]. The protein can exist in two forms liganded (azidomet- and oxyhemerythrin) and unliganded forms (met- and deoxyhemerythrin) when the iron complex does not bond to any additional molecules. The forms are determined by the iron center geometry which is different for the two forms.

When oxygen binds to the complex the form is called oxyhemerythrin and when azide takes the oxygen molecules place we speak about azidomethemerythrin.

The hemerythrin is colorless in deoxyhemerythrin form but it turns to a pink color when undergoes to the oxygenated state. The reversibly oxygen binding and releasing process [Kurtz 2003], is the proteins main biological function, with

the binding site being buried it undergoes to conformational changes to allow the oxygen to reach the site.

1.3. Hemoglobin

Hemoglobin (Hb) is a heme-containing oxygen (O_2) binding globular metalloprotein found in erythrocytes (red blood cells), in a order of hundred millions in each blood cell and with a function to transport oxygen from respiratory organs to tissues.

The protein is made up primarily by four polypeptides or subunits and has a size of around 62 kDa. Every hemoglobin molecule contains two identical alpha (α) subunits and two identical beta (β) subunits. These subunits in the case of hemoglobin are called globins; they have similarities but are not identical. The alpha chain has a length of 141 amino acids in case of humans and 142 amino acids in case of bovines. The beta chain has a length of 146 amino acids in case of humans and 145 amino acids in case of bovine hemoglobin.

In this work the measurements and the studies were carried out on bovine (*Bos taurus*) hemoglobin. The difference between the bovine hemoglobin and human hemoglobin consist in the amino acid sequence. While the human hemoglobin contains six native cysteines at the 105th position (α105C) in the alpha chain, at the 94th position (β94C) and at the 113th position (β113C) in the beta chain the bovine hemoglobin contains only a total of two native cysteines at the 92nd position (β92C) in the beta subunit. The native cysteines in the protein allow us to perform site directed spin labeling studies on the protein and to study by EPR spectroscopy without performing any mutation on the protein amino acid sequence.

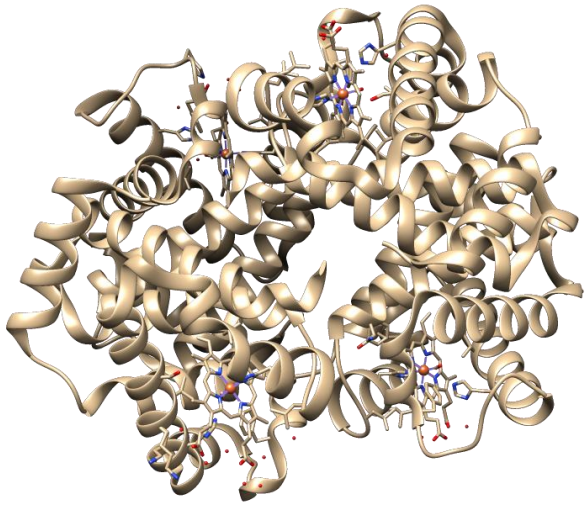

Figure 1.3.1. Representation of the Bos taurus hemoglobin quaternary structure with the heme group and the iron atom in the porphryin ring. The drawing was generated with the visualization software UCSF Chimera [Pettersen et al 2004] using the coordinates from 2QSS [Aranda et al 2009] entry of the Protein Data Bank.

The crystal structure was determined by [Perutz et al 1960, Perutz 1965] and both of the hemoglobin chains are formed mostly by α helices. There are 6

longer α helices and 2 short ones, and are labeled from A to H. The helices are connected by short non helical sections and are stabilized by hydrogen bonds. According to [Paoli et al 2004] the globular structure of the hemoglobin has a 50 - 55 Å diameter and the subunits have a 47 x 35 x 22 Å size.

The hemoglobin is being able to adapt and alternate between two states with distinct differences in its main quaternary structure [Perutz 1970, Perutz 1972]. The relaxed (R) state when he adapts the liganded oxy state and the tense (T) state when adapts the unliganded deoxy state. The T state contains extra stabilizing interactions between the subunits while the R state lacks these interactions and has a higher affinity.

The higher affinity of the R state is ensured by the location of the iron atom in the plane of the porphryin ring more accessible to oxygen, therefore its higher affinity to oxygen. In the T state the iron is pulled out of the porphryin rings plane and in this way being less accessible for oxygen binding.

The alternation between the R and T states is the result of a rotation of 15° between the dimmers and a translation of 0.8 Å causing a distance decrease between the irons atoms from 40 Å to 33 Å [Paoli et al 2004]. This transformation happens when hemoglobin reaches the lungs, where the amount of oxygen is higher; the oxygen binds to the T state causing the dimmers to rotate allowing oxygen to bind more easily. The hemoglobin regains its R state when he reaches the low oxygen environment and releases the carried oxygen.

2. PROTEIN STRUCTURE AND DYNAMICS INVESTIGATION TECHNIQUES

Various methods were applied for studying protein dynamics and structure over the last 30 years, some of them being more successful in some cases and some less. There is no exact approach to study protein dynamics and none of the methods can be called above the other although these techniques can be classified in two main categories: vibrational spectroscopic techniques (IR spectroscopy, Raman spectroscopy and circular dichroism spectroscopy) and resonance spectroscopic techniques (NMR spectroscopy and EPR spectroscopy).

In this work the chosen method for studying protein dynamics was with electron paramagnetic resonance (EPR) combined with the site directed spin labeling (SDSL) technique [Bordignon et al 2007]. Before giving an introduction to the above mentioned investigation method a few other useful tools and approaches will be presented which are currently used by the scientific community studying protein dynamics.

2.1. Vibrational spectroscopic techniques

The IR spectroscopy is based on molecules that absorb radiation from the electromagnetic spectrum at specific frequencies when the frequency equals with the energy transition of the structure.

The Fourier transformed infrared (FTIR) spectroscopy has proven to be a good technique in order to study proteins and lipids. A low resolution of the protein structure can be obtained and the technique allows the studied sample to be found in their native like environment.

As a complementary technique to IR spectroscopy the Raman spectroscopy exploits the weakness of the infrared spectroscopy. The theory of this method is based on the scattering of light by the vibrating molecules.

Contrary to IR spectroscopy the molecules do not need to possess a permanent dipole moment. We have a Raman active vibration when this causes changes in the polarisability. By using this investigation method we obtain information about the covalent character in the molecule. The Raman spectroscopy involves molecular and crystal lattice vibrations and is sensitive to composition, bonding, environment and crystalline structure.

The Raman spectrum of proteins, nucleic acids and lipids holds a large number of discrete bands which represent the molecular vibration modes and is a sensitive fingerprint of the three-dimensional structure, dynamics and interactions.

The circular dichroism (CD) spectroscopy is based on the differential absorption of left and right circularly polarized light.

The circularly polarized light is represented as a sum of two orthogonal but linearly polarized lights in which the amplitudes are equal and the phases have been shifted with $\pm\pi/2$.

In a medium which has optical activity the absorbance of the left circularly polarized light has a difference to the absorbance of the right polarized light. After both lights pass through the sample they remain polarized circularly but the

amplitude of the electric vectors of each is different than before. After the combination of the two oppositely directed circularly polarized light waves, we will obtain an elliptically polarized light due to the amplitude difference of the components.

The difference of the absorbance in the two components and the ellipticity of the resulted combination is the one studied and measured. Plotted against the wavelength the circular dichroism spectrum is obtained.

In case of proteins this technique has some limitations, although many proteins have been studied and their secondary structure was determined by circular dichroism [Greenfield 2006].

2.2. NMR spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a complex investigation technique based on the nuclear spin (I) resonance in an applied magnetic field (B_0) and microwave radiation.

In quantum mechanical terms the nuclear spin will align with or against an externally applied magnetic field in $2I+1$ ways. In terms of energy the preferred orientation will be parallel with the applied magnetic field this is called the low energy alignment while the high energy alignment is the anti-parallel alignment with the applied magnetic field. If the sample is irradiated with radio waved (MHz frequency range) the proton will absorb energy and will align with the less favorable energy state the higher one. The energy absorption is called resonance and the frequency of the applied radiation coincides (resonates) with the precession frequency.

The instruments for continuous wave NMR spectroscopy have an elevated cost due to the strong magnetic field required and maintained. These are superconducting magnets that require sensible cryogenic systems for cooling both by liquid helium and nitrogen. Although the elevated cost and sensible handling the technique of proteins dynamic investigation with NMR spectroscopy is widely applied and gives great results in structure determination. For studying proteins with NMR usually the sample is uniformly isotopically labeled with ^{13}C and ^{15}N. Although the method is a very efficient to use in protein study and gives an excellent resolution in structure and timescale there is somewhat limited by the size of protein. Proteins with molecular weight over 35 kDa are difficult to investigate, the rotational correlation time (τ_c) increases with the size of the proteins and larger rotational correlation times lead to peak broadening and also signal to noise decrease. Another aspect of large proteins is that they have an increased amount of atoms and therefore the NMR spectra contain more peaks which can often lead to peak overlapping.

2.3. EPR spectroscopy

The concept of electron paramagnetic resonance is similar to the nuclear magnetic resonance but instead of using the spins of the atomic nuclei to excite, the electron spins are the ones excited. The EPR spectroscopy over time has proven to

be a useful tool in a variety of fields. It is widely used in the field of biochemistry for protein dynamics studies because allows the sample to be investigated in its native like environment. Various types of EPR experiments, continuous wave and pulse were developed to study distances between two sites in a biomolecule or to study protein response to different environmental factors. Unfortunately not all proteins contain an active paramagnetic center, an unpaired electron, and since the method bases on the excitation of the electron spin, EPR silent samples will have no signal. To overcome this, the combination of site directed spin labeling with electron paramagnetic resonance spectroscopy was developed by Wayne L. Hubbell and coworkers [Altenbach et al 1989, Altenbach et al 1990]. With the site directed spin labeling method the insertion of an active paramagnetic center to a protein site is possible. These active paramagnetic centers are located on a nitroxide derivative spin label, an unpaired electron located along the N - O bond of the nitroxide radical (^{14}N, $I = 1$).

If we place the electron in a homogeneous magnetic field $B_0 = (0, 0, B_0)$, the electron spin will align parallel or anti-parallel to the magnetic field direction. The z-axis is considered the preferred direction because it is also the direction of the magnetic field, the spin quantum number m_s will reach the values $\pm 1/2$ due to the quantization orientation of an angular momentum. In this case the two values described by equation 2.3.1 are possible for the magnetic moment.

$$\mu_{s,z} = \pm\frac{1}{2}\cdot g_e \cdot \beta_e \tag{2.3.1}$$

The magnetic dipole $\vec{\mu}$ placed in a magnetic field has the energy described by equation 2.3.2, and allows two energy levels for the electron and an energy difference dependent on the magnetic field described by equations 2.3.3 – 2.3.5, represented on Figure 2.3.1

$$E = -\vec{\mu}\cdot B_0 = -\mu_z \cdot B_0 \tag{2.3.2}$$

$$E_\alpha = +\frac{1}{2}\cdot g_e \cdot \beta_e \cdot B_0 \tag{2.3.3}$$

$$E_\beta = +\frac{1}{2}\cdot g_e \cdot \beta_e \cdot B_0 \tag{2.3.4}$$

$$\Delta E = E_\alpha - E_\beta = g_e \cdot \beta_e \cdot B_0 \tag{2.3.5}$$

In order to obtain an electron spin transition from one energy level to another one, by EPR spectroscopy an electromagnetic radiation of an energy $h\nu = \Delta E$ has to be applied.

In case of nitroxide radicals the electron and nuclear spin system is characterized by $S = 1/2$ and ^{14}N $I = 1$, the electron Zeeman effect along with the hyperfine effect are taken into account due to their magnitude compared to the other interactions. The interaction between the electron spin and the externally applied magnetic field B_0 is described by the electron Zeeman effect which can be described by the equation 2.3.6.

$$\hat{H}_{EZ} = \frac{\beta_e}{\hbar}\cdot g \cdot B_0 \cdot \hat{S} \tag{2.3.6}$$

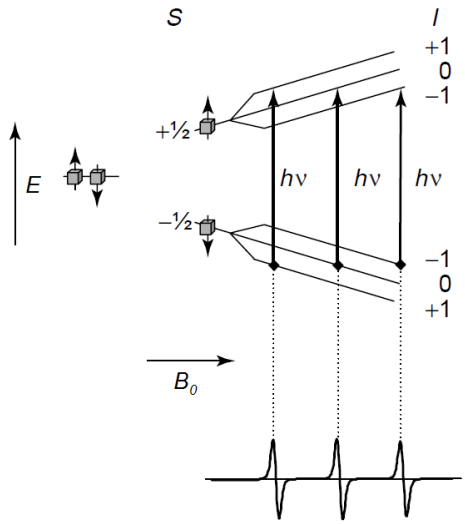

Figure 2.3.1. Electron Zeeman interaction and electron spin alignment to the external magnetic field along with the afferent hyperfine splitting and the allowed transitions. Picture adapted from [Fajer 2000] and slightly modified.

The *g* term describes the *g*-tensor and $\hat{S}$ the spin operator. The *g*-tensors serve as a fingerprint and include the symmetry of the system, which can be isotropic, axial and anisotropic.

$$\hat{H}_{HF} = \hat{S} \cdot A \cdot \hat{I} \tag{2.3.7}$$

In studies where nitroxide radicals are involved, by electron paramagnetic resonance spectroscopy, most of the information is obtained from the hyperfine interaction, this interactions is due to the coupling between the electron spin and the nuclear spin and results in the splitting of the EPR resonance lines due to the magnetic quantum numbers of the nucleus m_I. The hyperfine interaction is described by equation 2.3.7, where A is the hyperfine tensor.

The allowed quantum mechanical selection rules, $\Delta m_S = \pm 1$ and $\Delta m_I = 0$, in a nitroxide radical system, $S = 1/2$ and $I = 1$, will generate the allowed EPR transitions showed on the Figure 2.3.1, and give the specific line shape of the spectrum.

EPR experiment studies are characterized by dynamics and motion on the nanosecond scale, samples in aqueous solution are characterized by a rotational diffusion and a librational motion. These are random processes and are quantified on a timescale by the rotational correlation time τ_c. The rotational correlation time is defined by the Stokes-Einstein relation, equation 2.3.8, and is the characteristic time in which aligned spherical molecules in viscous solution lose their correlation.

$$\tau_c = \frac{4 \cdot \pi \cdot \eta \cdot a^3}{3 \cdot k_B \cdot T} = V \cdot \frac{\eta}{k_B \cdot T} \tag{2.3.8}$$

In the Stokes-Einstein relation the *a* represents the effective radius of the molecule, η is the viscosity of the solution the molecule is found, *T* is the absolute temperature and k_B is the Boltzmann constant.

3. NITRONE DERIVATIVES IN COMBINATION WITH EPR SPECTROSCOPY

Electron paramagnetic resonance (EPR) spectroscopy has a wide range of application including in vivo and in vitro studies of biological systems and free radical detection. In order to be able to perform these types of studies in systems that are EPR silent, special chemical compounds were developed that contain unpaired electrons in order to obtain an EPR signal. One of the main class of chemical compounds used in EPR spectroscopy for detection are the nitrones, they are compounds of organic chemistry containing an N-oxide, N-O bond, and a functional group. The nitrones can be classified in two sub classes the first class contains the spin labels or nitroxide radicals used for site directed spin labeling technique (SDSL) in combination with electron paramagnetic (EPR) spectroscopy.

The second sub class of nitrones contains the spin probes used in the spin trapping method in combination with electron paramagnetic resonance (EPR) spectroscopy. The nitrone spin traps have the property to react with the unstable and short lived free radicals and by this reaction they form a more stable nitroxide radical compound, called spin adducts, that can be detected by EPR spectroscopy.

3.1. Nitroxide radicals and the site directed spin labeling technique

The concept and method of the site directed spin labeling technique (SDSL) with the combination of electron paramagnetic resonance spectroscopy [Altenbach et al 1989, Altenbach et al 1990] overcomes the limitations of conventional spectroscopic techniques for the study of structural properties of biomolecules and nucleic acids by offering the possibility of investigating the behavior of proteins in their native environment.

Nitroxide spin labels can be attached to cysteine amino acid because it contains a thiol group that can react with the functional group of the spin label, creating disulfide bond (S-S bridge) [Klare et al 2009]. The functional group of the spin label can be of the following types: methanethiosulfonate [Berliner et al 1982], maleimide [Griffith et al 1966] or iodoacetamide. If a protein contains more cysteine and one would like to avoid labeling all of them, or the protein contains no native cysteine at all, the sample needs to undergo through a site directed mutagenesis process. By insertion the amino acid is replaced, at the selected position, with a cysteine and the attachment point for the spin label is created. If the investigated protein contains additional cysteine residues to the selected one, for the study, by site directed mutagenesis they are replaced by an alanine or a serine amino acid residue.

There is a long list of available spin labels for EPR studies, one of the most often used is the 1-oxyl-2,2,5,5-tetramehylpyroline-3methyl or methanethiosulfonate spin label (MTSSL) [Berliner et al 1982] is also the spin label of choice in this work du to its minimal disturbing effec on the protein [Klare et al 2009].

3.2. Molecular oxygen effect upon nitroxide radicals

The results and data presented in this subchapter were previously published in [Takacs et al 2011a, Takacs et al 2011b].

The oxygen amount in biological systems plays an important role in many of the physiological and pathological processes, associated with the reactive intermediates. Due to the gaseous nature of molecular oxygen and the chemical and physical properties it is possible to monitor the evolution and the consumption in biological systems [Renger et al 2009], one of the monitoring technique being with EPR spectroscopy.nAlthough the direct detection is impossible by using an indirect method based on spin-spin interaction the detection with EPR spectroscopy becomes possible [Hyde et al 1989]. The detection method, the evolution of the amount of molecular oxygen in a biological system, is based on the interaction of paramagnetic oxygen with non EPR silent spin probes placed in the sample. This interaction gives rise to changes in the relaxation times of these species due to the Heisenberg exchange effect [Hyde et al 1989]. The investigation method is called EPR oxymetry and is based on the line broadening study of the infused spin probe EPR signal which is related to the concentration of the triplet oxygen [Seeling et al 1976].

In this study TEMPO was used, a free state nitroxide radical, encapsulated in liposomes, found in aqueous solutions with different oxygen concentrations. The liposome samples were used for study had been prepared form lecithin, 5 mg/ml, in 10 mM phosphate buffer solution with a pH of 7.4. They were sonified for 5 minutes with an ultrasound bath and a concentration of 5 mM TEMPO was added to the resulted sample. The liposome and spin label mixture was incubated at 30°C for 20 hours and later centrifuged at 10 krpm, with a Beckman J21B centrifuge, for 20 minutes for the removal of the excess spin label amount.

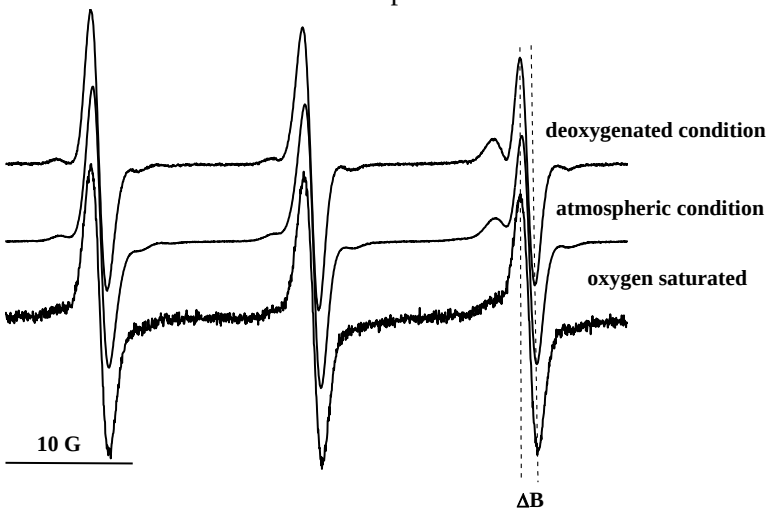

Figure 3.2.1. Recorded EPR spectra of TEMPO under different oxygen concentration. Image taken from [Takacs et al 2011a].

Three samples were prepared one for reduced oxygen concentration, one for normal atmospheric condition and a third with high concentration of molecular oxygen. For each sample a reference sample was prepared containing only free unencapsulated spin label in the same buffer used for the liposome samples.
According to [Pake et al 1959, Subczynski et al 2006] a relationship between the EPR line exchange broadening and the collision rate was defined by equation 3.2.1.

$$\Delta B = 4 \cdot \pi \cdot R \cdot k \cdot p \cdot \{D(O_2) + D(SL)\} \cdot ([\Delta O_2]) \qquad (3.2.1)$$

According to [Alexeeva et al 2002] the concentration of the oxygen for saturated samples under room temperature is 220 μM, with the help of this information and using the previously presented equation it is possible to determine the concentration of the oxygen in the samples and to represent the variation of the oxygen concentration in function of the line width variability.

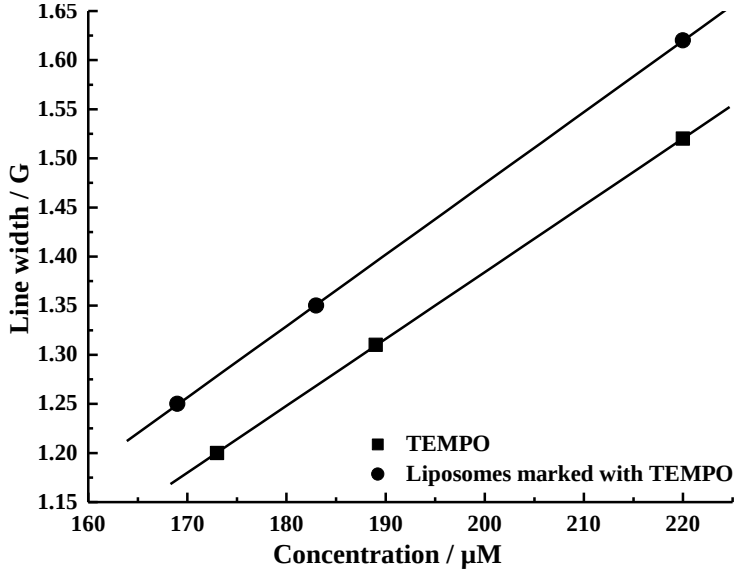

Figure 3.2.3. The variation of the line width according to the oxygen concentration changes. Image taken from [Takacs et al 2011a].

The changes in the EPR spectra due to the oxygen concentration variations in the solution is relative small but noticeable, as seen on the recorded spectra, it is clear that hyperfine line width can be influenced with the changes in the environment of the sample. As the resulted values clearly show the line width broadenings are proportional to the magnitude of the oxygen concentration and saturation found in the sample which has an influence also on the shape of the EPR spectra.

The oxygen concentration in the environment is one of the basic and most important variable in many of the physiological and biological processes and activities. Being able to detect it proportionalize it or even quantify it, represents a good option and an interesting method by using EPR spectroscopy in combination with nitroxide radicals.

3.3. Free radical detection by spin trapping

The results and the data presented in this subchapter were previously published in [Bolojan et al 2012].

In the process of cell metabolism it is possible that reactive oxygen species are formed and they are linked to human diseases [Hallivell 1997, Freeman 1982, Fantone 1985]. These free radical reactive oxygen species have a short lifetime, and therefore the best way of detecting them is indirectly by spin trapping. By using nitrone spin traps, the resulted adduct will have a higher stability and will be detectable by EPR spectroscopy [Spasojevic 2010]. The work [Bolojan et al 2012] presents the results obtained in the spin trapping study where DEPMPO (5-diethoxyphoshoryl-5-methyl-1-pyrroline-N-oxide) was used as trapping agent in order to monitor the superoxide radical generated from potassium superoxide in slightly alkaline buffers.

The main purpose of the potassium superoxide (KO_2) is the usage in respiratory equipments to produce oxygen from carbon dioxide and water vapor; it can be found in crystalline form and is soluble in DMSO. The potassium superoxide stock solution was obtained from 1 g of KO_2 in a volume of 1 ml of anhydrous dimethyl sulfoxide, DMSO, after a 5 minute mixing the excess potassium superoxide vas removed by centrifugation at 700 rpm. Three reactions were carried out in total according to previously described methods [Reiter et al 2000, Jia et al 2008]. The Reaction A was carried out by adding 1 µl KO_2 stock solution to 99 µL 50 mM PBS buffer containing 10 mM DEPMPO and 0.1 mM DTPA. The Reaction B was induced with 10 µL KO2 stock in 90 µl 50mM PBS buffer which contained 15 mM DEPMPO and 0.1 mM DTPA. The Reaction C was initiated with the addition of 20 µl KO_2 stock solution to 80 µl 50 mM PBS buffer containing additional 20 mM DEPMPO and 0.1 mM DTPA.

The superoxide O_2^- is generated after the potassium superoxide is dissolved in DMSO, after the addition of the dissolved potassium superoxide to the aqueous medium, the superoxide will form hydrogen bonds and according to [Afanas'ev 1989, Singh Evans 2006] will undergo to a disproportionation into HOO^- and HO^-.

$$2O_2^- + H_2O \rightarrow O_2 + HOO^- + HO^- \qquad (3.3.1)$$

These free radical superoxides are then confirmed with the presence of the DEPMPO spin trap in the solution, by EPR spectroscopic analysis because according to [Bacic et al 2008, Stolze et al 2000] the DEPMPO forms stable spin adducts and these oxygen radical species can be easily differentiated.

The simulation of the recorded EPR spectra was obtained with the software WINSIM 2002, the algorithm and the method of simulation is described by [Duling 1994]. The results of the simulation are summed up in the Table 3.3.1.

According to [Mojovic et al 2005, Ouari et al 2011] the values of the hyperfine constants obtained in the case of reaction A identifies the DEPMPO/OH spin adduct in two conformers 15 % respectively 85 %, and point to the dominance of the OH radicals in this case. In case of reaction B the DEPMPO/OH is identified in 83 % while the DEPMPO/OOH is found in 17 %. And in reaction C the

DEPMPO/OOH spin adduct is identified with two conformers 57 % and 35 % while the DEPMPO/OH spin adduct has a 7 % contribution to the spectrum. The hyperfine constant values, represented in the Table 3.3.1, determined by simulation are in great accordance with other studies in the literature [Frejaville et al 1995, Bacic et al 2008, Mojovic et al 2005].

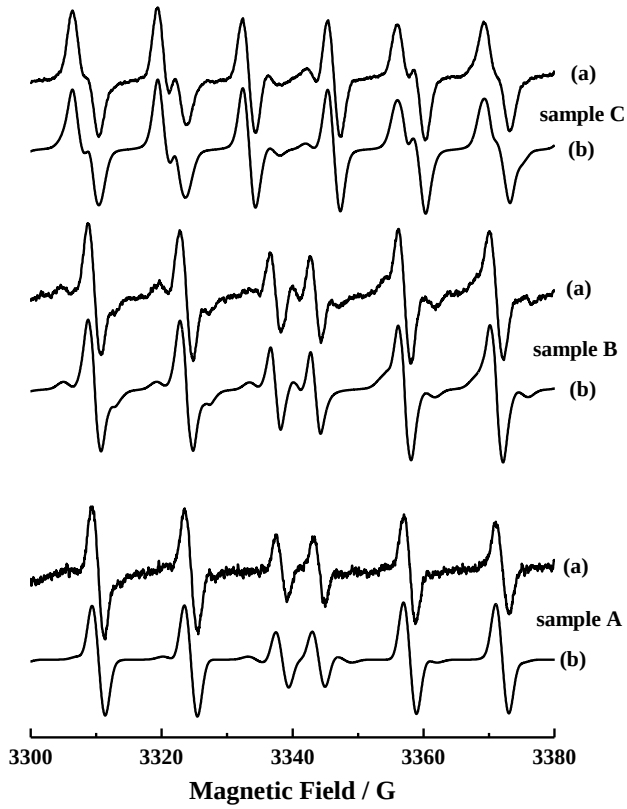

Figure 3.3.1. Representation of the experimental (a) and simulated (b) EPR spectra of the studied solutions. Figure taken from [Bolojan et al 2012].

Hyperfine (G)	DEPMPO/OH				DEPMPO/OOH			
	Sample A		Sample B	Sample C	Sample A	Sample B	Sample C	
	Conformer I	Conformer II					Conformer I	Conformer II
a_N	14.12	14.04	14.53	14.4	-	13.07	13.01	13.07
a_N^{β}	13.26	13.28	14.07	13.26	-	9.22	10.63	11.15
a_P	50.4	50.62	51.38	47.42	-	49.6	49.46	50.68
$a_H^{\gamma}(1H)$	0.89	0.96	1.07	1.01	-	0.91	0.87	0.96
$a_H^{\gamma}(6H)$	0.43	0.44	0.39	0.41	-	0.48	0.34	0.41

Table 3.3.1. The obtained hyperfine constants of DEPMPO-spin adducts for the three reactions. The values were taken from [Bolojan et al 2012].

The time evolution study of the three reactions showed a fast degradation, 10 minutes, of the spin adduct DEPMPO/OH in the reaction A, a more slowly decrease in the intensity of the B sample showing a more stability in the spin addicts for this reaction even measurable after 25 minutes. The C sample follows the DEPMPO/OOH type decay for 25 minutes after which the shape of the spectra takes the resemblance of a typical DEPMPO/OH spectrum.

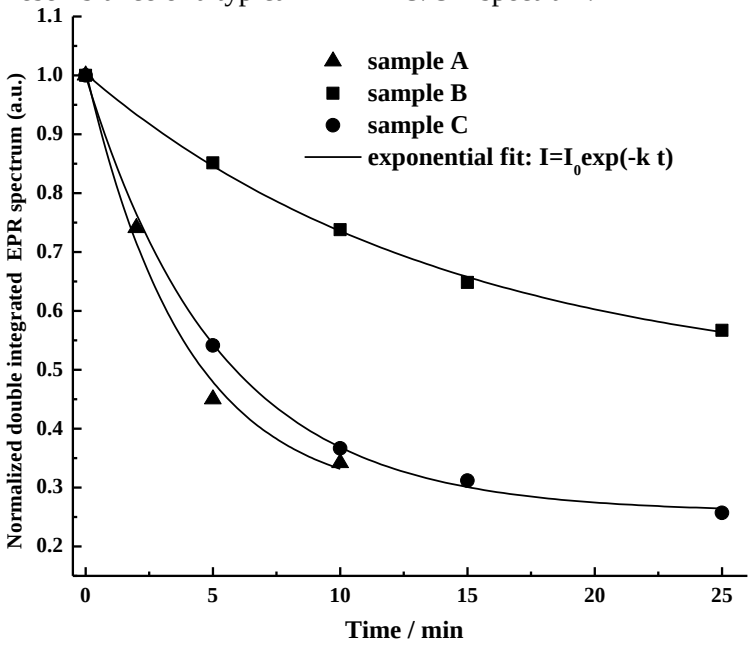

Figure 3.3.2. Representation of the obtained decay curves of the DEPMPO-superoxide spin adducts in the time evolution process. Figure taken from [Bolojan et al 2012].

The decay curves of the DEPMPO spin adducts are to be found in the Figure 3.3.2, the half-life time of the DEPMPO spin adducts were determined according to [Khan et al 2003, Bilsky et al 1996], for the reaction A a 2.7 min half-life time, for the reaction B the obtained value corresponds to a 10 min half-life time, while the reaction C has half-life timeof 3.6 min.

The spin adduct stability has a strong dependence to the concentration of the superoxide because of the interaction of the superoxide with the spin trap and the resulted reaction of the second superoxide with the spin adduct, pointing to that both, the decay and the formation, of the DEPMPO spin adducts have a dependence to the superoxide concentration.

According to [Samouilov et al 2004] the efficiency of the trapping process depends on the superoxide concentration, at low values the adduct formation rate is equal to the superoxide flux and at high values the adduct competes with the spin trap and decreases the spin trapping efficiency.

4. PROTEINS INVESTIGATED BY SITE DIRECTED SPIN LABELING

4.1. Materials and methods

The hemerythrin used in this study was extracted an purified according to the protocol described by [Mot et al 2010]. The hemoglobin used was extracted an purified by Dr. Cristina Bischin. The MTSSL spin label was dissolved in DMSO in a final stock of 100 mM.

The proteins were suspended in PBS buffer and prepared for spin labeling. The mixture of protein and DTT with a final concentration of 10 mM was incubated at 4°C and shaken for 2 hours in order to access the buried sites of the protein also. The DTT was washed from the system using 10 kDa Millipore filter in a Beckman J21B centrifuge. The sample was centrifuged 6 times at 4°C, 5000 rpm for an interval of 30 minutes each. After the washing and removal procedure of DTT was carried out, the MTS spin label stock solution was added to the sample in a 10 times excess to each cysteine and incubated overnight at 4°C. The excess spin label was removed by 10 kDa Millipore filters centrifugation at 5000 rpm at 4°C for 30 minutes for 5 times in total. In the following we will use the R1 abbreviation for the attached spin label [Czogalla et al 2007], so the resulting names are Hr51R1 and Hb92R1.

A spin labeling efficiency calculation was carried for both proteins and the results are the following 91 % for the spin labeled hemerythrin, 82 % for the hemerythrin where the DTT washing was omitted and 97 % for the spin labeled hemoglobin. The results and the spin labeling protocol was published at [Takacs et al 2013a]

The EPR measurements were carried out at room temperature with a continuous wave X-band (9 GHz), Bruker-Biospin EMXmicro EPR spectrometer from Bruker Biosciences Corporation equipped with a TE-102 resonator and a temperature controller.

The microwave frequency was set to 9.45 GHz, the used microwave power was set to 4 mW with a modulation frequency of 100 kHz, the modulation amplitude has been set to 1 G and the used microwave attenuation was 17 dB. The spin labeled protein samples were introduced in quartz capillary tubes with a final volume of 15 μL of each sample.

The obtained EPR spectra for the analyzed samples were subsequently simulated with the EPR tool program Multi-Component EPR Fitting v2 version 495, a LabVIEW software, developed by Dr. Christian Altenbach from University of California, Los Angeles, California (https://sites.google.com/site/altenbach/labview-programs/epr-programs/multicomponent). For the EPR spectra representations and calculations the software OriginPro 8.0 was used.

4.2. The librational motion of hemerythrin and hemoglobin

The EPR spectrum of the spin labeled hemerythrin (Hr51R1) is represented on the Figure 4.2.1.a, along with the afferent simulation spectra while the recorded and simulated spectra of the spin labeled hemoglobin can be found on the Figure 4.2.1.b. As previously mentioned the hemerythrin has an environment-sensitive conformation, mainly consists of eight identical monomers but it could also exist in other conformational states as a monomeric form [Langeman et al 1969] or in an intermediate conformation, a dimmeric [Sieker et al 1981], trimmeric [Smith et al 1983] or a tetrameric form [Sieker et al 1981]. This fact that the hemerythrin exist in more than one conformational state was confirmed by the simulation data. The EPR spectrum of the spin labeled hemerythrin was simulated with three components while the EPR spectrum of the spin labeled hemoglobin was simulated with two components. The resulted EPR parameters are to be found in Table 4.2.1 for the hemerythrin and in Table 4.2.2 for the hemoglobin, also these results are published at [Takacs et al 2013a].

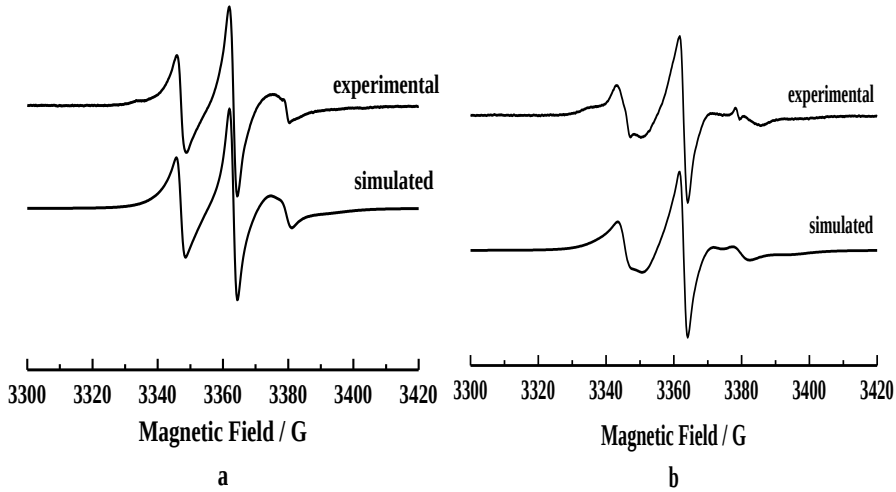

Figure 4.2.1.a. EPR spectra and simulated specra of the spin labeled hemerythrin. **b.** EPR spectra and simulated spectra of the spin labeled hemoglobin.

The three components used in the simulation of the spin labeled hemerythrin can be interpreted according to the possible conformations. The fast component corresponds to the monomeric structure, the slow component to the native octameric structure while the intermediate component is a mixture of a dimmeric, trimmeric and tetrameric conformations.

The spin labeled proteins were analyzed in a variety of controlled environments in order to see the behavior of the proteins [Takacs et al 2013b, Takacs et al 2013c]. A 0 – 85 % of glycerol was used as an agent with viscosity properties but small molecule with no interaction with the protein, 0 – 45 % PEG-4000 was uses as agent with viscosity properties but as a large molecule causing various interactions with the studied proteins. And finally BSA was used in a ratio of 0 – 200 mg/ml in order to see the interaction of the sample with other proteins in the environment and the crowding effect described by [Ellis 2001].

The spectral analysis led to the following changes in the dynamic and structure of the spin labeled hemerythrin in the presence of glycerol, the slow component has an increase of fraction from 68.34 % to 84.03 % while its rotational correlation time changes from 4.26 ns to 25.6 ns, the intermediate component has a fraction change from 22.57 % to 14.82 % with a mobility change from 3.5 ns to 10.1 ns while the monomeric conformation experiences a fraction change of 9.09 % to 1.15 % with a change in its rotational correlation time from 1.16 ns to 4.06 ns. In the presence of PEG-4000 the octameric conformation has a fraction change from 68.34 % to 86.02 % while the rotational correlation time change from 4.26 ns to 18.5 ns, the intermediate conformation shifts from 22.57 % to 13.98 % while its mobility changes from 3.5 ns to 9.19 ns while the fast component has a change in fraction from 9.09 % to 2.4 % and a mobility variation from 1.16 ns to 3.45 ns. In the presence of BSA the octameric structure has a change in fraction from 68.34 % to 59.23 % with a rotational correlation time change from 4.26 ns to 7.54 ns; the intermediate component changes in fraction from 22.57 % to 21.27 % and it correlation time from 3.5 ns to 4.28 ns, while the monomeric conformation changes from 9.09 % to 19.05 % and a mobility is varied by 1.16 ns to 2.16 ns.

Hr51R1	**Comp. fraction (%)**	**Rotational correlation time, τ_c (ns)**	**Hyperfine tensor A values**			**g- tensor values**		
			A_x	**A_y**	**A_z**	**g_x**	**g_y**	**g_z**
Comp. 1	68.34	4.26	7.35	7.65	33.02	2.0087	2.0067	2.0032
Comp. 2	22.57	3.5	8.25	8.55	32.05	2.0085	2.0065	2.0045
Comp. 3	9.09	1.16	8.55	8.85	31.56	2.0085	2.0065	2.0041

Table 4.2.1. EPR parameters obtained by simulation for the spin labeled hemerythrin.

Hb92R1	**Comp. fraction (%)**	**Rotational correlation time, τ_c (ns)**	**Hyperfine tensor A values**			**g- tensor values**		
			A_{xx}	**A_{yy}**	**A_{zz}**	**g_{xx}**	**g_{yy}**	**g_{zz}**
Comp. 1	21.21	3	9.83	10.13	33.68	2.00704	2.00504	2.00393
Comp. 2	78.79	4.73	5.9	6.2	34.76	2.00802	2.00602	2.00229

Table 4.2.2. EPR parameters obtained from simulation for the spin labeled hemoglobin.

In case of the spin labeled hemoglobin the following are the obtained results, in the presence of glycerol the first component has a drop in fraction from 21.21 % to 13.59 % while its rotational correlation time changes from 3 ns to 6.15 ns and the second component fraction changes from 78.79% to 86.41% with a shift in mobility from 4.73 ns to 9.45 ns. The protein in the presence of PEG-400 showed the following modifications in fraction for component 1 a change from 21.21 % to 19.63 % with a mobility change from 3 ns to 6.44 ns while the second component fraction changed from 78.79 % to 80.37 % and its mobility from 4.73 ns to 8.61 ns. The presence of albumin in the sample showed the following modifications a change in the component fraction from 21.21 % to 18.09 % with a rotational correlation time change from 3 ns to 3.56 ns while the second component was affected with a change from 78.79 % to 81.91 % and a 4.73 ns to 5.34 ns change in mobility.

4.3. Denaturation study of hemerythrin and hemoglobin

The term denaturation of proteins refers to the disruption and destruction of the secondary and tertiary structures in a protein conformation, this subchapter presents the obtained results in a denaturation study of the two spin labeled proteins hemerythrin and hemoglobin using EPR spectroscopy. With two approaches the determination of important thermodynamic parameters was possible, the Gibbs free energy in the absence of denaturant, the enthalpy, entropy and the heat capacity of the proteins. These studies are based on the study of denaturation curves, which are determined from a dependent variable plotted against the denaturant [Klug et al 1995, Klug et al 1998]. Two methods were used in order to determine the denaturation curves the firs method uses the double integrated spectral area difference of the various states compared to the native state. The second method uses the average rotational correlation time, with the consideration of the spectral component fractions, as dependent variable in order to form the denaturation curves. The denaturation curves obtained this way by fitting of a Boltzmann sigmoid curve to the individual points, f_D, representing this way the denatured fraction of the protein on a 0 to 1 scale in function of the denaturant.

The proteins were denatured using chemical method by guanidine chloride in a concentration of 0 – 5 M, after the denaturation curves are determined by using equations 4.3.1 – 4.3.2 the Gibbs free energy in the absence of denaturant can be obtained.

$$K_{eq} = \frac{f_D}{1 - f_D} \tag{4.3.1}$$

$$\Delta G = -R \cdot T \cdot \ln K_{eq} \tag{4.3.2}$$

$$\Delta G = \Delta G^0 - m[\text{denaturant}] \tag{4.3.3}$$

By using the approach of [Pace 1986, Kellies et al 1989] the system is found at equilibria at a transition point, C_m, where the equilibrium parameter, K_{eq}, is 1 so the Gibbs free energy is 0, with the help of the rest of equation 4.3.3 the Gibbs free energy in the absence of denaturant can be obtained representing the stability of the protein. The data obtained for the spin labeled hemerythrin are represented in the Table 4.3.1 while the values obtained for the spin labeled hemoglobin are showed in the Table 4.3.2.

Hr51R1	**ΔG^0 (kcal·mol^{-1})**	**m (kcal·mol^{-1}·M^{-1})**	**C_m (M)**
Method 1	6.26	2.53	2.47
Method 2	5.74	2.47	2.33

Table 4.3.1. Thermodynamic parameters obtained from the spin labeled hemerythrin with the two described methods.

Hb92R1	**ΔG^0 (kcal·mol^{-1})**	**m (kcal·mol^{-1}·M^{-1})**	**C_m (M)**
Method 1	2.21	1.07	2.06
Method 2	2.68	1.28	2.08

Table 4.3.2. Thermodynamic parameters obtained from the spin labeled hemoglobin with the two described methods.

The thermal denaturation resulted denaturation curves, on the temperature interval of 20 – 60 °C, can be analyzed using the van't Hoff equations 4.3.4 – 4.3.5,

van't Hoff analysis, in order to determine the van't Hoff enthalpy and entropy at the transition point (T_m) where the system is found at equilibria in order to use these values further with the Gibbs free energy in the absence of denaturant to obtain the value for the heat capacity, enthalpy and entropy at room temperature using the [Becktel and Schellman 1987] approach with equations 4.3.6 – 4.3.8.

$$\Delta G = \Delta H - T \cdot \Delta S = -R \cdot T \cdot \ln K_{eq} \tag{4.3.4}$$

$$\ln K_{eq} = -\frac{\Delta H}{R \cdot T} + \frac{\Delta S}{R} \tag{4.3.5}$$

The obtained values from the van't Hoff analysis for the two spin labeled proteins are represented in the Table 4.3.3 in case of the hemerythrin and Table 4.3.4 in case of hemoglobin.

Hr51R1	**ΔH_{vHoff} (kcal·mol^{-1})**	**ΔH (kcal·mol^{-1}) 293K**	**ΔS_m (cal·K^{-1}·mol^{-1})**	**ΔS (cal·K^{-1}·mol^{-1}) 293K**	**T_m (K)**	**ΔCp (cal·K^{-1}·mol^{-1})**
Method 1	104.70	39.26	325.81	112.62	321.36	2307.23
Method 2	107.65	26.67	335.42	71.45	320.95	2897.17

Table 4.3.3. Results of the van't Hoff analysis and the rest of the obtained thermodynamic parameters for the spin labeled hemerythrin by the two methods.

Hr51R1	**ΔH_{vHoff} (kcal·mol^{-1})**	**ΔH (kcal·mol^{-1}) 293K**	**ΔS_m (cal·K^{-1}·mol^{-1})**	**ΔS (cal·K^{-1}·mol^{-1}) 293K**	**T_m (K)**	**ΔCp (cal·K^{-1}·mol^{-1})**
Method 1	33.46	13.18	103.23	37.47	324.11	651.72
Method 2	35.21	23.23	109.10	70.16	322.80	401.98

Table 4.3.4. Results of the van't Hoff analysis and the rest of the obtained thermodynamic parameters for the spin labeled hemoglobin by the two methods.

$$\Delta H = \Delta H^0 + \Delta C_p \cdot (T - T_0) \tag{4.3.6}$$

$$\Delta S = \Delta S^0 + \Delta C_p \cdot \ln \frac{T}{T_0} \tag{4.3.7}$$

$$\Delta G(T) = \Delta H^0 - T \cdot \Delta S^0 + \Delta C_p \cdot (T - T_0 - T \cdot \ln \frac{T}{T_0}) \tag{4.3.8}$$

The proteins were investigated using various buffers with different pH values. The acidic range of the pH scale was obtained using citrate buffer for pH 3, 4, 5, 6; the pH values 7, 7.2, 7.6 , 7.8 were carried out with phosphate buffer and the basic range of the pH, 8, 9, 10, 12 were carried out using borate buffered saline. According to the literature [Bhadresh and Jayant 2001] around the midpoint, pH_m, the logarithm of the equilibrium parameter has a linear dependency to the pH and a function of the protonation and deprotonation reactions with the environment.

$$\log K_{eq} = \Delta\nu \cdot (pH - pH_{ref}) \tag{4.3.9}$$

This $\Delta\upsilon$ parameter representing the number of proton exchange in the denaturation process can be determined using the equation 4.3.10 by [Ibarra-Molero et al 1999].

$$\Delta G(pH) = \Delta G^0 + (\ln 10) \cdot R \cdot T \cdot \Delta\nu \cdot (pH - pH_{ref}) \tag{4.3.10}$$

By using the two denaturation curves separate processes can be determined for the acidic and for the basic range of the pH scale. The results obtained for the

protonation an deprotonation for the spin labeled hemerythrin are to be found in Table 4.3.5 while for the spin labeled hemoglobin in the chemical denaturation in Table 4.3.6.

Δυ	Method 1	Method 2
Acidic Range	7.33	8.12
Basic Range	-1.80	-3.51

Table 4.3.5. Proton exchange in the pH denaturation process for the spin labeled hemerythrin.

Δυ	Method 1	Method 2
Acidic Range	0.857	1.44
Basic Range	-2.42	-2.39

Table 4.3.6. Proton exchange in the pH denaturation process for the spin labeled hemoglobin.

The results and procedures presented and defined in this chapter will be published in the article [Takacs et al 2013d].

SUMMARY AND CONCLUSIONS

EPR spectroscopy has proved to be a very important tool in analyzing biological systems, due to its sensitivity and accuracy. By using site directed spin labeling in combination with EPR spectroscopy in protein investigation we were able to monitor the dynamics of the spin labeled hemerythrin and hemoglobin in various environments. The results were as expected; the environmental changes enhance the native behavior of the protein and we were able to distinguish the various components and monitor their activity.

Nitroxide radicals and nitrones have a wide range of application, they can be used for direct detection were this is possible, in case of spin labeled proteins and also in indirect detection, trapped free radicals and molecular oxygen concentration monitoring, in systems where direct detection was not possible.

The spin labeling of the hemerythrin is a first of this study, and has the advantage to monitor this protein, which shows a great interest since it was recently proposed as an alternative to hemoglobin based blood substitutes [Alayash 2004, Tsuchida et al 2009, Mot et al 2010, Fischer-Fodor et al 2011], by a different approach in it native like environment.

The denaturation study of the two oxygen-transport responsible proteins resulted in a new alternative method of determining protein stability and thermodynamic parameters. Both studied proteins have similar biological functions and structure. Both of them bind oxygen and transports to the cells and both of them have an oligomeric structure. The hemoglobin has a stabile quaternary structure compared to the hemerythrin where other conformations can be found besides the octameric one. The hemerythrin protein represents a great study subject, has a relative large size almost double compared to the hemoglobin and has an interesting structure, but also can contain other conformational states than its native one.

The study of the two proteins in various environment represents an important part in the EPR study of proteins because helps us understand the reaction of the studied protein to the environmental changes, also helps us see the dynamic of the protein behavior. Understand the motion of the protein in a theoretical environment where only the solutions viscosity is controlled and in actual biological environment where the protein collides with other macromolecules.

The denaturation study of these proteins is what completes the picture, with the help of this part we can understand how easily a protein can be disrupted from its function and influenced to undergo to structural changes which are often irreversible. In the denaturation study be approached the subject by two specific methods. Both methods are specific to EPR spectroscopy, Method 1 and Method 2, resulted values which are in the same range and relatively close to each other.

The denaturation curves obtained by using the spectral area remnant [Klug et al 1995, Klug et al 1998] and the average correlation time were similar to each other having only minor differences. These suggest that both approaches are correct and can be used in order to one determine the stability of a protein or to predict the outcome of a denaturation performed on a protein.

Although Method 1 seems easier to perform on a protein denaturation study, in reality needs more accurate work, the amount of the measured sample has

to be precise and identical for the titration process. Method 2 requires spectral simulation in order to obtain the required dependent variable, correlation time, which is used later.

The obtained values from the denaturation study are in agreement with the data from the literature proving that the used approaches are correct and applicable in the future. The thermodynamic parameters discussed in the previous chapters are highly protein and process specific, they are mostly dependent on the size and structure of the protein and also the number of residues found in its sequence. There is a large scale available for the enthalpy which can be placed between 10 – 150 $kcal \cdot mol^{-1}$. The entropy can be placed in the interval of 50 – 500 $cal \cdot mol^{-1}$. The heat capacity has a high dependency to the difference between the unfolded and folded accessible surface area and can take values between 0.2 – 4 $kcal \cdot mol^{-1}$. The Gibbs free energy of unfolding in the absence of denaturant is a parameter that quantifies the stability of the protein and also represents the energy needed in order to induce a complete denaturation it can vary on proteins from 1 – 20 $kcal \cdot mol^{-1}$.

The examples from the literature are in agreement with the thermodynamic parameters determined in this work, the parameters determined for various versions of human hemoglobin by [Ip and Ackers 1977, Valdes and Ackers 1977a, Valdes and Ackers 1977b] for the enthalpy 23.5 – 24.5 $kcal \cdot mol^{-1}$, and for the entropy 40.4 – 46.1 $cal \cdot mol^{-1}$ are close to the values obtained for the bovine hemoglobin. Although the Gibbs free energy in the absence of denaturant for bovine hemoglobin was slightly lower than 3.6 – 5.1 $kcal \cdot mol^{-1}$ found for human hemoglobin by [Haynie 2001], the values are still in an acceptable range taking in consideration the large number of factors and protein mutations that can be an influence in this case.

The enthalpy determined by [Pace et al 1998] for various mutants of ribonuclease is found in the range of 68 – 126 $kcal \cdot mol^{-1}$, its entropy in the range of 218 – 388 $cal \cdot mol^{-1}$, the heat capacity of various mutants of this protein was placed in the range of 1.3 – 1.9 $kcal \cdot mol^{-1}$ and the Gibbs free energy in native state of these mutants was placed between 2.8 – 9.3 $kcal \cdot mol^{-1}$. The results presented in [Klug et al 1998] show a variation of the Gibbs free energy in the absence of denaturant between 2.28 – 9.79 $kcal \cdot mol^{-1}$,for various mutants of the transmembrane β – strand located in the ferric enterobactin receptor, FepA.

The results obtained from [Santoro and Bolen 1992] in the equilibrium unfolding study of the oxidized form of Escherichia coli thioredoxin resulted in an unfolding Gibbs free energy in the absence of denaturant of 7.8 – 8 $kcal \cdot mol^{-1}$ when guanidinium chloride was used as denaturant and 8.6 – 9.5 $kcal \cdot mol^{-1}$ when urea was used as denaturant, also from thermal denaturation the resulted value was 8.1 – 8.2 $kcal \cdot mol^{-1}$.

According to [BinSheng 2011, Halim et al 2008, Kumar et al 2005, Greene and Pace 1974] β – lactoglobulin, composed of 101 amino acid residues, has a Gibbs free energy in the absence of denaturant of 11.7 – 12.5 $kcal \cdot mol^{-1}$, higher then the larger sized α – chymotrypsin, composed of 178 amino acid residues with only 8.3 – 8.7 $kcal \cdot mol^{-1}$, also for the human serum albumin (HSU), a protein with a size of 67 kDa and 585 amino acid residues, to be between 5.98 – 6 $kcal \cdot mol^{-1}$, and for bovine serum albumin only 4.04 – 4.6 $kcal \cdot mol^{-1}$.

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LIST OF PUBLICATIONS

A. ISI cited journals:

1. **Takacs IM**, Mot A, Silaghi-Dumitrescu R, Damian G, Site Directed Spin Labeling of Hemerythtin and Hemoglobin, Studia UBB Chemia, 2013, LVIII, 2:61-69.
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B. CNCS B+ cited journals:

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C. Conference participation:

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