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# THE HEMOGLOBIN DEFORMATION INDUCED BY NEAR INFRARED RADIATION – SPIN LABEL STUDY

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**The aim of presented study was to monitor the structural changes of hemoglobin induced by NIR radiation. We investigated the reaction between 4-isothiocyanato-2,2,6,6-tetramethylpiperdinooxyl spin label and hemoglobin sites: cysteines and N terminal groups by means of EPR spectroscopy. The reactivity of mentioned groups depends on the protein structure. We observed lower reactivity of N terminal sites for 15 and 30 min exposition to NIR. The accessibility of the hemoglobin's cysteine increases with irradiation time. Analysis of nitrogen hyperfine splitting indicates decreasing polarity in environment of both sites for samples 15 min. exposed to NIR.**

## INTRODUCTION

Isothiocyanates are used in protein chemistry as reagents for the determination of primary structure of peptides and proteins (Britto, Knipling, & Wolff, 2002, Ramachandran, Rami, & Udgaonkar, 2000). These substances with antimicrobial and cytotoxic effect are also known as outstanding SH reagents (Wang & Ashby, 2008). Electron spin resonance (ESR) analysis of spin labeled human hemoglobin with 4-isothiocanato-2,2,6,6-tetramethylpiperdinooxyl indicated a partially resolved five-line spectrum, suggesting that the label was attached to at least two different binding sites. One binding site was attributed to the sulfhydryl group of cysteine (immobile) and the α-amino group of the N-terminal valines (mobile) (Giagrande & Kim, 1975, Wajnberg *et al,* 1978). For each molecule of hemoglobin, there are four N-terminal ends and two reactive sulfhydryl groups (Manoharan, Wang, Alston, & Rifkind,1990). These features make isothiocyanate a convenient probe for analysis of the local environment of the spin labeled hemoglobin and its changes during processes due to physical factors like near infrared radiation (NIR). The intend of presented study was to monitor the structural changes of hemoglobin induced by NIR radiation. In this study we investigated the reaction of isothiocyanate nitroxide spin label with SH and NH groups of hemoglobin by means of EPR spectroscopy.

## MATERIALS AND METHODS

### *Erythrocytes*

All experiments were carried out on human erythrocytes taken from Regional Center of Blood Donation and Blood Therapy in Wrocław, not older than 8 days mainly of 0 Rh+ blood group but also with A Rh+ and B Rh+. The erythrocytes were isolated by centrifugation at 1750x*g* for 4 min. at 4°C. Erythrocytes, washed three times in phosphate buffered saline (PBS), pH=7.4, were diluted to obtain 10% hematocrit in PBS. The PBS isotonic solution contained (in mM) 0.5$MgCl_2$, 1.2 $CaCl_2$, 1.7 $Na_2HPO_4$, 4.2 $NaH_2PO_4$, 4.4 KCl, 13.5 $Na_2CO_3$, and 117.8 NaCl

### *Irradiation procedure*

Erythrocyte suspension in PBS, pH=7.4 (hematocrit 10%) were exposed to the radiation of halogen lamp equipped with a 720-2000 nm filter. The power density incident light was 6.9 $Wm^{-2}$. During exposure, the suspension was gently stirred and cooled. Irradiation temperature was kept constant 293±2 K by means an air- and water-cooling system. Samples were irradiated for 15, 30, and 60 min. The control cells were similarly processed except irradiation (kept at 293±2K in darkness).

*Preparation of hemolysate*

After exposition to NIR, erythrocytes were isolated by centrifugation at 1750x*g*, supernatant was removed and then the hemolysate was obtained by centrifugation for 30 min at 20000x*g* ($8^0$C). After dilution the final concentration of hemoglobin was $6.4 \pm 0.8$ g/100cm$^3$ (Drabkin' s method) in all experiments.

*Spin labeling*

The spin label used in this study was 4-Isothiocyanato-2,2,6,6-tetramethylpiperidine 1-oxyl (ITC-Tempo), chemical formula is presented on the Fig.1. The 2.5 µl of spin label ($10^{-2}$ M) was added to 50 µl of hemolysate ($6.4 \pm 0.8$ g/100cm$^3$ ) and EPR measurements were performed as quickly as possible.

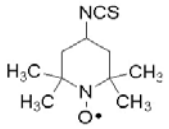


Fig.1. Chemical formula of 4-Isothiocyanato-2,2,6,6-tetramethylpiperidine 1-oxyl (ITC-Tempo).

EPR spectrum of ITC-Tempo bonded to the hemoglobin is presented on Fig. 2. Nitroxides attached to the SH are less mobile than those attached to the $NH_2$ site. The signal intensity of mobile and less mobile fraction (see Fig.2) was measured as a function of time. The signal was registered automatically every 1 minute for approximately 1h. Time t=0 was a moment when spin label was added. All spectra were recorded at $27^0$C using a standard SE/X-28 ESR spectrometer operating in the X-band (manufactured by the Wroclaw University of Technology). under the following experimental conditions: modulation amplitude 0.1 mT, time constant 0.01 s, sweep width 10 mT, and sweep time 64 s. The hyperfine coupling constant ($2A_{\|}$) and intensity of spin label fraction attached to SH and $NH_2$ site (see Fig. 2) were calculated from EPR spectra.

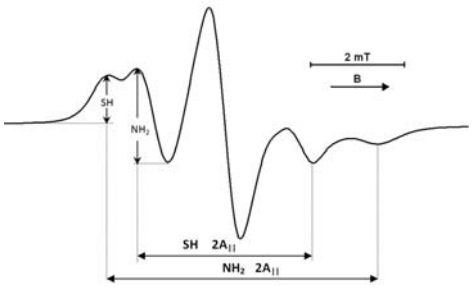


Fig.2. EPR Spectrum of 4-Isothiocyanato-2,2,6,6-tetramethylpiperidine 1-oxyl (ITC-Tempo) bonded to hemoglobin. Arrows show calculated intensity of spin label reacting with SH and $NH_2$ groups.

## RESULTS AND DISCUSION

Results of our study are presented in the Table 1 and Figs.3-4. In buffered solutions at pH 4-6, isothiocyanates react primarily with the deprotonated SH groups of cysteines (Podhradsky, Drobnica, & Kristian, 1979). Hemoglobins contain a cysteine residue at position 93 on the β chain. ITC-Tempo spin labels located on the (SH) β-93 are less mobile than those on the N-terminal end. Nitrogen hyperfine splitting ($2A_{\|}$) for the immobile site is 6.4 mT but for mobile $2A_{\|}$ equals 3.8 m (Giagrande & Kim, 1975, Wajnberg *et al*, 1978, Buehler, Haney, Gulati, Ma, & Hsia, 2004). The values of $2A_{\|}$ presented in the Table 1 show that spin label is bond to both mentioned sites: SH group of cysteine and $NH_2$ terminal group of the protein. The hyperfine coupling constant for products of reaction depends on the irradiation time and the reaction time. For 15 min. exposition time to NIR, $2A_{\|}$ for mobile ($NH_2$) and immobile (SH) fraction reached the minimal value during the first 5 minutes of reaction. The width of spin label spectra bonded to the $NH_2$ groups ($2A_{\|}$) increases with the increasing reaction time to the value 3.84 mT independently on the radiation time.

Table 1. Values of hyperfine coupling constant $2A_{\|}$ calculated from EPR spectra for spin label bonded to NH and SH groups at the beginning (after 5 min.) and after 1h. reaction time. SD (standard deviation) = 0.01mT.

| sample | —$NH_2$ $2A_{\|}$ 5 min reaction mT | —$NH_2$ $2A_{\|}$ 60 min reaction mT | —SH $2A_{\|}$ 5 min reaction mT | —SH $2A_{\|}$ 60 min reaction mT |
|---|---|---|---|---|
| Hb control | 3.80 | 3.84 | 5.59 | 5.57 |
| 15 min irr. | 3.76 | 3.84 | 5.53 | 5.60 |
| 30 min irr. | 3.82 | 3.85 | 5.69 | 5.69 |
| 60 min irr | 3.80 | 3.85 | 5.80 | 5.80 |

When N- terminal of hemoglobin is spin labeled its lengths increases by approximately 5Å (Manoharan, *et al*, 1990). The polar nitroxide radical becomes a proton acceptor for hydrogen bonding, resulting in interactions

of elongated terminal with other side chains. The increasing width ($2A_{\|}$) of spectra illustrates that effect. After 60 min. labeling the values are slightly higher compare to the first 5 min. of reaction (see table 1), what can be explained as a growing polarity of the label environment and formation of hydrogen bonds.

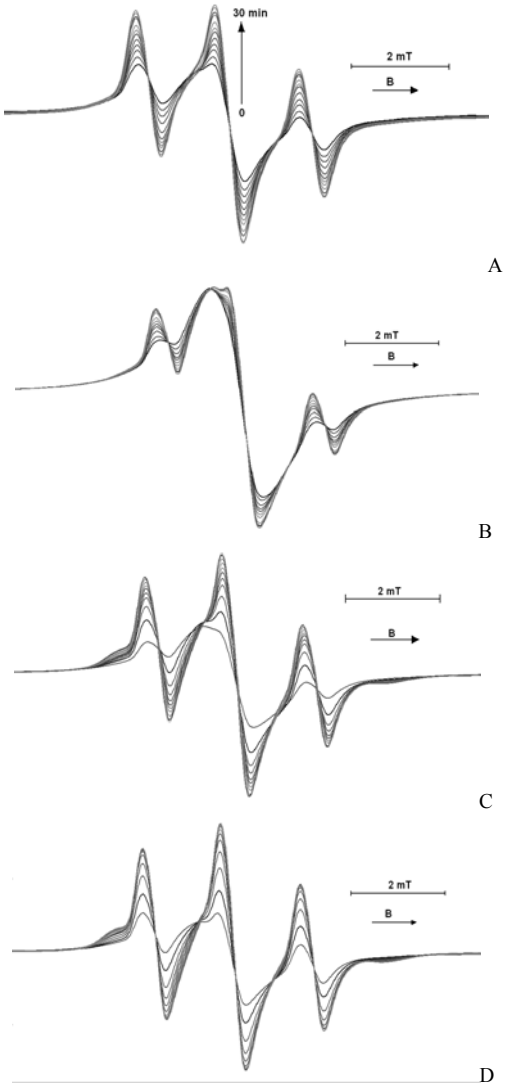


Fig.3. EPR spectra of ITC-Tempo spin label monitored during reaction with hemoglobin: A – unirradiated, B – 5 min. exposition to NIR, C – 30 min exposition, D – 60 min exposition. Spectra were registered during 30 min, every 1 minute.

The rapid decrease of the $2A_{\|}$ parameter for 15 min. irradiation for both binding sites indicates decreasing polarity at the beginning of the reaction but only for short irradiation time. 15 min. exposition time to NIR markedly influences on the protein structure. The 2A|| parameter for SH bonded spin label increased with increasing irradiation time and values are independent on the reaction time (see Table 1), excluding 15 min. irradiated sample during the first 5 minutes of reaction.

Analysis of the hyperfine coupling constants indicates that NIR effectively modifies polarity of the neighborhood of the both sites but its changes are dependent on the irradiation time only for SH site.

Considerable difficulty exists in the interpretation of the accessibility of the cysteine and $NH_2$ residues of hemoglobin. The signal intensity of spin labeled sites and the rate of bonding is a measure of the sites accessibility. All modified by NIR, including the control samples, cysteine residues reacted very rapidly during the first 3 minutes of reaction (the signal intensity of SH bonded spin label strongly increases). After that, control sample reached the equilibrium state, while the signal intensity for NIR modified samples linearly increases with the reaction time (see Figs. 3 and 4).

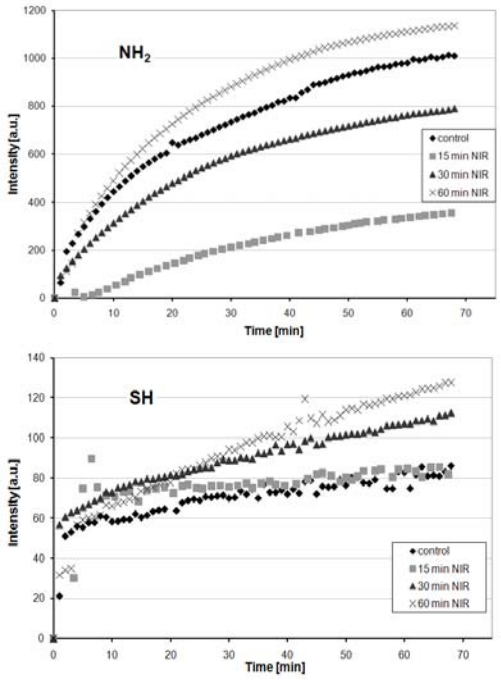


Fig.4. EPR signal intensity measured as a function of a reaction time. Diamonds-control sample; squares-5 min. irradiated; triangles-30 min.irradiated; crosses-60 min.irradiated.

The signal intensity of N terminal bonded spin label increases in logarithmic manner with a reaction time for control, 30 and 60 min. irradiated samples. After 1h reaction signal intensity reached 78% and 113% of control samples intensity in the order: 30 min and 60 min irradiated samples. Behavior of 15 min. irradiated sample strongly differentiate from remaining. During the first 5 minutes of reaction N terminal groups did not react. Later, reactivity of mentioned groups slowly

increases (intensity of EPR signal increased) with the reaction time but after 1h signal reached only 35% of control sample signal (see Fig. 5).

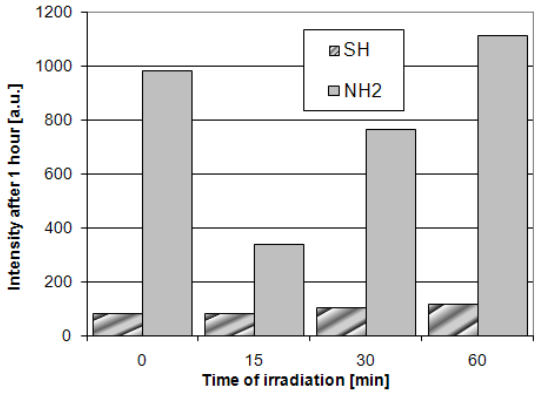


Fig.5. Signal intensity of spin label attached to SH and $NH_2$ sites as a function of irradiation time (dose of NIR radiation)

The signal intensity of spin label attached to SH groups, monitored after 1h reaction, slightly increased with increasing radiation time. In contradiction, signal intensity of spin labeled $NH_2$ sites dropped to 35% of the control sample signal and increased to higher values for longer exposition to NIR (see Fig. 5). In this study we showed that NIR modified both sites, however N terminal ends are more sensitive to NIR than cysteine residues. The hemolysate was not dialysed thus solution contained two substrates which reacted with ITC - Tempo spin label: hemoglobin and glutathione (GSH). The EPR signal increase of the weakly immobilized spin label is attributed to both components. It is known from literature (Kao, &Sheen 2003) that NIR radiation induced a significant increase in GSH concentration and GSH reductase activity but for cells 48 hours incubated after exposition to NIR. Our measurements were done immediately after NIR exposition what means that the signal increase coming from GSH is stable and independent on the NIR radiation. Noticed differences in the reactivity of spin label with hemolysate are mainly due to structural changes of hemoglobin.

One of the two hemoglobin cysteines is probably outside exposed and accessible for spin label for unmodified by NIR protein, while the second deeper buried is not reactive. Changing by NIR protein structure slightly increases reactivity of the inactive cysteine in the dose dependent manner.

Cysteine's reactivity has long been known to be altered by the conformational state of the hemoglobin (Giagrande & Kim, 1975, Wajnberg *et al,* 1978, Manoharan, *et al*, 1990). In the R (relaxed, oxygenated) structure, the cysteine faces inward toward the heme moiety and is positioned between a histidine and an aspartate residue. However, in the T (tense, deoxygenated) structure, this cysteine is rotated outward toward the salt bridge and away from the heme. In this structure, the positioning between the histidine and aspartate residues is lost (Gow, 2006).

In our previous papers, we studied effects (in vitro) of NIR on bovine and human erythrocytes. After red cells exposition to NIR the concentration of deoxyhemoglobin increased by about 30% (Komorowska, Cuissot, Czarnołęski,& Białas, 2002). Our data presented in this paper confirmed previous work where we discussed possible molecular mechanism of hemoglobin transition from R to T state.

The transition of the oxyhemoglobin to the deoxy-state could be induced probably by dehydration of the protein followed to NIR radiation because the energy of dehydration, estimated as 0.8 kJ/mol is comparable with NIR energy (Komorowska *et all* 2002). Decreasing polarity is a sign that the first stage of NIR radiation is partially dehydration of protein and after that a secondary processes are possible.

## ACKNOWLEDGMENTS

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