

# Interactions of $\beta$ -Carotene with Red Blood Cells: Its Regulatory Role on Hemoglobin Functioning

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Carotenoids are structurally and functionally a very diverse group of natural pigments. They are produced exclusively by organisms capable of photosynthesis and serve as important exogenous antioxidants for all living organisms. One of the most widespread carotenoids is  $\beta$ -carotene. *In vitro* studies demonstrated its ability to integrate with red blood cells' membranes affecting their physical and functional properties. Here, the results of experiments conducted on isolated red blood cells treated with  $\beta$ -carotene at concentration  $\approx 10^7$   $\beta$ -carotene/cell are presented. In particular, the Mössbauer spectroscopy was used to monitor hemoglobin states and its ability to reversibly bind oxygen in red blood cells incubated in the presence of  $\beta$ -carotene. The results showed that even at concentrations slightly above the physiological level (in plasma:  $10^4$ – $10^5$   $\beta$ -carotene/cell)  $\beta$ -carotene may affect not only the morphometric parameters of red blood cells but also modulate hemoglobin-oxygen affinity.

topics:  $\beta$ -carotene, heme-iron, hemoglobin, red blood cells

## 1. Introduction

Typical red blood cells (RBCs) from healthy humans have biconcave discoid shapes. They contain a globular oxygen-carrying protein called hemoglobin (Hb). The main form of Hb present in adult human RBCs consists of four subunits ( $\alpha_2\beta_2$ ), each composed of one polypeptide chain and one heme group. The prosthetic group is formed by protoporphyrin IX coordinating via its nitrogen atoms an iron ion which can bind an oxygen molecule. The heme-iron (HFe) is additionally bound to two histidine residues of the Hb subunit in the physiological deoxygenated hemoglobin (deoxyHb). In the case of oxygenated hemoglobin (oxyHb),  $O_2$  is the sixth ligand. The main functions of Hb, the transport of  $O_2$  and  $CO_2$ , buffering of  $H^+$  ions and NO metabolism, are already well-established. Hemoglobin is an allosteric protein: binding of one  $O_2$  to one HFe increases the oxygen affinity within the remaining heme groups. Such a cooperativity results in a significant reorganization of its quaternary conformation. There are various allosteric modulators that affect HFe- $O_2$  binding equilibrium. The most apparent are pH,  $pCO_2$  and 2,3-diphosphoglycerate. Likewise, other effectors such as signaling molecules or some other

types of natural and synthetic compounds, also used as drugs, are capable to shift equilibrium toward either high or low Hb- $O_2$  affinity [1].

The RBCs have a specific membrane skeleton which contains about 20 major proteins and  $\approx 850$  minor ones [2]. The main function of skeleton proteins (spectrin, actin, ankyrin, protein 4.1R and many others) is to strengthen the lipid bilayer and provide flexibility in response to RBCs deformation [3]. Any changes in the physical and chemical properties of the membrane skeleton may affect RBCs functioning and, in extreme cases, even induce occurrence of clinical symptoms [4].

Beta-carotene ( $\beta$ -Crt) belongs to carotenoids, natural pigments of polyene type highly diverse with respect to their structure and function [5]. They are synthesized by all types of phototrophic organisms, whereas humans acquire them exclusively from food [6]. From about 700 already known carotenoids, less than 30 including  $\beta$ -Crt have been found in human blood samples. Beta-carotene (see Fig. 1), as a non-polar compound, is known to be easily incorporated into cell membrane [7, 8]. *In vitro* study revealed its capability of integrating into RBCs membranes' outer layer [9, 10], or acting as an inhibitor of Hb oxidation induced by external peroxyl radicals' generators [11].

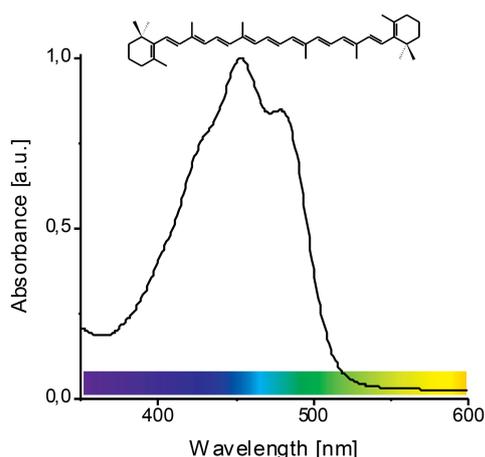


Fig. 1. Chemical structure of  $\beta$ -carotene and its absorption spectrum in acetone.

These discoveries inspired us to undertake research on the effect of  $\beta$ -Crt on the functioning of RBCs and possible changes of physiological forms of Hb that may influence the  $O_2$  dissociation curve. In the course of the study, UV-VIS absorption and the Mössbauer spectroscopies were applied. The latter one is a unique tool which allows to determine the valence and spin states of Fe-ions and any structural changes within its first coordination sphere (conformational and ligand changes) [12–15]. In this way, one may distinguish different forms of Hb inside RBCs. The osmotic stability of RBCs untreated and treated with  $\beta$ -Crt was investigated using UV-VIS absorption spectroscopy.

## 2. Materials and methods

All procedures described in the manuscript that involved the use of human blood were conducted in accordance with the recommendations of the World Medical Association and approved by the local Bioethical Commission of Physicians (317/KBL/OIL/2019).

### 2.1. Sample preparation

Blood samples were obtained from three healthy 25–35 years old male donors at the John Paul II Hospital in Kraków. After receiving their written consent to the experimental protocol, fasting blood (5 ml) was collected by vein puncture to tubes containing sodium heparinate as an anticoagulant. Red blood cells were isolated and purified as described previously [16]. In each case, RBCs concentration was established to be about  $1.65 \times 10^9$  cells/ml. The suspensions were divided into two equal parts: the control samples containing untreated RBCs and RBC<sub>100 $\beta$ -Crt</sub> containing RBCs incubated in the presence of  $\beta$ -Crt ( $\approx 3.5 \times 10^7$   $\beta$ -Crt/cell). Beta-carotene (95% all-*trans*, Sigma) was solubilized in ethanol and titrated to RBCs to get its final concentration of 100  $\mu$ M. The samples were incubated

for 10 min at room temperature in the dark. Subsequently, each of them was further divided into two parts in order to check their osmotic stability and Hb affinity to  $O_2$ .

### 2.2. Sample characterization: RBCs size, shape, and osmotic stability

The size and shape of RBCs were determined using an optical microscope (Olympus IX71, Japan). One hundred randomly selected control RBCs and those subjected to  $\beta$ -Crt treatment were analyzed.

RBCs osmotic fragility was assessed by the application of a series of NaCl solutions (0.9%–0%) as described [17]. The hemolysis rate was determined based on absorption spectra (Cary50Bio spectrophotometer, Varian) of the respective supernatants measured in the range of 460–700 nm and analyzed using the combination of exponential and Gaussian functions. Normalized curves were further fitted with a basic Boltzmann function (OriginPro 2019b).

### 2.3. Mössbauer spectroscopy

The Mössbauer spectroscopy was applied to investigate the impact of  $\beta$ -Crt on Hb ability to rebind  $O_2$  in RBCs untreated and treated with  $\beta$ -Crt. After dark incubation, the samples were washed thrice with phosphate buffer (5 mM, pH 7.4, 0.15 mM NaCl). After that, all samples were concentrated to  $\approx 8.9 \times 10^{10}$  RBC/ml, frozen down with liquid nitrogen and stored at  $-80^\circ\text{C}$  until measurement [18]. The measurements were performed at  $85 \pm 0.1$  K in a home-made cryostat enabling gas exchange. A source of  $\gamma$ -radiation (14.4 keV) was 50 mCi  $^{57}\text{Co}$ (Rh). In each case, the spectra were recorded every 30 min during 24 h of a total measurement and analyzed with Recoil software [19].

## 3. Results and discussion

### 3.1. Morphometric analysis

Healthy human RBCs are biconcave disks with flexible membranes that facilitate deformations of RBCs during passing through vessels and narrow capillaries. Typically, they are 6–8  $\mu$ m in diameter and  $\approx 2$   $\mu$ m in thickness. In the course of the present study, the average values of normalized diameter  $d$  and longitudinal to lateral ratio  $k$  of control RBCs were estimated to be, respectively,  $6.21 \pm 0.04$   $\mu$ m and  $1.09 \pm 0.01$ . Incubation in the presence of 100  $\mu$ M  $\beta$ -Crt led to a decrease in RBCs size ( $d = 5.96 \pm 0.03$   $\mu$ m) together with a change of their shape ( $k = 1.13 \pm 0.01$ ). The observed changes are of statistical significance as verified by a Wilcoxon test at probability  $p < 0.0001$  for  $d$  value, and for  $k$  value at  $p < 0.005$ . The effect of shape alteration is associated with the elongation of RBCs as presented in Fig. 2.

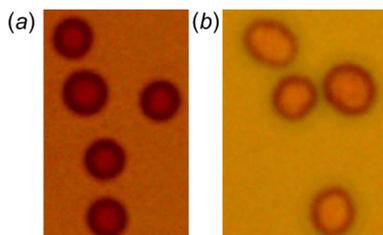


Fig. 2. Exemplary images showing changes of RBCs shape: (a) control sample, (b) RBCs treated with  $100 \mu\text{M}$   $\beta\text{-Crt}$ . The images are shown at different scale.

The effect of shape alteration may result from a random distribution of  $\beta\text{-Crt}$  molecules within lipid bilayers and their interactions with membrane proteins involved in forming a skeleton. The RBCs membrane-skeleton is a two-dimensional network of different types of proteins including spectrins which are largely responsible for characteristic RBCs morphology and their unique elastic and biorheological properties [2]. Hence, any disruptions that affect connectivity between spectrins and other components of the membrane-skeleton may lead to its reorganization and finally influence the shape of RBCs. For example, interactions between actin filaments and rod-like tropomyosin molecules or tropomodulin at the central junctions as well as between spectrin and its ankyrin binding site were recognized to be important for maintenance of the tetra-, penta-, hexa-, or heptagonal structures of RBCs membrane-skeleton [20–22]. Moreover, these shape and surface changes are accompanied by a modification of the functioning of whole cells as well as the formation of different forms of Hb or its affinity to  $\text{O}_2$  [18, 23, 24].

### 3.2. Osmotic fragility analysis

Transfer of RBCs to the hypotonic environment causes the formation of pores in the membrane and leakage of Hb into the solution. The amount of released Hb corresponds to RBCs osmotic stability. In Fig. 3, the normalized exemplary osmotic fragility curves of  $\text{RBC}_{100\beta\text{-Crt}}$  and the corresponding control sample are shown. They have a sigmoidal shape.

For the control sample,  $H_{50}$  (the level at which hemolysis reaches 50%) was observed at a NaCl concentration of about  $0.394 \pm 0.001\%$ . For  $\text{RBC}_{100\beta\text{-Crt}}$ ,  $H_{50}$  has shifted towards higher salt concentration ( $0.411 \pm 0.001\%$ ). At the same time,  $H_{90}$  (the level at which hemolysis reaches 90%) has shifted from  $0.350 \pm 0.001\%$  to  $0.336 \pm 0.001\%$ , while  $H_{10}$  (the level at which hemolysis reaches 10%) from  $0.435 \pm 0.001\%$  to  $0.451 \pm 0.001\%$ . Thus, one sees that the incubation of RBCs with  $\beta\text{-Crt}$  resulted in the modification of the shape and position of the sigmoid.

Analyzing the osmotic fragility curves for three different pairs of samples (RBCs treated and untreated with  $\beta\text{-Crt}$  from three different donors), we

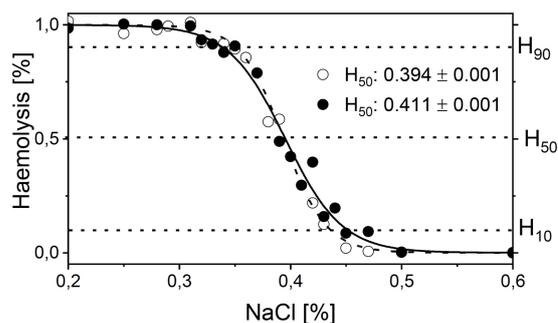


Fig. 3. Normalized exemplary osmotic fragility curves of control (open circle) and  $\beta\text{-Crt}$  treated RBCs (full circles). Inset: calculated  $H_{50}$  values.

observed shifts of  $H_{50}$ ,  $H_{90}$  and  $H_{10}$  values, the latter one being the most significant within the measurement uncertainty. These changes suggest that  $\beta\text{-Crt}$  in RBCs at the applied concentration reduces RBCs stability. One of the mechanisms responsible for this behavior is the modification of the permeability of their membranes to ions.

### 3.3. Mössbauer spectroscopy

The Mössbauer spectroscopy is a well-known technique extremely useful in studies of iron porphyrins [13]. In the course of the present study, the effect of  $\beta\text{-Crt}$  on oxygen reversible binding properties of HFe in Hb embedded in RBCs was investigated. The measurements were performed following the procedure described in [18]. Briefly, the samples were subjected to high partial pressure of nitrogen, put into a dedicated vessel, frozen down with liquid nitrogen and placed in a cryostat enabling gas exchange. The exemplary Mössbauer spectra of control RBCs and  $\text{RBC}_{100\beta\text{-Crt}}$  are shown in Fig. 4.

The spectrum of a control sample (see Fig. 4a) was resolved into two components. The major one, designated as oxygenated Hb (oxyHb, hemoglobin saturated with oxygen), at the steady state has characteristic hyperfine parameters  $\text{IS} = 0.15 \pm 0.01 \text{ mm/s}$  (IS — an isomer shift) and  $\text{QS} = 2.17 \pm 0.02 \text{ mm/s}$  (QS — a quadruple splitting). In turn, a non-physiological deoxyhemoglobin (deoxyHbOH, hemoglobin with HFe having OH/ $\text{H}_2\text{O}$  as the sixth ligand) has  $\text{IS} = 0.15 \pm 0.03 \text{ mm/s}$  and  $\text{QS} = 1.62 \pm 0.08 \text{ mm/s}$ . One would also expect the presence of a physiological deoxyhemoglobin (deoxyHb, HFe bound to histidine residues via its axial ligands) characterized by hyperfine parameters typical for a high spin ferrous ion. However, it is not always visible in a spectrum as it is known to convert to oxyHb within the first two hours of measurement (see Fig. 5).

To obtain a good fit of the spectrum of RBCs treated with  $\beta\text{-Crt}$ , it was necessary to take into account two to five components with a different contribution in the spectrum along with

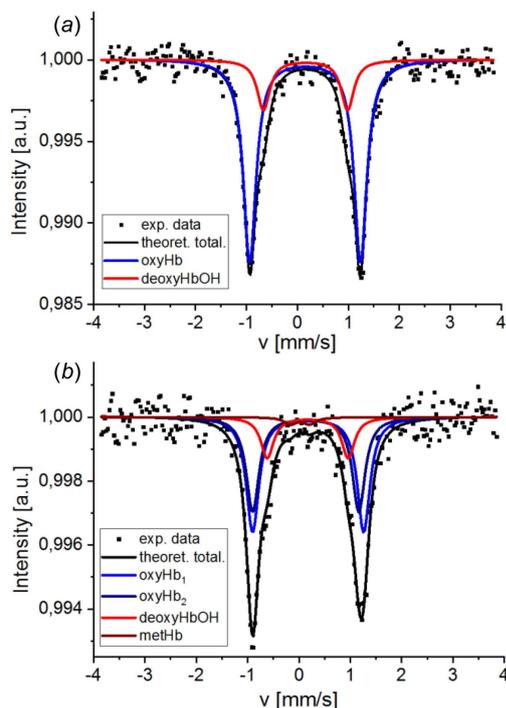


Fig. 4. Mössbauer spectra of (a) control RBCs and (b) RBCs subjected to  $\beta$ -Crt treatment. Experimental data are indicated by points, theoretical curves by lines including subspectra.

the duration of the measurement (see Figs. 4b and 5). At the beginning, a component with high IS  $\approx 0.42$  mm/s and QS  $\approx 1.71$  mm/s was observed (called deoxyHb<sub>t</sub>). During the first 3 h of measurement, it converted partially to oxyHb (in the case of RBCs<sub>100 $\beta$ -Crt</sub> oxyHb<sub>1</sub>) and deoxyHbOH. Later, it additionally evolved into a component called oxyHb<sub>2</sub> (Fig. 5). Furthermore, after  $\approx 6.5$  h of measurement, a methemoglobin (metHb, Hb containing oxidized HFe<sup>3+</sup>) was detected. However, its contribution did not exceed 3%. The hyperfine parameters of deoxyHb<sub>t</sub> suggest that it may contain mixed valence and spin states of HFe [12, 15]. However, the most intriguing component is the one that occurred after 4 h of measurement. Its hyperfine parameters and increasing share in the spectrum at the beginning at the expense of deoxyHb<sub>t</sub> and then oxyHb<sub>1</sub> along with the measurement time suggest that it is oxyhemoglobin with changed symmetry of the oxygen binding to HFe (Fig. 5c). This component was named oxyHb<sub>2</sub>, while the one present from the beginning — oxyHb<sub>1</sub>. Both components differ in isomeric shifts and quadrupole splittings. OxyHb<sub>2</sub> has about twice lower IS and by about 0.12 mm/s smaller QS than oxyHb<sub>1</sub> (Fig. 5a and b). The appearance of metHb along with a decrease in the total contribution of deoxyHbOH indicates the interplay between different oxygenated forms of Hb that are present in the sample.

The applied experimental procedure of RBCs incubation under high N<sub>2</sub> pressure that led to the

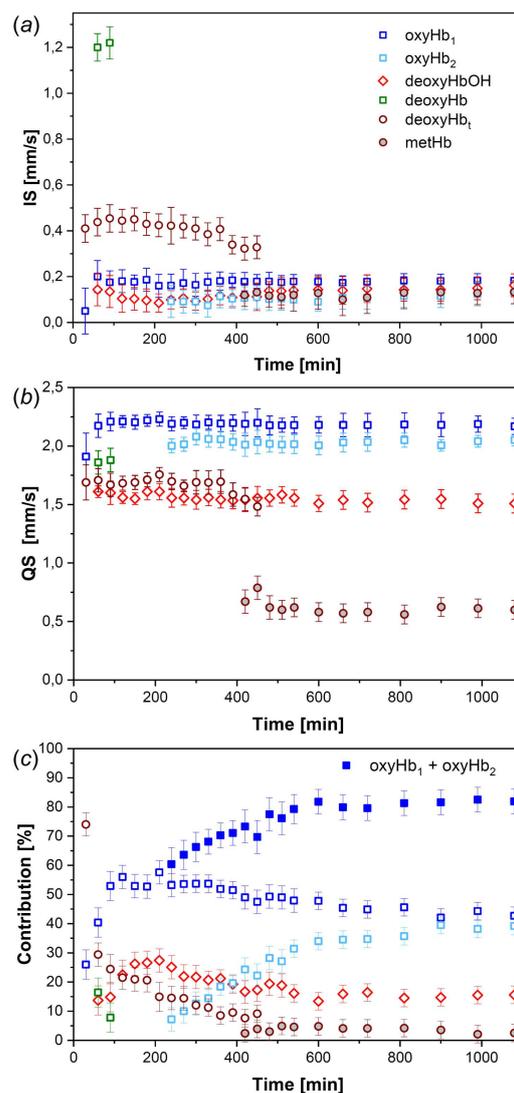


Fig. 5. Time evolution of the (a) isomeric shift, (b) quadrupole splitting, and (c) contribution of various Hb components in RBCs subjected to  $\beta$ -Crt treatment.

release of attached O<sub>2</sub> allowed for monitoring of the process of its reversible binding by Hb inside RBCs. The time evolution of the oxyHb saturation in RBCs untreated (control sample) and treated with  $\beta$ -Crt is presented in Fig. 6.

It can be clearly seen that the process of oxygen saturation by Hb is considerably slower in RBCs treated with 100  $\mu$ M  $\beta$ -Crt. After 30 min of measurement, the oxygen saturation level in a normalized control sample reached as much as  $71 \pm 3\%$  and ultimate saturation was achieved after about 5 h. In the case of RBCs<sub>100 $\beta$ -Crt</sub>, it was respectively  $32 \pm 3\%$  and the maximum saturation was reached after about 13 h of measurement. In both cases, the final recorded oxygen saturation level of Hb was on the similar 80% level. The obtained results indicate the change in Hb-O<sub>2</sub> affinity as a consequence of  $\beta$ -Crt presence.

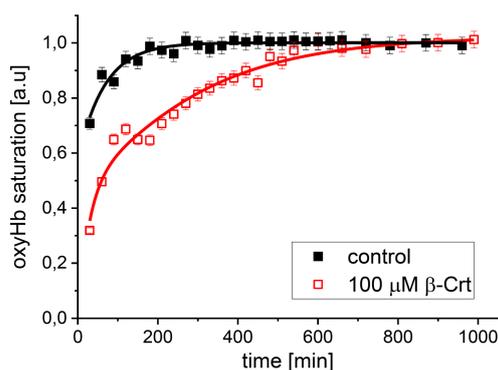


Fig. 6. Time evolution of the normalized oxyhemoglobin contribution in RBCs subjected to  $\beta$ -Crt treatment in relation to control.

#### 4. Conclusions

The presented results provided experimental evidence that  $\beta$ -Crt at the concentration used causes changes in RBCs, modifying their structural and functional properties. In particular, this hydrophobic carotenoid: (i) modifies RBCs membrane protein network leading to the alteration of a cell's shape, (ii) leads to increased sensitivity of RBCs to osmotic shock, (iii) affects Hb forms stabilized inside RBCs, and (iv) decreases Hb affinity to  $O_2$  reversible binding. These findings show that overloading the diet with beta-carotene can have serious effects on the cardiovascular system. Further systematic studies are planned.

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