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Interplay Between Carotenoids and Red Blood Cells: What Can We Learn from Mössbauer Study?

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Hemoglobin is a tetrameric protein composed of four polypeptide chains with embedded prosthetic heme groups that, in particular, are binding sites for O_2. There are various modulators, both natural and synthetic, that can influence the affinity of hemoglobin for molecular oxygen. Carotenoids are natural pigments that are well known for their beneficial health effects. In model studies, they have been shown to integrate with the membranes of red blood cells, altering their properties and functionality, and act as protective agents against induced hemoglobin oxidation. Due to their significance, the properties of a model carotenoid, β-carotene, as a potential modulator of Hb–O_2 interactions, were investigated. This article briefly reviews the effect of β-carotene on altering the properties of hemoglobin inside red blood cells that were exposed to it *in vitro*.

topics: carotenoid, erythrocyte, heme-iron, hemoglobin

1. Introduction

Red blood cells (RBCs, erythrocytes) are the most common type of blood cell in the human body. Their primary function is the transport of O_2 and CO_2. Furthermore, RBCs maintain the acid-base balance, regulate blood pressure, and participate in physiological processes, such as interactions with minor blood components and regulation of temperature. The erythrocyte of a healthy adult has a biconcave discoid shape [1]. It is stabilised by the membrane skeleton, which consists of a number of proteins suspended in the lipid membrane and located on its inner side. These include, for example, the anion-exchange channel protein (band 3), spectrin, actin, and ankyrin [2]. They ensure the flexibility and durability of the RBC in response to its deformation during microcirculation [3]. Across the red blood cell membrane, respiratory gases and some small molecules such as glucose and urea are exchanged. The interior of the RBC is filled with oxygen-binding hemoglobin (Hb), which is responsible for the delivery of O_2 to cells. Hemoglobin is a tetrameric protein made up of four globular subunits (see Fig. 1) [4]. Adult human Hb consists of two α chains and two β chains ($\alpha_2\beta_2$), each of which binds a prosthetic group — heme. Heme is the binding site for O_2, CO, and NO [5]. The heme consists of a ferrous ion held in the centre of a protoporphyrin IX and coordinated by its four nitrogen atoms. In physiologically deoxygenated Hb (deoxyHb), heme iron (HFe) is also directly bound to Hb globin via the so-called proximal and distal histidine (His), located on helices F and E of the polypeptide chains, respectively. This arrangement results in the octahedral coordination of six HFe ligands [6]. In oxidised Hb (oxyHb), the sixth HFe ligand is O_2-stabilised by hydrogen bonding to distal His (see Fig. 2) [4, 7, 8].

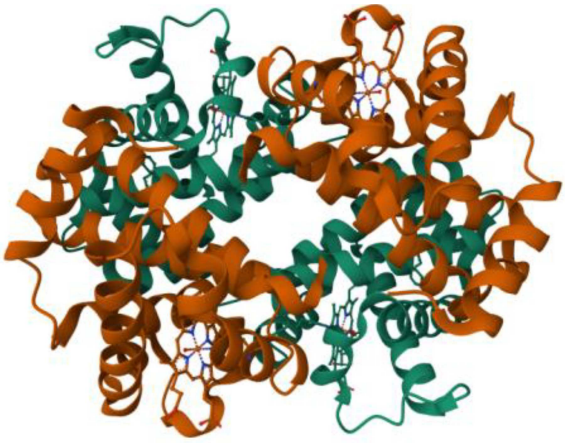

Fig. 1. Hemoglobin tetrameric helical structure in the oxygenated form; α-subunits are marked in green, β-subunits in brown. Protein Data Bank (PDB) identifier: 2DN1 [4].

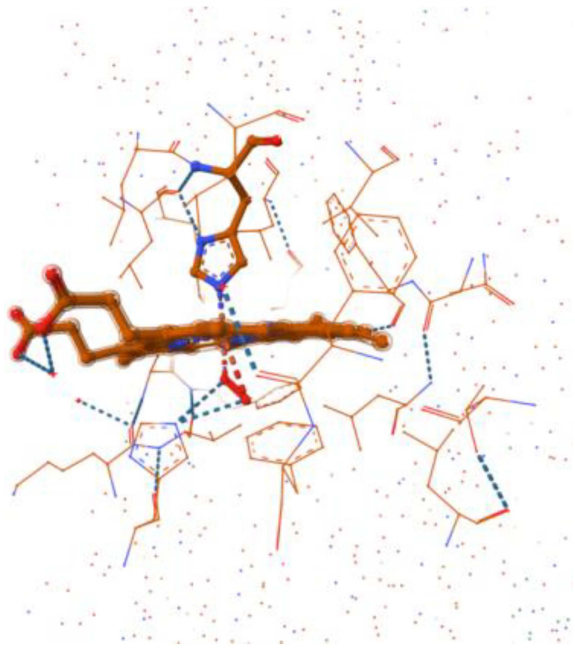

Fig. 2. Schematic representation of the heme moiety encompassing HFe (brown ring) with proximal (above the ring) and distal (below the ring) histidines and oxygen (red) non-covalently bound to HFe. Protein Data Bank (PDB) identifier: 2DN1 [4].

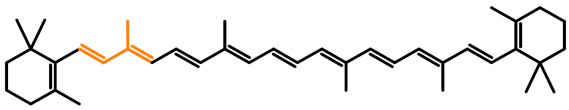

Fig. 3. Chemical structure of all-*trans* β-carotene as an example of model carotenoid. An isoprene unit is marked in orange.

Hemoglobin is an allosteric protein, i.e., a binding of an O_2 to one of the HFe sites (allosteric site) results in an alteration of its subunit's conformation and an"increase in oxygen affinity within the remaining heme moieties [5]. There are various allosteric modulators that can modify the affinity of HFe–O_2 [9]. Some of them, such as 2,3-diphosphoglycerate or inositol hexaphosphate, are well known to bind to both liganded and unliganded forms of Hb, leading to modulation of its dynamics, inducing changes in the quaternary structure and lowering the affinity of Hb for O_2 [10–12]. Other allosteric effectors, including natural and synthetic compounds, sometimes used as drugs, are also capable of changing the binding equilibrium toward either high or low affinity for Hb–O_2 [8].

Carotenoids are a group of natural pigments, mainly yellow, orange, and red, widely distributed as constituents of fruits and vegetables [13]. They are tetraterpenoids formed out of eight isoprenoid subunits (see Fig. 3). Of the more than 750 carotenoids already known, less than 30 have been found in human blood samples [14]. They are efficiently delivered as food components in a normal diet and, more recently, also as food supplements. As antioxidants, they are believed to offer significant health benefits [14, 15]. However, as was demonstrated under certain experimental conditions [16] and in population studies [17], they can act as pro-oxidants. In model studies, carotenoids were incorporated into cellular membranes [18]. They have been shown to affect the physicochemical properties of membrane components by increasing the rigidity and stability of membranes and reducing their permeability to ions and oxygen [19, 20].

β-Carotene (β-Crt; see Fig. 3) is one of the most abundant carotenoids. As demonstrated in model studies, it can integrate into the outer layer of RBC membranes [21, 22]. β-Carotene was also shown to protect Hb against oxidation induced by external radical generators [23]. These and other recent findings on β-Crt properties prompted us to explore more deeply its effect on the functioning of RBCs. In particular, interactions that caused changes in physiological forms of Hb that can affect the affinity of Hb–O_2 were studied [24, 25]. Here, on the basis of the results obtained by means of Mössbauer spectroscopy, a brief overview is given of the effect of β-Crt on the properties and functioning of Hb inside the RBC exposed to it *in vitro*.

2. Discussion of Mössbauer spectroscopy data

Mössbauer spectroscopy turned out to be a very useful technique in biomedical research. It is applied in a wide range of studies on the qualitative and quantitative changes of biomolecules containing Mössbauer nuclides in normal and pathological processes, the effect of environmental factors (physical, chemical, biological) on these biomolecules or metabolic processes by analysis of their pathways in tissues [26–30]. It is sensitive to the valence and spin state of probing nuclei (example isotopes of use in biosystems: ^{57}Fe, ^{119}Sn, ^{153}Sm, ^{197}Au, or ^{129}I) and its chemical environment in its first coordination sphere [28, 31–33]. In particular, the hyperfine interaction parameters of heme iron in Hb allow the differentiation of hemoglobin states. Here, we review the findings of the effect of an external biological factor, the model carotenoid β-Crt, on Hb molecular properties. Data subjected to the current analysis came from Mössbauer measurements carried out on erythrocytes from healthy donors, which were incubated in the presence of different concentrations of β-Crt, as described in [24, 25]. In view of the presence of additional physiological forms of Hb that were reported as a consequence of the RBC treatment with β-Crt, the contribution and time evolution of the oxygenated forms of Hb are discussed. The experiments were performed according

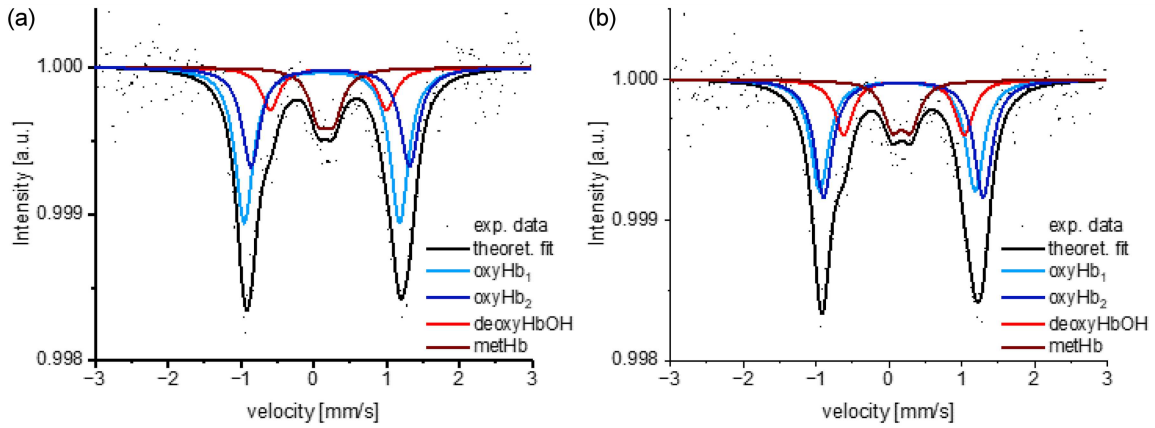

Fig. 4. Exemplary Mössbauer spectra of erythrocytes from a healthy donor, treated with β-Crt (500 μmol/dm^3), collected after (a) 8.5 h and (b) 14.0 h. Measurements were performed at 85 K. The experimental data are indicated with black symbols, and the theoretical curve with a black line. The characteristic doublets for the different forms of Hb are represented by solid lines (see figure legend). Hyperfine interactions parameters of subspectra (a) oxyHb$_1$ with IS $= 0.11 \pm 0.03$ mm/s and QS $= 2.11 \pm 0.04$ mm/s, $\sim$ 45.6% contribution; oxyHb$_2$ with IS $= 0.23 \pm 0.03$ mm/s and QS $= 2.17 \pm 0.05$ mm/s, $\sim$ 29% contribution; deoxyHbOH with IS $= 0.20 \pm 0.04$ mm/s and QS $= 1.60 \pm 0.06$ mm/s, $\sim$ 12.3% contribution; metHb with IS $= 0.17 \pm 0.04$ mm/s and QS $= 0.20 \pm 0.07$ mm/s, $\sim$ 13.1% contribution; (b) oxyHb$_1$ with IS $= 0.12 \pm 0.03$ mm/s and QS $= 2.13 \pm 0.03$ mm/s, ~34,5 % contribution; oxyHb$_2$ with IS $= 0.20 \pm 0.03$ mm/s and QS $= 2.19 \pm 0.04$ mm/s, $\sim$ 36.1% contribution; deoxyHbOH with IS $= 0.21 \pm 0.03$ mm/s and QS $= 1.63 \pm 0.05$ mm/s, $\sim$ 16.3% contribution; metHb with IS $= 0.17 \pm 0.04$ mm/s and QS $= 0.25 \pm 0.07$ mm/s, $\sim$ 13% contribution. Here, IS is an isomer shift and QS is a quadrupole splitting. The spectra were always fitted with a constant half-line-width for all components $\Gamma = 0.15$ mm/s. To obtain consistent changes in the spectra over time, it was sufficient to include two oxyHb components.

to the protocol described in [34], which allows for studying the affinity of Hb for O_2 and the reversible binding of an oxygen molecule to HFe.

In Fig. 4, exemplary Mössbauer spectra of erythrocytes incubated in the presence of β-Crt at a concentration of 500 μmol/dm^3 are shown. To achieve a good fit with the experimental data, four components with typical parameters were required that stayed in line with the published data [32, 34]. The two fractions that were the most abundant were assigned as oxygenated Hb (oxyHb, hemoglobin saturated with oxygen). The remaining two components are characterised by hyperfine interaction parameters corresponding to non-physiological deoxyhemoglobin (deoxyHbOH, hemoglobin with HFe having OH^-/H_2O as the sixth ligand) and methemoglobin (metHb, hemoglobin with five-coordinated HFe^{3+}). Physiological deoxyhemoglobin (deoxyHb with HFe bound to distal His residues via its sixth axial ligand) is not present in these spectra.

In the spectra of erythrocytes treated with a lower concentration of β-Crt (50 μmol/dm^3), the same major forms of Hb were observed as in the case of control, namely only two components: oxyHb and deoxyHbOH [24]. However, in the spectra of samples incubated with higher β-Crt concentrations (100 μmol/dm^3 and 500 μmol/dm^3, respectively), two forms of oxyHb could be resolved (see Figs. 4, 5b, and 5c). These components differ in their hyperfine interaction parameters. For the sample treated with 100 μmol/dm^3, differences in both the isomer shift (IS) and the quadrupole splitting (QS) were observed for the two forms of oxyHb. In this case, hemoglobin, called oxyHb$_1$, had an IS of about 0.10 ± 0.02 mm/s and a QS of about 2.04 ± 0.03 mm/s, while the second form, oxyHb$_2$, had an IS of approximately 0.17 ± 0.03 mm/s and a QS of approximately 2.19 ± 0.04 mm/s [24]. Following the changes within the measurement time, it was observed that the first new component was partially converted to oxyHb$_1$ and deoxyHbOH, which later eventually evolved into oxyHb$_2$.

Incubation of RBC with 500 μmol/dm^3 also resulted in the appearance of an additional component, oxyHb$_2$. However, in this case, it was detectable from the beginning of the measurement (see Fig. 5c). The hyperfine interaction parameters of this component and its increasing contribution to the spectrum at the expense of oxyHb$_1$ during the course of the measurement indicated similar changes in the binding site of the oxygen molecule within Hb as in the case of erythrocytes treated with 100 μmol/dm^3 β-Crt. Interestingly, contrary to the described observations, at a lower β-Crt concentration (50 μmol/dm^3), only one state of oxyHb was observed.

In control samples, in the absence of β-Crt, the process of oxygen saturation by Hb was significantly faster (3-4 hours) than in the case of all erythrocyte

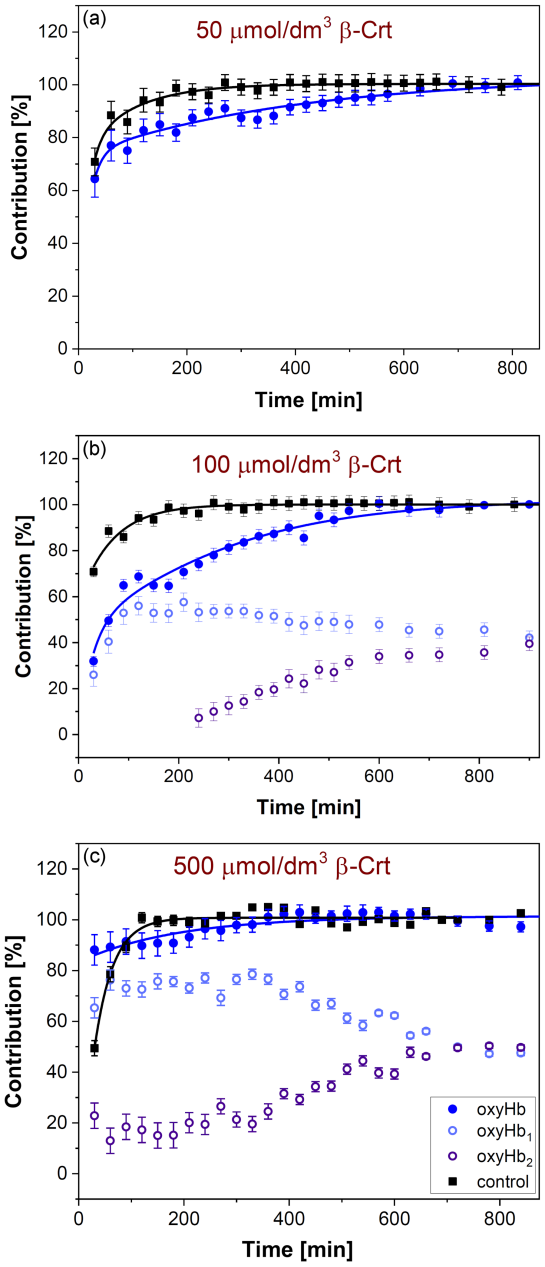

Fig. 5. Time evolution of the normalised contribution of oxyHb in erythrocytes treated with (a) 50 μmol/dm^3 β-Crt, (b) 100 μmol/dm^3 β-Crt, (c) 500 μmol/dm^3 β-Crt with respective controls. The increasing time scale corresponds to the increasing partial pressure of O_2 in erythrocytes. Labels: filled black square — control; filled blue circle — oxyHb; light blue open circle — oxyHb$_1$; violet open circle — oxyHb$_2$. More details can be found in the main text and in [24, 25].

samples that were incubated with carotenoid. After treatment of RBC with lower β-Crt concentrations, the level of oxygen saturation equal to the respective controls was reached after about 11 h. In the case of the highest applied concentration of β-Crt, it was considerably accelerated — the plateau was achieved after about 6 h. Furthermore, a higher contribution of oxygenated Hb was observed for the first two measurement points than for the corresponding control.

3. Conclusions

On the basis of the previous results obtained during studies of erythrocytes treated with different concentrations of β-Crt, carried out by means of Mössbauer spectroscopy and described in this work, a series of observations were made at the molecular level regarding the interactions between the carotenoid and Hb. At the lowest concentration tested (50 μmol/dm^3), this carotenoid affected the ability of Hb to rebind to O_2, mainly by altering the physicochemical properties of the RBC membrane skeleton. A twofold increase in the concentration of β-Crt resulted in a further decrease in the affinity of Hb for oxygen, accompanied by the appearance of an additional form of oxyHb. This means that β-Crt was not only incorporated into the RBC membrane but also penetrated inside the cell, directly affecting the hemoglobin states. However, only oxyHb, namely the relaxed form of Hb, was found to be susceptible to the influence of β-Crt, which caused changes in the symmetry of the HFe bond with O_2 and simultaneously affected its rigidity. This is confirmed by the increase in the presence of additional oxyHb already in the initial stage of monitoring the oxygen saturation of Hb for the highest carotenoid concentration, i.e., 500 μmol/dm^3. However, in this case, the affinity of Hb for oxygen increased significantly, which may be due to (i) the effect of β-Crt on the allosteric process itself by modifying the mobility and stabilisation of the quaternary structure of Hb and/or (ii) the change in the permeability of the erythrocyte membrane to O_2 under the influence of β-Crt.

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