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Lowell T. Duncan
Editor

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Contents

Preface

This volume contains nine chapters that detail recent advances in health and disease. Chapter One discusses the roles and structure of hemoglobin, the iron-containing metalloprotein responsible for oxygen transport. Chapter Two analyzes the understanding that systemic inflammation underlies comorbidity in chronic obstructive pulmonary disease (COPD). Considering the many biological activities and medicinal uses of Bauhinia spp., Chapter Three presents an overview of the genus, providing an update on the botanical characteristics, chemical properties, and antimicrobial activity. Chapter Four details the pharmacokinetics of ace inhibitors and their role in diseases. Chapter Five proposes an alternative treatment of psycho-emotional disorders in limonene. Chapter Six discusses loop-mediated isothermal amplification (LAMP), a technique for the diagnosis of tuberculosis and several other bacterial, fungal, parasitic and viral infections. Chapter Seven aims to address the role and regulation of Heme Oxygenase-1 and the signaling pathways modulated by it toward gaining a comprehensive knowledge of this critical regulator of macrophage defense. Chapter Eight provides a comprehensive overview of the structure and functional insights on renin, shedding light on its role in the physiological pathways involved in blood pressure control. Lastly, Chapter Nine analyzes the studies of ceftazidime-avibactam carried out to date.

Chapter 1

Hemoglobin – Its Molecular Structure and Biological Roles

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Abstract

Hemoglobin is the iron-containing metalloprotein responsible for oxygen transport. It was found in most vertebrates' erythrocytes (red blood cells), except the fish family *Channichthyidae*. The term hemoglobin comes from Greek and Latin and means αἷμα (haîma, "blood") + globulin (from globus ("ball, sphere"), respectively. Hemoglobin is used for oxygen transport from the lungs to the tissues, and partially carbon dioxide from the lungs (20-25%). The hemoglobin content in every 100 ml of blood is 12 to 20 grams for a healthy human. The oxygen released in tissues permits aerobic respiration for energy generation needed in mainly the organism's functions, described as metabolism.

Keywords: Fe, Fe-containing proteins, heme, metalloprotein, oxygen transport, porphyrin

Introduction

Interestingly, hemoglobin can also carry other gases, for instance, nitric oxide (bounded to a globin protein thiol group), which is part of the necessary regulatory process of this metalloprotein [1]. The oxygen-binding capacity of

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hemoglobin is 1.35 mL O_2 per gram. The oxygen-binding capacity of hemoglobin *in vitro* and *in vivo* samples is comparable for non-smokers (1.365 ± 0.010 and 1.366 ± 0.007 ml per gHb) [2], which is seventy-fold more than oxygen dissolved in the blood. In smokers, these values were less: 1.26 and 1.20 [3], or comparable 1.39 ml per gHb [4] or higher [5]. Carbon dioxide is carried mainly as bicarbonate ions than as attached to the heme protein. For mammals, the hemoglobin molecule can transport four oxygen molecules. The protein total and dry content in erythrocytes are circa 35% and 96% as to weight, respectively [6]. Bernard explained hemoglobin's role in the blood: every heme group contains one iron atom, which can attach one oxygen molecule [7, 8]. Mostly hemoglobin in mammals has four heme groups.

The oxygen transport is not only one function of hemoglobin. It was also found in the A9 dopaminergic neurons in the substantia nigra, macrophages, alveolar cells, lungs, retinal pigment epithelium, hepatocytes, mesangial cells, endometrial cells, cervical cells, and vaginal epithelial cells [9]. In these tissues, hemoglobin plays the role of a regulator of iron metabolism and an antioxidant. Also, hemoglobin can attach to excessive glucose in the blood and convert into hemoglobin A_{1c}.

Hemoglobin and its derivatives were found in fungi, plants, and many invertebrates [10]. They are also used for oxygen transport or CO_2, NO, H_2S, and S^{2-}. For selected plants or bacteria (for instance, cyanobacteria), oxygen is toxic and should be removed. Leguminous plants use leghemoglobin for scavenging O_2 away from anaerobic systems.

From a medical point of view, the hemoglobin concentration and its condition in the blood plasma could indicate whether an organism is healthy or not. For instance, too low hemoglobin concentration causes anemia and high hemoglobinemia. It is related to separating hemoglobin from red blood cells (intravascular hemolysis). It also led to anemia, in consequence.

The first known research on hemoglobin was done by Engelhart in 1825. He found that the ratio of iron to protein is very close in several kinds of hemoglobins [11]. He determined protein's molecular mass: calculated the molecular mass of hemoglobin to n × 16000 (n = number of iron atoms per hemoglobin, now known to be 4 [12]); the atomic mass of iron was known, Hüfner and Gänsser estimated this mass as 16,700 in 1907 [13]. These results were unacceptable for many scientists who could not accept that any molecule could be that big. Engelhart's results were confirmed by Adair, who measured hemoglobin solutions' osmotic pressure in 1925 [14]. Hünefeld discovered the most crucial hemoglobin property – the possibility of oxygen transport in 1840 [15], and the fact that oxygenation of hemoglobin is reversible was confirmed

by Hoppe-Seyler, several years later [16]. Funke found that hemoglobin is built of proteins in 1851 [17]. The hemoglobin was investigated by Pauling and Coryell (its magnetic properties), who published two papers in 1936 [18, 19]. Perutz identified the molecular structure of hemoglobin by x-ray crystallography in 1959 [20] and won the Nobel Prize in Chemistry in 1962 with Kendrew for research on the structures of globular proteins [21]. Perutz estimated the hemoglobin mass as 67,000.

Hemoglobin is built from protein subunits, and the hem, a non-protein subunit, consists of the iron atom in the center and porphin rings. These proteins or polypeptides are built from different amino acids and folded chains. Their chemical properties and function are determined by appropriate amino acids – the kind and their sequence. DNA stretches called genes determine the order (sequence) of the amino acids in polypeptide chains. Hemoglobin must be coded by more than one gene; for instance, in humans, hemoglobin A is coded by three genes, *HBA1*, *HBA2*, and *HBB* [22]. The most common hemoglobin sequences in humans, chimpanzees, and bonobos, are identical (the similarity of these living organisms is high, i.e., their genes), and potential differences consist of exchanging one-two amino acid in protein chains [23]. Possible differences in amino acid sequence grow for less closely related living organisms.

Potential differences were found in central African people and described as sickle-cell disease, a kind of hemoglobinopathy [24]. This illness is caused by gene mutation and is related to the exchange of one amino acid (glutamine acid into valine), which has changed the erythrocyte's shape. Still, it provides higher resistance to malaria in consequence. This disease was not found in humans, for instance, in Europe, Northern America, or Asia. However, many mutations in the genes for the hemoglobin protein cause no illness. Selected mutant forms of hemoglobin are classified as hereditary diseases named hemoglobinopathies. In general, globin gene regulation problems and mutations can cause too low or abnormal hemoglobin production levels; this phenomenon was described as thalassemias [25]. As a consequence, a patient has anemia.

Hemoglobin is mainly built from two α and two β chains. Potential differences could be related to adaptation to living in areas at high altitudes because the atmospheric (oxygen) pressure decreases if the altitude increases [26, 27]. Then hemoglobin should bind proportionally more oxygen from the arbitrary volume unit (as a percentage, the mass of oxygen is comparable) in comparison to organisms living in areas at low altitudes (lowland prairies in contrast to the mountains). It was found that any changes were observed in the

genes coding the oxygen-carrying capacity of their hemoglobin; other genes were the same. The researchers argued that in highland mice, the genetic difference had caused more efficient use of their oxygen. Some mutations were found in mammoth hemoglobin, allowing for oxygen delivery at lower temperatures, and living in present-day northern Canada or Siberia. These mutations in *A. castelnaudii*, *Oreotrochilus*, *A. viridicuada*, *P. gigas*, and *C. violifer*, have caused a lower affinity for inositol hexaphosphate (IHP) to proteins. IHP plays a similar role in birds as 2,3-BPG in humans and binds oxygen at lower partial pressures. Also, birds' circulatory lungs are unique, and efficient use of oxygen at low partial pressures of O_2 is observed (i.e., during a flight on relatively high attitudes).

Similar adaptational changes were also found in humans; for instance, in Tibetan women residing at 4,000 m height above sea level, a higher offspring survival rate is observed [28, 29]. In detail, the authors measured the number of pregnancies and lived births. They found no evidence of mean genotypic differences in fecundity. Also, they observed high saturation genotypes caused lower offspring mortality and more surviving offspring. Also, the mortality rate of offspring is meaningfully higher for women with lower hemoglobin-oxygen affinity than the mortality one of offspring from women with high hemoglobin-oxygen affinity. This change is crucial, and these women can better sustain critical metabolic processes.

Hemoglobin is synthesized in a series of steps:

1. heme: in the mitochondria and the cytosol of undeveloped red blood cells;
2. globin: in the cytosol by ribosomes;
3. transformation of hemoglobin occurs in the bone marrow, from the proerythroblast phase to the reticulocyte (early development of hemoglobin). It is observed that residual ribosomal RNA allows for further synthesis of Hb until the reticulocyte loses its RNA soon after ingoing the vasculature. Then, the nucleus is missing in mammalian red blood cells; however, not for birds and many other living organisms.

Structure of Hemoglobin

Like many multi-subunit globular proteins, hemoglobin is built from four globular protein subunits and has a quaternary structure. Short non-helical

parts connect α-helices. The helical sections inside this protein are stabilized by hydrogen bonds and the folding of every polypeptide chain into a specific shape. Hemoglobin's quaternary structure could be described as a tetrahedral arrangement. The subunit is built of a protein chain closely connected with a non-protein prosthetic heme group. The protein chain arrangement could be defined as an α-helix structural parts coupled with a globin. It is worth mentioning that an identical folding motif is found in other heme/globin proteins like myoglobin. Hemoglobin saturated with O_2 is known as oxyhemoglobin and desaturated with O_2 molecules – deoxyhemoglobin.

The iron ion is localized in a porphyrin center – a heterocyclic ring defined as a heme group. The porphyrin ring is built from four pyrrole molecules cyclically coupled with the iron ion with the four nitrogen atoms by methine bridges, which all lie in one plane. The iron is covalently bound via the nitrogen atoms from the imidazole ring to the globular protein. These rings, which could be treated as F8 histidine residues, are localized below the porphyrin ring. The oxygen molecule could be reversibly bonded by creating a coordinate covalent bond at a sixth position. Then the octahedral group of six ligands is created. The oxygen molecule binds as a distorted one: one oxygen atom is attached to Fe, and the second protrudes at an angle. When oxygen is absent, the H_2O molecule is very weakly bonded, and a distorted octahedron is created. In opposite to O_2, CO_2 is attached not to iron. The amine groups of the protein chains are coupled with the heme groups. Oxygen transport is impossible if the iron ion is oxidized from ferrous to ferric state ($Fe^{2+} \rightarrow Fe^{3+}$). Hemoglobin with Fe^{3+} ion is known as ferrihemoglobin (methemoglobin). A reduction system realizes the protection from the oxidation of iron in hemoglobin in red blood cells.

This phenomenon suggests that iron ion is oxidized (temporarily and reversibly) if an oxygen molecule is attached. Also, the oxygen turns into the O_2^- ion (the superoxide).

$$Fe^{2+}\,(\text{hem}) + O_2 \rightarrow Fe^{3+}\,(\text{hem}) + O_2^-$$

$$Fe^{3+}\,(\text{hem}) + O_2^- \rightarrow Fe^{3+}\,(\text{hem})\text{-}O_2^-$$

$$H^+ + O_2^- \rightarrow HO_2$$

Fe^{3+} (hem) + HO_2 → *a reaction is not occurred (binding oxygen is impossible)*

Fe^{3+} (hem) + HO_2 + methemoglobin reductase (the enzyme) → Fe^{3+} (hem)-O_2^-

The most predominated hemoglobin type (in adult humans) is hemoglobin A. It is built from two α and two β subunits non-covalently bound ($\alpha_2\beta_2$), consistent from 141 and 146 amino acid residues, respectively. Both subunits are structurally close, and their size is comparable. A particular subunit's molecular weight is 16 kDa, and the total molecular weight – is 64 kDa or 64,458 g/mol (1 g/dL = 0.1551 mmol/L) [30]. Because of its prevalence, hemoglobin A is the most intensively investigated of all hemoglobin molecules. Conversely, in human infants, the hemoglobin molecule is built from α and γ chains ($\alpha_2\gamma_2$); however, as an infant grows, the γ chains are progressively replaced by β chains. In the hemoglobin molecule, the polypeptide chains are coupled by salt bridges, hydrogen bonds, and hydrophobic interactions: a phenomenon of aggregation of nonpolar substances in an aqueous solution with water exclusion molecules is observed.

Oxyhemoglobin is created in physiological respiration in the pulmonary capillaries adjacent to the lungs' alveoli. The oxygen is transported to cells in the bloodstream. Next, it is utilized as a terminal electron acceptor during oxidative phosphorylation in ATP formation. It is worth mentioning that cyanide's toxicity is related to blocking the enzyme-like cytochrome oxidase – the oxygen transport is undisturbed from the alveoli to the cells, but the binding has become not reversible. Interestingly, the oxygen transport does not change the blood pH (this value is equal to 7.35-7.45) compared to CO_2 when pH is shifted. Only a minority of carbon dioxide is transported as a bind to iron ion, a majority – as carbonate ions.

The form of hemoglobin is dependent on many factors such as pH, and CO_2, 2,3 BPG levels:

- a taut (tense) form (T) – for a low pH value, high CO_2, and high 2,3 BPG; in this case, oxygen affinity is low, and it is released in the tissues; also, this state is found at low partial pressures;
- a simple form (R) – is the opposite of a high pH value, low CO_2, or low 2,3 BPG; in this condition, it is favored oxygen binding and is observed at high partial pressures of oxygen.

A slight conformational shift in the iron versus the porphyrin ring plane was found when binding oxygen to the iron ion. This shift induces oxygen to bind to the next heme units within hemoglobin. In other words, oxygen binding is cooperative.

If the amount of oxygen in a patient's blood is measured, or when the blue to purplish color, tissues are observed in hypoxia for cyanosis detection. The absorption spectra of deoxyhemoglobin and oxyhemoglobin are different: the bands are found at 660 nm and 940 nm, respectively. This change is used in an instrument called a pulse oximeter.

Probably, splitting the gnathostome common ancestor from jawless fish became circa 450–500 million years ago, and at this moment, the predecessors of these genes arose through another duplication event. It is assumed that the separation of myoglobin from hemoglobin occurred when lampreys diverged from jawed vertebrates [31-33]. Then, the functions of myoglobin and hemoglobin were different – oxygen storage and oxygen transport, respectively. Every subunit of the protein chain in hemoglobin is encoded by other genes (the α- and β-like globin). The formation of genes coding the α- and β-like globin let a physical composition central to hemoglobin's ability to bind oxygen in consequence. Next, a duplication of the gene and the formation of the *HBA1* and *HBA2* genes occurred [34-36].

Further duplications and divergences allow for creating a diverse range of α- and β-like globin genes. Sometimes hemoglobin genes have been lost as an adaptation to living in the environment, such as the crocodile icefish or white-blooded fish *(Channichthyidae)* living in cold water found in the Southern Ocean around Antarctica. These fish are the only known vertebrates with no hemoglobin in their blood as adults [37].

Iron's Oxidation State in Oxyhemoglobin

It is interesting what is the iron's oxidation state in oxyhemoglobin; however, its investigation is not simple. Oxyhemoglobin and deoxyhemoglobin are diamagnetic and paramagnetic, respectively [38]. Pauling [39] and Weiss [40] proposed an end-on angular bond as a bent Fe-O_2 configuration. According to Chen et al. the O_2 is bonded His_{64} by the protein's hydrogen atom / amino group. This complex is sensitive to the bulk polarity of the protein. If the protein is removed, significant changes in the complex are observed [41]. As mentioned above, because oxyhemoglobin is diamagnetic, it has no net unpaired electrons. However, unpaired electrons are present in both oxygen and iron. Ground-state or the lowest-energy electron configurations are expected to be paramagnetic, and at least one unpaired electron in the Hb-O_2. The lowest-energy form of O_2, and the lowest energy forms of the appropriate oxidation states of Fe, are as follow:

- the lowest-energy of molecular oxygen is a triplet, and two unpaired electrons are present in antibonding π* molecular orbitals;
- it was detected that Fe(II) is present in a high-spin (HS) $3d^6$ configuration when four unpaired electrons are observed;
- the iron radius decreases from 92 nm to 75 nm;
- for Fe(III), a configuration such as $3d^5$ is found, and one to five unpaired electrons, independence of the electronic state.

These structures are not diamagnetic, only paramagnetic (have unpaired electrons). Another distribution of electrons in the combination of oxygen and iron should explain the detected diamagnetism and lack of unpaired electrons. Also, higher energy for one molecule or both could not be excluded. We can explain these observations, no net spin Hb-O_2, as:

- low-spin (LS) of Fe^{2+} + singlet of O_2, they have zero unpaired electrons, and both are diamagnetic; however, singlet of O_2 is the higher-energy of O_2, the lowest is triplet because two unpaired electrons exist; bio-inorganic chemists postulated it primarily;
- LS of Fe^{3+} + O_2^- (the superoxide ion), and the two unpaired electrons couple antiferromagnetically exists, and the complex of [LS(Fe^{3+}) + O_2^-] is diamagnetic; the iron has one unpaired electron, and the oxygen, too (the iron/oxygen has lost/gained one electron);
- the iron has been oxidized to Fe^{4+} (LS) and binds to peroxide, O_2^{2-}; the iron has two unpaired electrons, and the oxygen zero, and the iron-oxygen complex is paramagnetic in consequence.

According to experimental data:

- oxidation state of iron is approximately 3.2 (x-ray photoelectron spectroscopy);
- the O-O bond length is coming from rather superoxide than oxide (133 nm and 121 nm, from IR spectra), and a bond order is about 1.6 (for superoxide – 1.5); also, the iron the O-O stretching frequency decreases from 1580 cm^{-1} to 1105 cm^{-1} when O_2 is attached, for O_2 and O_2^-, respectively [42];
- the XANES spectra – *K*-edge derivatives *K*-absorption edge shapes are different for oxygenated, reduced, and oxidized forms [43]. The energy shift between deoxyhemoglobin and oxyhemoglobin is 3.5 eV

(XANES at the Fe *K*-edge, measured by Bianconi et al.) [44], and the local charge is closer to Fe^{3+} than Fe^{2+}. The XANES spectra of the Hb molecule are also very sensitive on an attached molecule such as O_2, CO, N_3^- [45].

According to Chen et al. calculations (CASSCF/MM and DFT/MM), the oxyheme complex of Hb behaves as an electronic chameleon, changing its bonding according to its protein host and axial ligand.

Both iron and oxygen have a single unpaired electron, and no net spin to the entire configuration is given. Therefore, the formal oxidation state of Fe in Hb-O_2 is the +3 state, and for oxygen, the −1 state (superoxide O_2^-) and the complex of [Fe^{3+}+$O_2^{\bullet-}$] is present. It is in agreement with diamagnetic oxyhemoglobin found experimentally.

Singlet oxygen can exist in an unrealistically high energy state (then Hb-O_2 is diamagnetic) and its probability is low. Similarly, the iron ion as Fe^{4+} is also low probable: the iron charge change could modify the interaction between the Fe ion and the porphyrin ring. In this case, the Hb-O_2 complex is paramagnetic (it is not detected experimentally). The iron atom's size in Hb-O_2 is smaller than in Hb (Fe^{3+} in comparison to Fe^{2+}), and the iron ion is located in the plane of the porphyrin ring. It can induce allosteric changes in the globulins. The iron oxidation state in methemoglobin is III, and it cannot bind the oxygen from the air. The main difference is that for methemoglobin, superoxide O_2^- is absent.

Initially, it was postulated that the binding of O_2 is located in HS Fe(II) in an octahedral field of strong-field ligands. Then the crystal field splitting energy was increased, and Fe electrons were paired into the LS configuration. It was diamagnetic, agreeing with the experimental data. This forced LS pairing is found in iron after the oxygen binding; however, this model does not explain its size change. In Hb-O_2, removing an additional electron from iron by oxygen is required because iron's smaller size as an increased oxidation state and oxygen's weaker bond is found.

However, the oxidation state of iron and oxygen is a formalism, and the covalent bonds are not needed for detailed bond orders in the Fe-O_2 complex in Hb-O_2 involving whole electron transfer. We can say that the iron model in Hb-O_2 being Fe(III) is more probable than the classical one when the iron oxidation state does not change after oxygen binding.

Transport of Oxygen and Other Molecules

It is worth mentioning that after binding oxygen to the iron ion initiates the conformation change in the heme group, shifting from the T state to the R state. Also, Fe moves back toward the center of the plane of the porphyrin ring. Then, the imidazole side-chain of histidine residue is moved toward the porphyrin ring. This interaction between Fe and the histidine residue forces the sideways ring plane toward the outside of the tetramer. A strain in the histidine-containing protein helix is observed and moves closer to the iron ion. This strain is transferred to the following monomers in the heme group, analogous conformational changes in the heme groups are observed, and oxygen binding is easier. Consequently, this phenomenon promotes the saturation process of the hemoglobin molecule with oxygen.

The Hb-O_2 creation process is cooperative, and the binding of the first oxygen molecule stimulates the shape of the coupling sites and favors binding the following oxygen molecules. The conformational changes are observed in the oxygen binding curve, which is sigmoidal (S-shaped); in case of no cooperative binding, the curve is hyperbolic. In other words, the binding affinity of hemoglobin for oxygen correlates with the oxygen saturation of the molecule.

We consider the Hb-O_2 as a form of an iron complex. It is easy to deduce that also other molecules could attach to the iron ion, for instance, CO, and CN, which are competitive inhibitors, or NO, and CO_2, described as allosteric ligands:

1. Hb-CO_2 is known as carbaminohemoglobin, and CO_2 is bound to amino groups of the globin proteins [46]. However, only about 10% of CO_2 is transported in this way.
2. In the case of NO, it is bound to specific thiol groups in the globin protein, and an *S*-nitrosothiol is created. The *S*-nitrosothiol dissociates into thiol and free nitric oxide if oxygen is released from its heme site. NO can probably help in oxygen transport in peripheral tissues, and in case of low oxygen levels in tissues, NO is released for vasodilatory [47]. It is worth mentioning that NO may oxidize a small portion of hemoglobin to methemoglobin ($Fe^{2+} \rightarrow Fe^{3+}$) in red blood cells. It is assumed that a similar reaction comes from a remnant activity of globins' more ancient NO dioxygenase role. It is also observed the transport of NO by hemoglobin, in detail, the appropriate globin part; a specific cysteine residue (binding is reversible), is related to the

state (R or T) of the hemoglobin. As mentioned above, NO improves O_2 transport in the periphery and allows for respiration control. The *S*-nitrosylated hemoglobin allows for many NO-related activities like blood pressure, vascular resistance, and respiration control. Nitrogen oxide is not released in the cytoplasm of erythrocytes, instead carried out by an anion exchanger known as AE1 [48].

3. CO is also attached to the heme-binding site, similar to oxygen. Carbon monoxide comes from tobacco smoking, incomplete combustion in furnaces, and exhaust gas. This gas is odorless, tasteless, colorless, and toxic, described as a potentially fatal threat. It is estimated that hemoglobin's binding affinity for CO is 210 times greater than O_2 [49, 50]. Consequently, small amounts of CO dramatically decrease hemoglobin's ability to transport oxygen to the target tissue. The color of Hb-CO is a very bright red and is known as carboxyhemoglobin. It may change CO poisoning victims' skin to pink in death, not white or blue. Concentrations of CO > 0.02% induce headache and nausea; at higher as 0.1%, unconsciousness follows. It is observed that circa 20% of the oxygen-active sites may be blocked by CO in heavy smokers.
4. The toxic effects of the chemical compounds such as CN^-, SO, S^{2-}, and H_2S are similar to CO; they bind to iron ions, and which oxidation state has been unchanged. Also, oxygen-binding is blocked, causing toxicity.

As mentioned above, CO_2 is attached to a different site on the hemoglobin compared to O_2. Also, only a minority of carbon dioxide is transported by hemoglobin, and mostly as dissolved in deoxygenated blood – carbonic acid is formed in the presence of the enzyme carbonic anhydrase

$$CO_2 + H_2O \rightarrow H_2CO_3 \rightarrow HCO_3^- + H^+$$

According to Le Chatelier's principle, if any molecule can accept the proton produced, the reaction is shifted to the right. Histidine residues in hemoglobin may attach the protons and play the role of buffers. The phenomena related to increased affinity for carbon dioxide by the venous blood is defined as the Haldane effect

$$H^+ + HbO_2 \rightleftharpoons H^+Hb + O_2$$

CO_2 is attached to the α-amino group of amino acid residues (i.e., $–NH_2^+ + CO_2 \rightarrow –NH–CO–OH^+$) and protons – at various places on the protein. Dissolving of CO_2 in the blood causes the bicarbonate and hydrogen ions to appear, and pH is decreased (becomes more acidic) [51]. It is observed to bind protons and carbon dioxide to hemoglobin, and a conformational change in the protein part occurs. Also, the binding of carbon dioxide to hemoglobin and lowering of pH causes decreasing hemoglobin's affinity for oxygen. This phenomenon is described as the Bohr effect [52], and T state favoring rather than the R state is observed. In other words, the O_2-saturation curve is shifted to the right. In the lung capillaries, where carbon dioxide and protons are released from hemoglobin, in the presence of the enzyme, then increasing pH induces growth of the oxygen affinity in hemoglobin. Also, hemoglobin's total binding capacity to oxygen is reduced by lower pH, known as the root effect. It is assumed that more efficiency in binding (in lungs) or unloading (in tissues) of O_2 by hemoglobin is observed for the sigmoidal curve.

A higher concentration of 2,3-bisphosphoglycerate (2,3-BPG) in the blood of people living acclimated to high altitudes is observed. 2,3-BPG allows transporting a more significant amount of oxygen to tissues if oxygen tension is lower. Also, the R state of the protein part is favored in the presence of 2,3-BPG (then hemoglobin can bind oxygen more readily). The phenomena related to stimulating a molecule A by a molecule B by binding A to B to a transport molecule C is known as a heterotropic allosteric effect [53]. Humans must use other molecules than animals to attach oxygen to hemoglobin, and the O_2 affinity changes depending on conditions. For example, ATP and GTP are used both by fish. Also, ATP and GTP are bound to a phosphate "pocket" on their hemoglobin molecule. Then the tense state is stabilized, and oxygen affinity is lowered. The reduction of hemoglobin-oxygen affinity is higher for GTP than ATP, probably due to creating an additional hydrogen bonds to stabilize the tense state [54, 55]. It is interesting that the concentration of both ATP and GTP is lower in fish erythrocytes under hypoxic conditions for increasing oxygen affinity [56]. It is worth noting that higher blood Hb concentrations raise the O_2 capacitance coefficient [57].

Oxygen transport is observed from maternal blood to fetal blood in the placenta. Usually, a basic form of hemoglobin found in humans is $\alpha_2\beta_2$; however, another hemoglobin called fetal one (Hb F, $\alpha_2\gamma_2$) was found in the developing fetus. The affinity of Hb F is higher than adult hemoglobin, and at lower oxygen tension, a higher percentage of hemoglobin can attach oxygen. It is observed that the oxygen binding curve for fetal hemoglobin is left-shifted if we compare it to that of hemoglobin in the adult human.