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Reversible Oxygenation of Oxygen Transport Proteins

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A lecture demonstration illustrating changes in the visible spectra of oxygen transport proteins upon reversible oxygen binding is reported. The coloration upon exposure to oxygen is dramatic. Deoxyhemoglobin changes from purple to red, deoxyhemocyanin is transformed from colorless to blue, while colorless deoxyhemerythrin turns red. This demonstration illustrates an interesting example of a chemically reversible reaction and is used in the discussion of basic thermodynamic properties. A comparison of the physical characteristics of these oxygen storage and transport proteins is provided to illustrate the structural diversity associated with these proteins having a similar biological role.

Organisms with a high surface-area-to-volume ratio obtain oxygen by diffusion; however, for large organisms diffusion is too inefficient. Coevolution of a dioxygen transport system to support respiration in the inner tissues is characteristic of higher organisms. Three distinct dioxygen transport and storage proteins have evolved: hemoglobin (Hb) and its monomer myoglobin (Mb), hemerythrin (Hr), and hemocyanin (Hc). The dioxygen transport and storage proteins have different active sites as well as protein sequences that vary substantially between species.

The prefix "heme" is derived from the Greek word for blood. Thus, confusion arises since the term "heme group" is associated with an iron porphyrin system, which actually is found in hemoglobin, but not in hemerythrin or hemocyanin. The active-site structure and dioxygen-binding geometry of hemoglobin (1-3), hemerythrin (4-7), and hemocyanin (8, 9) has been reviewed extensively. Significant investigative efforts indicate that there is a wide range of active site structures found in oxygen transport metalloproteins, as illustrated in the table and Figure 1. One of the most interesting aspects of these dioxygen transport proteins is that the geometry of the dioxygen bond to the metal(s) differs in each case. There are two possible metal-dioxygen geometries in a 1:1 complex, the end-on (10) geometry and the side-on (11) structure. For 2:1 metal-dioxygen complexes, there

Comparison of Oxygen Transport Proteins

Property	Hemoglobin	Hemerythrin	Hemocyanin
metal	Fe	Fe	Cu
metal/dioxygen	1/1	2/1	2/1
metal coordination	porphyrin	protein	protein
number subunits	1, 4 to 180	1, 2, 3, 4, 8	6, 12 to 120
molecular weight	64,000 ^a	108,000 ^b	10 ⁷ ^c
color			
oxygenated	red	burgundy	blue
deoxygenated	purple	colorless	colorless
mL O ₂ /100 mL blood	9-25	2	2-8

^a Tetramer.

^b Octomer.

^c 150 subunits.

Figure 1. Active site structures for the oxygenated respiratory proteins: (a) hemoglobin and myoglobin, (b) hemerythrin, (c) hemocyanin. Histidine (H), aspartic acid (D), glutamic acid (E).

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are five possible bonding geometries, which are illustrated in Figure 2. The pressure required for one-half of the protein active sites to be coordinated ($P_{1/2}$) measures the binding efficiency. Figure 3 illustrates the range of $P_{1/2}$ values for each protein.

Comparison of Oxygen-Binding Proteins

Myoglobin and its tetramer, *hemoglobin*, can be found in almost all vertebrates, some invertebrates, and even legumes and yeasts. Human myoglobin consists of a single polypeptide chain (globin) of 153 amino acid residues. The active site contains one iron atom coordinated by a porphyrin (heme) with a histidine in the axial position. The intense purple color in the deoxygenated state is due to the conjugated porphyrin system and is shifted to red when oxygenated.

Figure 2. For proteins with two metal atoms in the active site, there are five possible dioxygen binding geometries (a through e). Hemerythrin binds dioxygen in geometry (a), and hemocyanin binds dioxygen with geometry (c).

Figure 3. Efficiencies of the respiratory protein compared by the range of dioxygen pressure in which half of the active sites are oxygenated, $P_{1/2}$. (Hb(T) and Hb(R) refer to hemoglobin before and after the protein conformation change. Myoglobin is similar to Hb(T).)

There are no X-ray studies providing an unequivocal bondlength for the bound dioxygen in oxyhemoglobin; however, the bond angle is known for both oxymyoglobin (12) and oxyhemoglobin (13). These studies indicate the dioxygen is bound end on as in Figure 1a. Much of our understanding of hemoglobin comes from simple model compounds such as the picket fence porphyrin (14), which exhibits thermodynamic and spectroscopic properties similar to the myoglobin and hemoglobin active sites.

Hemerythrin is isolated from the erythrocytes in the coelomic fluid of sipunculids (spoonworms), priapulids (tubeworms), brachiopods (lampshells), and polychaete annelids (15). Hemerythrin is usually found as an octomer of molecular weight 108,000 that can be converted to the monomer by chemical modification of the cysteine sulfhydryl group (16). Each subunit contains an active site with two iron atoms coordinated directly to the protein amino acids that participate in binding dioxygen. Upon binding dioxygen, the colorless ferrous deoxyhemerythrin turns a bright salmon red. There are no X-ray structures of the oxygenated protein; however, resonance Raman studies indicate the dioxygen is bound to one iron atom in an end-on fashion (Fig. 2a) (17-19). X-ray crystallographic studies of azido-methemerythrin indicate the iron atoms are bridged by two amino acid carboxyl groups with an oxobridge between the iron atoms (Fig. 1b).

Hemocyanin can be found in the blood of gastropods (abalone, whelk, snails, slugs), cephalopods (squids, octopi, cuttlefish), crustaceans (lobsters, shrimp, crabs), arachnids (spiders, scorpions), and bivalve mollusks. Hemocyanin is an exceptionally large molecule with a molecular weight of up to 10^6 amu. Both copper atoms in the active site of hemocyanin are important in the binding of dioxygen. In the oxygenated state the protein is blue and in the deoxygenated state it is colorless. The study of several met (irreversibly oxidized proteins) forms (20-23) support an active site geometry shown in Figure 1c where the copper atoms in the deoxygenated protein are coordinated by three histidine groups and a phenolate (R group) from tyrosine. The copper atoms in the oxygenated protein are in a nearly square pyramidal environment, and the coordination of the copper atoms changes from four to five upon oxygenation. Resonance Raman (24) and electronic (23) studies indicate dioxygen is bound by both copper atoms (see Fig. 2c).

Cooperativity

The most striking feature of hemoglobin is its cooperative binding of dioxygen. Once a subunit of hemoglobin binds an oxygen molecule, it is thought the accompanying distortion of the active site causes potential energy to build in the protein. When a sufficient number of subunits bind dioxygen, the accumulated potential energy triggers a protein conformation change. The remaining deoxygenated subunits exhibit a significant increase in oxygen affinity resulting in a cooperative affect for dioxygen binding (2, 25). The degree of cooperativity in hemoglobin depends on numerous factors, including pH, ionic strength, and the concentration of anions such as 2,3-diphosphoglycerate, chloride, and ATP. Since the mechanism for cooperativity in hemocyanin is thought to be significantly different from that for hemoglobin (8, 26, 27), other factors are likely to be important. However, cooperativity is not yet demonstrated for hemerythrin and certain hemocyanin proteins.

Preparation of Materials

General

All glassware and water used is sterilized. Caution should be employed while handling the scalpel and broken whelk shells. Broken whelk shells are sharp and the bacteria on them can cause infection.

Isolation of the Bloods

Hemoglobin from bovine blood is obtained from a veterinary clinic; 30 ml is filtered through a coarse glass frit and diluted with 60 mL sterilized deionized water.

Hemerythrin is obtained from the sipunculid marine worm *Phascolopsis gouldii*² by placing them in a tray and making an incision toward the center of the body and draining the coelomic fluid. The fluid from six worms, about 18 mL, is filtered through a coarse glass frit and diluted with 30 mL of sterilized, deionized water.

Hemocyanin is obtained from American whelks *Busycon canaliculatum*² and is obtained by placing the whelk upside down in a cool moist place for 4–6 h to drain the sea water. The whelk is bled by making a large incision parallel to the foot and draining the ensuing fluid into a clean tray. The foot is the tough, cartilagelike part protruding from the shell. Three whelks provide about 36 mL of blood, which is subsequently diluted with 20 mL of sterilized, deionized water.

Demonstration

The blood obtained is oxygenated. Completely deoxygenating the protein is accomplished by the rapid bubbling of argon or nitrogen through the solution for 45 min. Avoid excess foaming of the blood solutions. Placing a hypodermic syringe needle at the bottom of the blood–water solution contained in an Erlenmeyer flask fitted with a rubber septum cap provides a satisfactory arrangement. The syringe needle is attached to the gas supply by Tygon tubing. The container is vented by a short syringe needle with the tip placed well above the solution. Oxygen or air is bubbled through the solution to effect oxygenation.

The color change associated with oxygenation occurs within a minute. After noting the color changes, the reversibility of dioxygen binding can be demonstrated during the course of the lecture by bubbling nitrogen or argon through the solutions as described above. It takes about 15 min to deoxygenate these proteins to the point where reoxygenation creates a noticeable color change. To illustrate the differences, the UV–vis spectra of the three proteins in the oxygenated and deoxygenated state can be presented.

This procedure can be repeated many times before any loss of color for the oxy- and deoxy-forms due to denaturation of the protein. The protein solutions can be saved for

² These animals are obtained from Marine Biological Labs, Woods Hole, MA. Suitable animals may also be obtained from Pacific Bio-Marine Labs, Venice, CA.

several months by storing the solutions in a freezer at –15 °C. They will survive up to three freeze–thaw cycles.

Conclusions

Since many chemistry students have biological interests, a lecture demonstration emphasizing biologically active compounds is appropriate. This demonstration can be supplemented by observing the colorimetric effects of an oxidant on ferrous and cuprous ions. The cost of this demonstration is less than \$30, and some materials can be reused. Several experiments using hemoglobin and myoglobin suitable for introductory, organic, or analytical chemistry lab courses have been developed (28–36).

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Responsibility

Experiments, laboratory exercises, lecture demonstrations, and other descriptions of the use of chemicals, apparatus, and instruments are presented in *this Journal* as illustrative of new, novel, or improved ideas or concepts in chemistry instruction and are directed at qualified teachers. Although every effort is made to assure and encourage safe practices and safe use of chemicals, the *Journal of Chemical Education* cannot assume responsibility for uses made of its published materials. We strongly urge all those planning to use materials from our pages to make choices and to develop procedures for laboratory and classroom safety in accordance with local needs and situations.