This student paper was written as an assignment in the graduate course

Free Radicals in Biology and Medicine

(77:222, Spring 2005)

offered by the

Free Radical and Radiation Biology Program
B-180 Med Labs
The University of Iowa
Iowa City, IA 52242-1181
Spring 2005 Term

Instructors:
GARRY R. BUETTNER, Ph.D.
LARRY W. OBERLEY, Ph.D.

with guest lectures from:
Drs. Freya Q . Schafer, Douglas R. Spitz, and Frederick E. Domann

The Fine Print:

Because this is a paper written by a beginning student as an assignment, there are no guarantees that everything is absolutely correct and accurate.

In view of the possibility of human error or changes in our knowledge due to continued research, neither the author nor The University of Iowa nor any other party who has been involved in the preparation or publication of this work warrants that the information contained herein is in every respect accurate or complete, and they are not responsible for any errors or omissions or for the results obtained from the use of such information. Readers are encouraged to confirm the information contained herein with other sources.

All material contained in this paper is copyright of the author, or the owner of the source that the material was taken from. This work is not intended as a threat to the ownership of said copyrights.

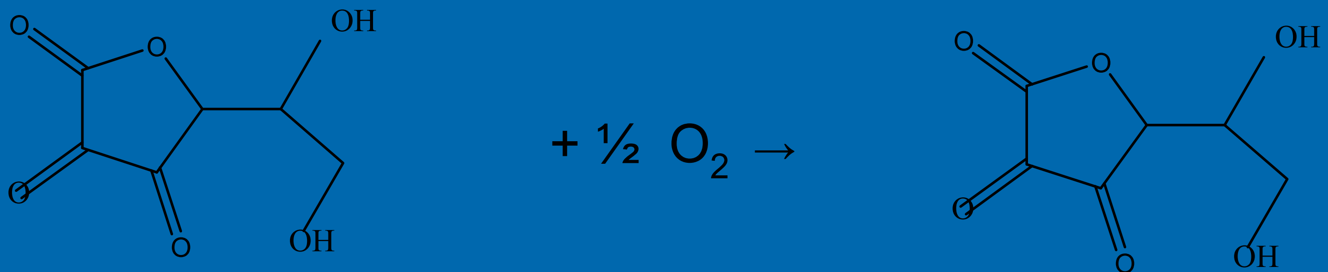
Ascorbate Oxidase

An important plant enzyme

By Irma Nydegger

The function of AO

AO is found in plants and it catalyzes the oxidation of ascorbic acid (AA) to dehydroascorbic acid (DHA) and the four-electron reduction of O_2 to produce water according to the reaction scheme below [1, 2].



AO and cell metabolism

- Ascorbic acid (AA) is believed to be the most important defense mechanism system of plants against oxidative stress caused by pollutants such as SO_2, NO_2 and oxidants like ozone [3-4].
- AA in plants is mainly found in the cytoplasm, but it gets transported to the apoplast where it can reach mM ranges of concentration [2-4].
- AO converts AA to monodehydroascorbate (MDHA, also known as ascorbate radical $Asc^{\bullet -}$), by a one-electron reduction. MDHA dismutates rapidly to give dehydroascorbic acid (DHA) and AA [2-4].
- DHA then gets transported into the cytosol by plasma membrane carriers ensuring a continuous flow of reducing power in the cell wall [2-4].

AO and cell expansion

- AO is believed to play a role in cell elongation and differentiation [2-4].
- AO regulates ascorbic acid levels, which in turn stimulates the production of hydroxyl radicals that cause cleavage of cell wall polysaccharides and hence cell elongation [2-4].
- AO can also catalyze the formation of MDHA, which causes an increase in cell wall acidity by influencing the H^+-ATPase levels [2].

AO and fruit development

- Fruit ripening has been linked to an increase in activity of reactive oxygen species (ROS) and a decrease in AA levels [2].

- An increase in AO activity is observed in the last ripening phase of melon fruit [2].

- In zucchini a loss of activity of AO is observed during fruit ripening [2].

- Fruit development and AO activity seem to be dependent on the type of plant [2].

The structure of Ascorbate Oxidase

- Ascorbate oxidase (AO) belongs to the family of blue copper proteins and is a homodimer with a molecular weight of approximately 70 kDa per monomer [1, 2, 5].

- The active site of each monomer comprises of a Type I mononuclear Cu that is 12.5 Å away from a trinuclear cluster containing Type II and Type III Cu atoms [2].

- Type I Cu proteins in general exhibit a strong blue color which corresponds to a charge transfer from a thiol containing group (in ascorbate oxidase it would be either Cysteine or Methionine) to the Cu(II). Their main function is electron transfer‡.

- Type II Cu proteins have Cu centers that have either square planar or pyramidal geometry and coordinate either an oxygen or nitrogen‡.

- Type III Cu proteins contain a pair of Cu atoms that exhibit no EPR activity‡.

‡From http://www.chem.qmul.ac.uk/iubmb/etp/etp6t11.html accessed on 3/16/05

Structure of AO continued…

- AO contains Type I, II and III Cu in its active site.
- Type I Cu gives ascorbate oxidase the blue color and is coordinated to one methionine, one cysteine and two histidine ligands [2].
- The two Type III Cu atoms coordinate to six histidine residues and contain an OH^- (or O_2^-) bridge. The strong coupling of the atoms caused by the OH^- bridge does not allow this center to be EPR active [2].
- The three Type II Cu atoms are approximately 3.7 Å from each other and they are trigonally coordinated to two histidine ligands and an OH^- (or H_2O) ligand [2].
- The reduction potentials of the Type I and III Cu at 298 K and pH 7.0 are the same and they are measured to be approximately 350 mV [5].

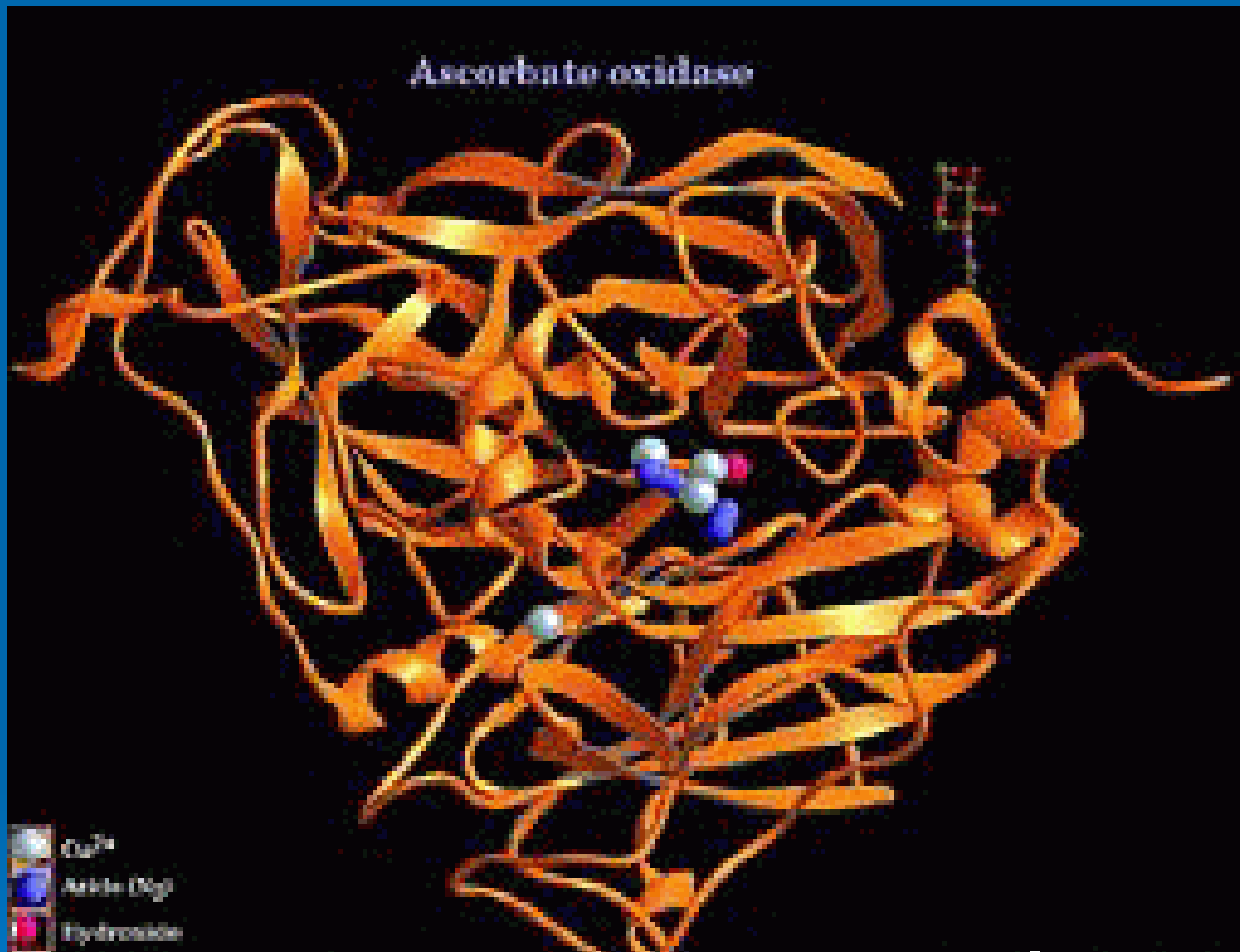


Figure 1: AO 3D structure. Light blue balls represent the coppers and the pink ball represents the hydroxide bridge of the Type II & III trinuclear copper cluster.
(from http://chem.ch.huji.ac.il/~eugeniik/mediator12.htm accessed on 3/16/05)

Intramolecular electron transfer

- Part of the catalytic cycle of AO consists of several electron transfer steps [6].
- The first step of this electron transfer occurs when an electron is transferred from the substrate (ascorbic acid or any other reducing agents) to the Type I Cu reducing it from $Cu^{(II)}$ to $Cu^{(I)}$ [2, 5, 6, 7].
- The second step consists of the electron transfer from the Type I Cu center to the Type III Cu center [2 , 5, 6, 7].
- The O_2 as a substrate is coordinated to the Type III Cu center. The electrons flow from the Type III Cu center to the O_2 reducing it to water [2 , 5, 6, 7].
- A possible through bond mechanism of electron transfer is proposed to be from the Type I Cu to the Cys-509 that is coordinated to this copper and then to His-508 or His-510, which are coordinated to the Type III Cu [7].

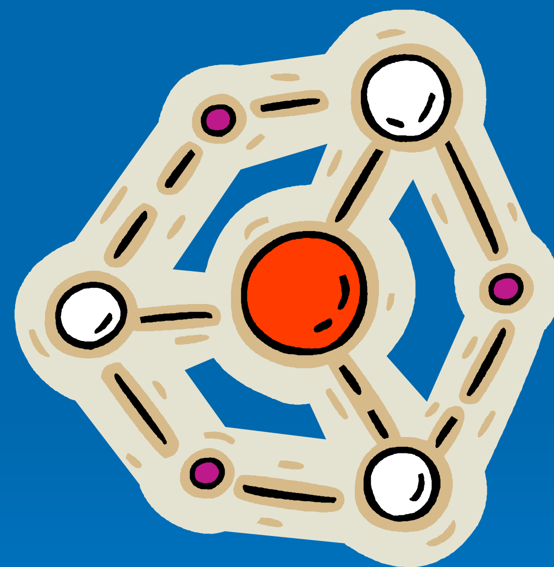
O_2 and Intramolecular Electron Transfer

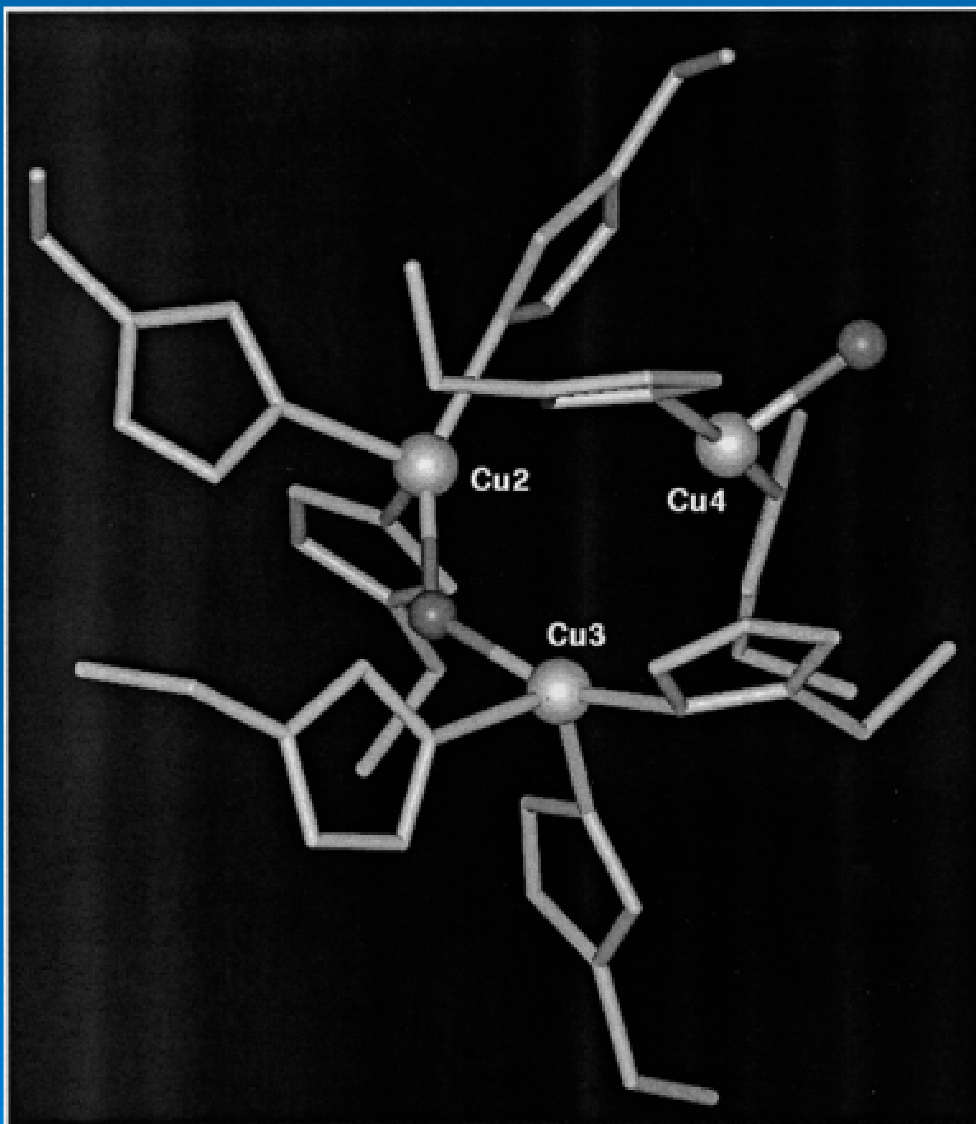
- The electron transfer in AO depends on the medium and the substrate [5-7].
- In excess O_2 the intramolecular electron transfer between Type I and Type III Cu is increased [7].
- The rate constant of the electron transfer under aerobic conditions

  (k =1100 ± 300 s^{-1}) is five times the rate constant under anaerobic conditions [7].
- A difference of 15 mV in reduction potentials of Type I and III Cu is observed (under anaerobic conditions) after the initial reduction of the Type I Cu by a substrate. This difference in reduction potential can jump up to 100 mV under aerobic conditions [7].

N_3^- and Intramolecular Electron Transfer

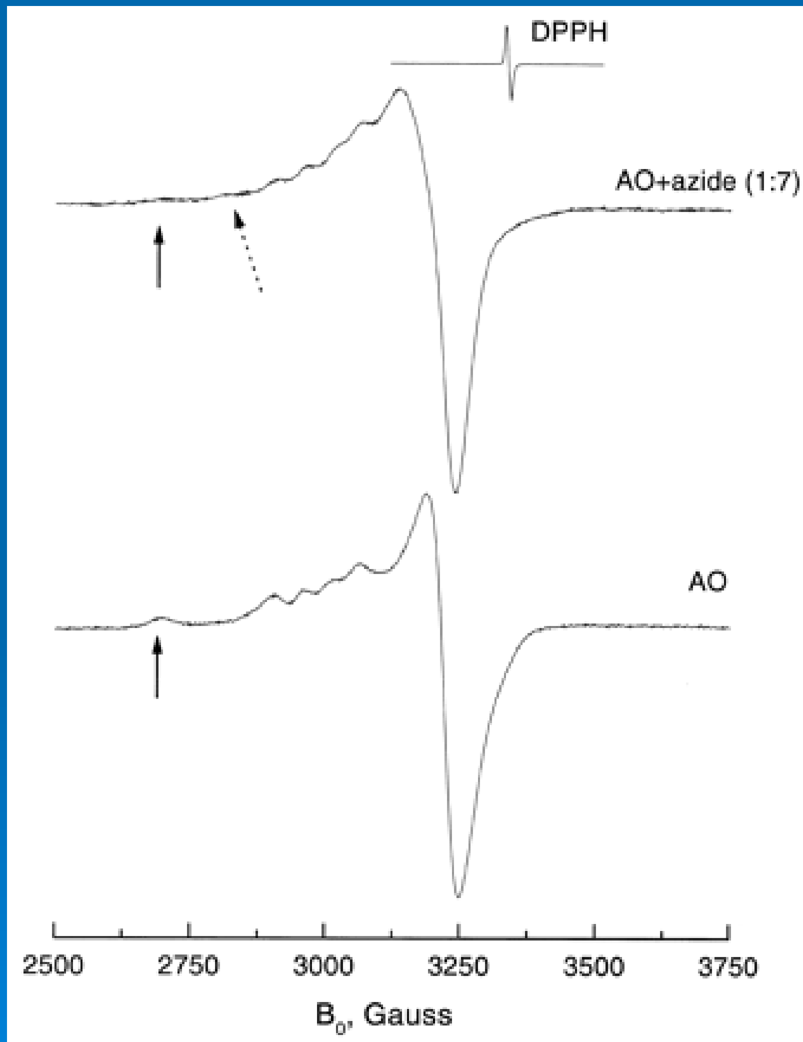
- Azide reacts with AO forming three different species [8].
- The first species is formed when the azide binds to either one or both of the Type III Cu ions and disturbs their anti-ferromagnetic coupling [8].
- The second species is formed when the azide binds to the Type II Cu and it does not disrupt the coupling of the Type III Cu pair [8].
- In the third species the azide either does not bind to the centers at all, or it binds to the Type III Cu pair without disturbing the EPR signal [6]. The binding of azide to Type III Cu removes the bridging oxygen of OH^- (or H_2O) [8].





- The 3-D structure of the trinuclear copper center in AO. Cu2 and Cu3 form the Type III Cu center and Cu4 represents Type II Cu. Adapted from [8].

EPR spectra of AO and AO-Azide



- CW-EPR spectra of AO and AO-Azide (1:7) solutions (1.3 mM, pH = 6). Measurements were made at 80 K and *v* = 9.277 GHz. The solid arrows represent the position where the Type II Cu is the only contributor to the EPR signal and the dotted arrow points to the appearance of new signals. Adapted from [8].

Enzyme activity and substrates

- **The activity of AO is influenced by the type of substrate that binds to it. O_2, as mentioned before, is shown to enhance the enzyme's activity.**

- **Treatment of AO with ferricyanide will decrease its reactivity because the cyanide will bind to the Type III Cu center disrupting the electron transfer from Type I to Type III Cu [2, 9].**

- **Steady state activity of AO seems to be inhibited by several metal ions including Cu^{2+}, Au^{3+}, Ni^{2+}, Pd^{2+}, Pt^{2+} and Ag^{+}. Ag^{+} is shown to form an Ag^{+}-AO complex preventing the intramolecular electron transfer between Type I and III Cu from occurring [2].**

- **Any other cations or anions that can fit into the active site of the enzyme and bind to the trinuclear cluster of Type II and III Cu will inhibit the intramolecular electron transfer thus inhibiting the enzyme activity [2].**

A little bit of history

- Purified AO from the cortex of Japanese cucumbers was used in the 1960s to determine kinetic rates in the formation of ascorbic acid free radical [10].

- Ascorbic acid transfers one electron to AO forming the ascorbate radical [10].

- Ascorbate radical dismutates to produce ascorbate and dehydroascorbic acid [10].

Monitoring the formation of ascorbate radical by AO

Ascorbate Oxidase reaction (adapted from [10]).

AO concentration based on Cu (M)	v ($M \times s^{-1}$)	Ascorbate Radical concentration (M)	k_d/K ($M^{-1} \times s^{-1}$)
4×10^{-8}	2.75×10^{-5}	5.4×10^{-7}	4.7×10^{7}
2×10^{-8}	1.6×10^{-5}	4.4×10^{-7}	4.1×10^{7}

Mean Value of $k_d/K = 4.4 \times 10^{7}\ M^{-1}\ s^{-1}$

Summary

- AO is a plant enzyme that catalyzes the oxidation of AA to DHA followed by the four electron reduction of O_2 to H_2O.
- AO activity is linked to cell elongation and differentiation as well as fruit development in plants.
- AO contains Type I, II and III Cu in its active site. Extensive intramolecular electron transfer studies have shown that Type I Cu is the electron acceptor (from the substrate) and Type III Cu is the reducing site for O_2.
- Intramolecular electron transfer can be influenced by the surrounding environment.

References

1. Asard H, May JM, Smirnoff N. (2004) The role of ascorbic acid in defense networks and signaling in plants, and Ascorbate and plant growth: from germination to cell death. In: Asard H, May JM, Smirnoff N, ed. *Vitamin C: functions and biochemistry in animals and plants*. Oxon, UK: Garland Science/BIOS Scientific Publishers. **4&5**:65-92.

2. Santagostini L, Gullotti M, Hazzard JT, Maritano S, Tollin G, Marchesini A. (2005) Inhibition of intramolecular electron transfer in ascorbate oxidase by Ag+: redox state dependent binding. *J Inorg Biochem*. **99**: 600-605.

3. Pignocchi C, Fletcher JM, Wilkinson JE, Barnes JD, Foyer CH. (2003) The function of ascorbate oxidase in tobacco. *Plant Phys*. **132**: 1631-1641.

4. Smirnoff N. (1996) The function and metabolism of ascorbic acid in plants. *Annal Bot*. **78**: 661-669.

5. Farver O, Pecht I. (1992) Low activation barriers characterize intramolecular electron transfer in ascorbate oxidase. *Proc Natl Acad Sci USA*. **89**: 8283-8287.

6. Farver O, Pecht I. (1997) The role of the medium in long-range electron transfer. *J Bio Inorg Chem*. **2**: 387-392. Gromov I, Marchesini A, Farver O, Pecht I, Goldfarb D. (1999) Azide binding to the trinuclear copper center in laccase and ascorbate oxidase. *Eur J Biochem*. **266**: 820-830.

7. Farver O, Wherland S, Pecht I. (1994) Intramolecular electron transfer in ascorbate oxidase is enhanced in the presence of oxygen. *J Biol Chem*. **269**(37): 22933-22936.

8. Gromov I, Marchesini A, Farver O, Pecht I, Goldfarb D. (1999) Azide binding to the trinuclear copper center in laccase and ascorbate oxidase. *Eur J Biochem.* **266**: 820-830.

9. Meyer TE, Marchesini A, Cusanovich MA, Tollin G. (1990) Direct measurement of intramolecular electron transfer between Type I and Type III copper centers in the multi-copper enzyme ascorbate oxidase and its Type II copper-depleted and cyanide-inhibited forms. *J Biochem.* **30**: 4619-4623.

10. Yamazaki I, Pietter LH. (1961) Mechanism of free radical formation and disappearance during the ascorbic acid oxidase and peroxidase reactions. *Biochim Biophys Acta.* **50**: 62-69.