## O20

## X-ray Absorption Studies of Laccase and Ceruloplasmin

DARLENE J. SPIRA, ARTURO G. PORRAS, EDWARD I. SOLOMON*, JAMES E. HAHN, MAN SUNG CO and KEITH O. HODGSON*

*Department of Chemistry, Stanford University, Stanford, Calif. 94305, U.S.A.*

Determination of oxidation state is essential to interpretation of the spectroscopy and chemistry of metal ions. We will describe the use of X-ray absorption edges to quantitatively determine the percent of Cu(I) in samples of mixed Cu(I)/Cu(II) composition, and specific applications of this technique to characterization of copper oxidation states in various derivatives of the multicopper oxidases laccase and ceruloplasmin.

It has long been recognized that the energy and the shape of an X-ray absorption edge are correlated, respectively, with the effective charge on the absorbing atom and with the geometry of the absorbing site. Cu complexes, in particular, have X-ray absorption edges which change dramatically with metal oxidation state. Cuprous complexes show an intense transition at approximately 8984 eV which is absent for complexes in the +2 oxidation state. In addition, the peak absorbance for Cu(I) complexes is less intense than for Cu(II) complexes. The different edge shapes for Cu(I) and Cu(II) have previously been used as a qualitative indicator of the presence of Cu(I) in a variety of metalloproteins [1–4], and in particular, in a study of the multicopper oxidase, *Rhus vernicifera* laccase [5].

Laccase, which contains four copper ions at its active site, is the least complicated of the multicopper oxidases and is the current focus of our binuclear copper research. While study of the coupled binuclear site in this enzyme is complicated by the presence of blue and normal copper centers, a reversible procedure exists for T2 copper removal to yield type 2 depleted laccase (T2D) [6], wherein the binuclear site is only complicated by the additional blue copper site. Much controversy has ensued over the chemical and spectroscopic properties of the prepared T2D laccase [7]. Our preliminary X-ray absorption edge studies [5] indicated that T2D contained approximately 70% cuprous copper, while $H_2O_2$ treated (met) T2D and native laccase contained essentially no Cu(I). Together with EPR evidence that the T1 copper remains oxidized in T2D laccase, these edge studies demonstrated that T2D laccase contains a reduced T3 site which can be reoxidized by peroxide.

We will present evidence that the properly normalized difference between two Cu X-ray absorption edges, *under certain circumstances*, can also be used to *quantitatively* determine the Cu(I) concentration in a sample [8]. We have studied an extensive series of Cu model complexes in order to determine the limitation of the difference-edge technique. We have then used this technique to quantitatively determine the Cu(I) concentration in native and T2D laccase as well as their ferricyanide, nitrite, and hydrogen peroxide treated forms. Preliminary results on ceruloplasmin will also be presented.

An Extended X-ray Absorption Fine Structure (EXAFS) study of met and met-$N_3^-$ T2D laccase is also discussed. The EXAFS data are analysed to determine the average environment of the three Cu atoms in the T2D laccase derivatives, and also compared with the EXAFS data for plastocyanin, which is an appropriate model for the blue copper site in laccase [9].

*Acknowledgement.* This work was supported by grants from the National Institutes of Health (NIH) (AM 31450-1, to EIS) and the National Science Foundation (NSF) (PCM 82-08115, to KOH). JEH is the recipient of an NSF predoctoral fellowship. The synchrotron radiation used in these studies was provided by the Stanford Synchrotron Radiation Laboratory which is supported by the Department of Energy, Office of Basic Energy Sciences; the NSF Division of Materials Research; and the NIH Biotechnology Resource Program, Division of Research Resources.

1 V. W. Hu, S. I. Chan and G. S. Brown, *Proc. Nat. Acad. Sci., U.S.A., 74,* 3821–3825 (1977);
V. W. Hu, S. I. Chan and G. S. Brown, *FEBS Letts., 84,* 287–290 (1977).
2 L. Powers, W. E. Blumberg, Y. Ching, P. Eisenberger, B. Chance, C. Barlow, J. S. Leigh, Jr. J. C. Smith, T. Yonetani, J. Brown, T. Spiro, J. Peisach, S. Vik, J. Hastings and M. Perlman, *Biophys. J., 25,* 43a (1979);
L. Powers, W. E. Blumberg, B. Chance, C. Barlow, J. S. Leigh, Jr., J. C. Smith, T. Yonetani, S. Vik and J. Peisach, *Biochem. biophys. Acta, 546,* 520–538 (1979).
3 W. E. Blumberg, J. Peisach, P. Eisenberger and J. A. Fee, *Biochemistry, 17,* 1842–1846 (1978).
4 J. M. Brown, L. Powers, B. Kincaid, J. A. Larrabee and T. B. Spiro, *J. Am. Chem. Soc., 102,* 4210–4216 (1980).
5 C. D. LuBien, M. E. Winkler, T. J. Thamann, R. A. Scott, M. S. Co, K. O. Hodgson and E. I. Solomon, *J. Am. Chem. Soc., 103,* 7014–7016 (1981).
6 M. T. Graziani, L. Morpurgo, G. Rotilio and B. Mondovi, *FEBS Lett., 70,* 87–90 (1976).
7 L. Morpurgo, M. T. Graziani, A. Finazzi-Agrò, G. Rotilio and B. Mondovì, *Biochem. J., 187,* 361–366 (1980).
B. Reinhammar and Y. Oda, *J. Inorg. Biochem., 11,* 115–127 (1979);
L. Morpurgo, A. Desideri, G. Rotilio and B. Mondovì, *FEBS Lett., 113,* 153–156 (1980).
8 D. Spira, J. E. Hahn, M. S. Co, K. O. Hodgson and E. I. Solomon, *Biochem. Biophys. Res. Comm.,* (1983) in press.

9 J. E. Hahn, M. S. Co, K. O. Hodgson, D. Spiro and E. I. Solomon, *Biochem. Biophys. Res. Comm.*, (1983) in press.

## O21

## Ceruloplasmin–Diethyldithiocarbamate (DTC) Interaction. Evidence of DTC Chelation to Two Non Enzymatic Cu(II)

MARTINE HERVE, ARLETTE GARNIER

*Laboratoire de Chimie Bioinorganique, UER de Médecine et Biologie Humaine, Université Paris XIII, 93000 Bobigny, France*

and L. TOSI

*Département de Recherches Physiques, L.A. No. 71, Université Pierre et Marie Curie, 75230 Paris Cedex 05, France*

Ceruloplasmin (Cp) is a plasma copper oxidase containing several copper ions. Among them five are essential to enzymatic activity and are classified into three types [1]: two cupric ions are of type I ($I_a$ and $I_b$ [2]) or 'blue', one cupric ion of type II or 'non blue' and two cupric ions of type III which are not EPR detectable.

In this communication we report evidence suggesting the presence of two extra cupric ions that can bind chelating agents such as diethyldithiocarbamate (DTC).

The addition of DTC to Cp (pH 5.5) yields an absorption at 445 nm in the Cp spectrum. When increasing equivalents of DTC are reacted with the protein no further spectral changes are appreciable at DTC/Cp $>$ 4 and the absorptivity at 445 nm indicates the chelation of two Cu(II) forming two $Cu(DTC)_2$. The absorption at 610 nm is not modified and the circular dichroism (CD) spectrum exhibits only a slight decrease. On the other hand the band at 445 nm due to the DTC $\rightarrow Cu^{2+}$ charge transfer transition is not optically active indicating that the $Cu(DTC)_2$ complex is not coordinated to the protein. Addition of an inhibitor, such as $N_3^-$, to a Cp–DTC solution gives rise to the absorption at 380 nm and to the CD spectrum characteristic of $N_3^-$ bound to type II Cu(II). Moreover, the Cp–DTC solution exhibits the same enzymatic activity as Cp free of DTC.

From these results we can infer that the five cupric ions necessary to the enzymatic activity of Cp are not modified by the addition of DTC which instead chelates two extra cupric ions hereafter labeled X and Y.

Both X and Y Cu(II) can be removed from Cp, by centrifugation, after addition of DTC, if this is performed within ten minutes following the addition of DTC. On the other hand, when Cp is incubated for several hours with DTC a very slight precipitate appears and the solution recovers the blue color of native Cp. If DTC is then added to the supernatant the absorption at 445 nm due to both DTC-bound X and Y reappears. These results suggest that as time elapses the $Cu(DTC)_2$ complexes dissociate and that X and Y Cu(II) reenter the protein.

1 M. A. Foster, T. Pocklington and A. A. Dawson, in 'Metal Ions in Biological Systems', Vol. 10, 129–166 (1980).
2 M. Hervé, A. Garnier, L. Tosi and M. Steinbuch, *Eur. J. Biochem., 116,* 177 (1981).

## O22

## The Active Site of Bovine Serum Amine Oxidase

SHINNICHIRO SUZUKI, TAKESHI SAKURAI, AKITSUGU NAKAHARA

*College of General Education, Osaka University, 1-1 Machikaneyama-cho, Toyonaka, Osaka 560, Japan*

TAKASHI MANABE and TSUNEO OKUYAMA

*Department of Chemistry, Faculty of Science, Tokyo Metropolitan University, Fukazawa, Setagaya-ku, Tokyo 158, Japan*

Bovine serum amine oxidase (BSAO) is a type II copper-containing protein. The enzyme has a molecular weight of 190 000 daltons and 2 gram atoms of copper per mol protein which is composed of two electrophoretically equivalent subunits. Native BSAO exhibits a yellowish pink color by virtue of a broad band around 476 nm ($\epsilon$ = 3800) [1]. Replacement of Co(II) [2], Ni(II) and Zn(II) for the inherent Cu(II) which is coordinated by two *cis*-arranged imidazole nitrogens and two oxygens [3] revealed that the pink color comes from the organic prosthetic group. Electronic, CD and ESR spectra of native and metal substituted BSAO treated with phenylhydrazine suggested that the unknown prosthetic group is located very close to the copper(II) ion, but probably is not directly coordinated to the metal ion. The presence of a metal ion affects sensitively the electronic state of the prosthetic group whose role is considered to bind a substrate and catalyze the subsequent deamination. At the following step, two electrons are passed from the reduced chromophore to molecular oxygen probably through the copper ion. Combined cooperation of the chromophore and copper(II) ion during the whole enzymatic process corresponds to the catalytic role of a flavin group. We are now working on the prosthetic group to clarify its structure and properties