Vol. 112, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 29, 1983 Pages 737-745

QUANTITATIVE CU(I) DETERMINATION USING X-RAY ABSORPTION EDGE SPECTROSCOPY: OXIDATION OF THE REDUCED BINUCLEAR COPPER SITE IN TYPE 2 DEPLETED RHUS LACCASE

James E. Hahn, Man Sung Co, Darlene J. Spira,
Keith O. Hodgson*, and Edward I. Solomon*

Department of Chemistry, Stanford University, Stanford, CA 94305

Received March 22, 1983

We report a procedure, through difference comparison of X-ray absorption edge spectra, for the quantitative determination of Cu(I) content in copper complexes of mixed oxidation state composition. This technique is tested on copper model systems and then used to quantitatively determine that untreated T2D Rhus laccase contains 70±15% Cu(I). Whereas excess ferricyanide is demonstrated not to alter the Cu(I) content of the untreated T2D, aqueous peroxide and nitrite at pH 6.0 are shown to oxidize the cuprous type 3 site and generate met T2D protein forms.

An important derivative of Rhus laccase [1] (one Blue or T1, one normal or T2 and one coupled binuclear or T3 center) has been prepared [2] in which the T2 copper is selectively removed (type 2 depleted: T2D). Chemical perturbation and spectroscopic study of this simplified protein form have enabled us to further prepare and define a series of stable T3 binuclear copper derivatives of this T2D laccase. In the presence of an oxidized T1 center, protein forms are now accessible wherein the T3 coppers are reduced [3], mixed valent [4], and oxidized [3], which are defined, in analogy to hemocyanin and tyrosinase chemistry [5,6], as untreated or deoxy, half-met, and met T2D, respectively. However, anion binding studies [4,7] of these derivatives have indicated that, in strong contrast to hemocyanin and tyrosinase, exogenous ligands do not appear to bridge at the coupled binuclear copper site in laccase.

Procedural variations, which apparently relate to pH [8] and perhaps extent of metal removal [8-10], by different groups in preparing the

*To whom all correspondence should be addressed.

0006-291X/83 $1.50

simplified T2D protein form have added significant complication to this derivative. The resultant T2D forms display different physical properties and chemical reactivity [2,8,10-13], the incomplete characterization of which has further confused the problem. Determination of copper ion oxidation state is a critical point in understanding these initial differences in T2D, as both Cu(I) and the coupled binuclear cupric T3 site lack characteristic optical and EPR spectral features. In our preliminary X-ray absorption spectroscopy (XAS) edge studies [3], this was accomplished on the T2D derivative, which we prepare in 0.05 M NaOAc at pH 5.2, following the procedure of Graziani et al. [2] (untreated T2D) and which exhibits physical properties and reactivity as described in their literature [2,8,10,11,14]. XAS can be used to directly probe copper ion oxidation state[3], and showed this T2D protein to contain a reduced T3 site and an oxidized T1 site.

Herein we develop a procedure which, under certain circumstances, allows us to quantitatively determine Cu(I) concentrations in samples of mixed Cu(I)/Cu(II) composition. This technique is tested on copper model systems and then used to quantitate Cu(I) content in a number of important reactions of this well characterized (T3:[Cu(I)Cu(I)]; T1:[Cu(II)]) untreated T2D laccase derivative. Aqueous H_2O_2 and HNO_2 at pH 6.0 are shown to oxidize the cuprous T3 site in T2D and generate met proteins forms containing coupled binuclear cupric centers. Finally, while reaction of T2D with excess $Fe(CN)_6^{3-}$ has been reported [13] to result in a fully oxidized T2D laccase form, quantitative edge analysis demonstrates that $Fe(CN)_6^{3-}$ does not alter the ~70% Cu(I) composition of the untreated T2D.

MATERIALS AND METHODS

All XAS edges were measured at the Stanford Synchrotron Radiation Laboratory using a Si[220] double crystal monochromator. To ensure consistent energy determinations, the internal calibration method [15] was used. To allow proper normalization, the absorption was measured for at least 300 eV below and 200 eV above the Cu K edge. At least two scans were averaged for each sample, after which the pre-edge region was fitted with a second-order polynomial which was subtracted from the data to correct for residual absorption.

Laccase was purified [12,16] from the acetone powder (Saito and Co.,Japan) of Rhus vernicifera. Type 2 copper was removed by the modified [10] procedure of Graziani [2]. Reagent grade chemicals were used in 0.1 M potassium phosphate buffer, pH 6.0.

RESULTS AND DISCUSSION

Quantitative XAS Edge Analysis: Technique and Application to Model Complexes.

Copper complexes in the +1 oxidation state show an intense transition at ~8984 eV which is absent for complexes in the +2 oxidation state [17]. While this change in edge shape relates to geometric and electronic structural differences which are yet to be fully elucidated, it can be used as a qualitative indicator of the presence of Cu(I) in a variety of metalloproteins [3,18-21]. However, due to difficulties in normalization, background removal, and effects of instrumental resolution on edge structure, there have been few attempts to use X-ray absorption edges quantitatively [22].

In the absence of EXAFS effects, the absorbance above the edge would be proportional to [Cu] in a sample and could be used to normalize edge spectra [18]. Rather than normalize based on the absorbance at a single energy, we fit a straight line to the EXAFS region (starting at ~9050 eV) and extrapolate this line to 9000 eV to avoid any error caused by incomplete background removal. The data are normalized by scaling the pre-edge subtracted data to give an extrapolated absorbance of 1.0 at 9000 eV. This linear fit removes any potential normalization errors due to the EXAFS modulations.

Background removal is necessary since the 8984 eV transition is not resolved, but occurs as a shoulder on a rapidly increasing absorption background. The apparent area of the 8984 eV transition thus depends on the background which is used to model the intense transition. Our difference technique, avoids this difficulty by not requiring calculation of this area.

Resolution has been suggested as being responsible for apparent differences in the edge structure of nominally identical samples [23]. Since the current data were all collected with significantly better resolution (~2eV) than the width of the 8984 eV transition (FWHM ~ 5eV), small resolution differences between data sets should not affect our results.

Examination of the properly normalized edges reveals that in addition to the 8984 eV transition, Cu(I) edges differ from Cu(II) edges by having a weaker peak absorption at ~9000 eV. Subtraction of a typical Cu(II) edge from a Cu(I) edge thus yields the characteristic derivative pattern shown in Fig. 1.

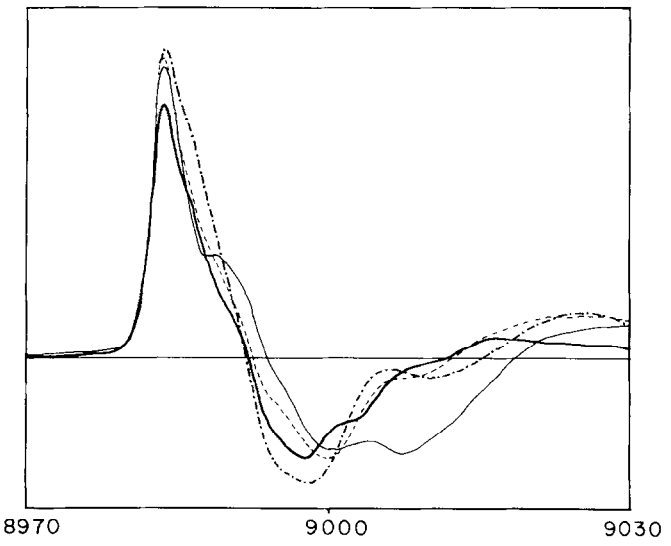

Fig. 1 Normalized difference edge spectra for one Cu(I) model (N_2,S coordination) minus a variety of Cu(II) models (N_4,O_1 (—,·–·); N_2,O_2 (– –); and N_2,O_2,S (▬) coordinations). Similar shapes and amplitudes were obtained for several Cu(I) and Cu(II) coordinations.

The values ε_1 and ε_2 are defined to be the normalized absorbance of Cu(I) and Cu(II), respectively. A sample containing mole fraction f of Cu(I) will thus have a normalized absorbance ε given by $f\varepsilon_1 + (1-f)\varepsilon_2$. For samples X and Y with mole fraction Cu(I) of f_X and f_Y respectively, the normalized difference of X-Y, $\Delta\varepsilon$, will be given by

$$\Delta\varepsilon = (f_X - f_Y)(\varepsilon_1 - \varepsilon_2) \quad (1)$$

Thus, from a knowledge of $(\varepsilon_1 - \varepsilon_2)$ one can determine change in % Cu(I), $(f_X - f_Y)$, from a measurement of $\Delta\varepsilon$. Although expression (1) will be valid for all points on the absorption edge, the most accurate results will be obtained for the maximum values of $(\varepsilon_1 - \varepsilon_2)$, e.g. at ~8984 eV and ~9000 eV (Fig. 1).

This analysis implicitly assumes that ε_1 is a constant for all Cu(I) edges (and similarly for ε_2). Although this is not strictly true, we find that for a wide variety of Cu(I) and Cu(II) ligation and site structures, the differences $(\varepsilon_1 - \varepsilon_2)$ at ~8984 and ~9000 eV are surprisingly constant, the relative variation being ~10%.

As shown in Fig. 2, the absorption edges are nearly identical for two samples, each containing an ~1:4 mixture of Cu(I):Cu(II). The difference edge

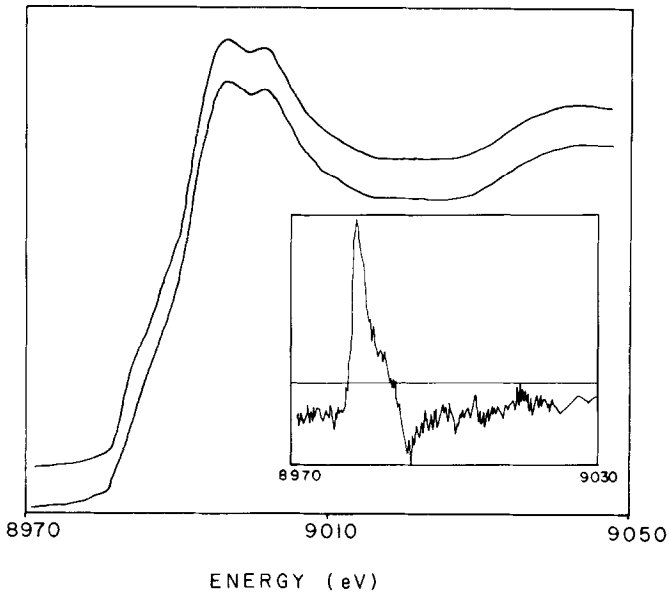

Fig. 2 Normalized edge spectra for two different oxidation state mixtures of a Cu model complex. Inset shows the difference edge spectrum (upper edge - lower edge), indicating Δ% Cu(I) = 6%. Using Eq. 1 and a Cu(II) reference, the upper spectrum represents a 26:74 mixture of Cu(I):Cu(II), the lower, a 20:80 mixture.

(see inset), however, clearly reveals the different compositions. This sensitivity suggests that an important application may be the detection of photo-reduction or photo-oxidation. We estimate that single scans of ~1mM protein samples have a sufficiently large signal/noise ratio to allow detection, by comparing the first and last scans, of a 5% change in [Cu(I)] during data collection.

T2D Laccase and Its Reactivity with Potential Exogenous Oxidants

Through difference edge analysis, comparing protein edges to those of Cu(II) model compounds, the untreated T2D form studied here and in [3] is clearly defined to contain 70±15% Cu(I). As EPR and optical data indicate T1 Cu(II), this requires that ≥90% of the T3 centers are reduced in deoxy T2D. Thus, O_2 does not reoxidize the binuclear coppers in the T2D laccase form (as prepared in references 2-4,7-8, and 10-11). However, >95% of the cuprous binuclear copper is reoxidized by peroxide (met T2D, data not shown) as also demonstrated in our initial qualitative X-ray absorption edge work [3].

Before presenting further quantitation of Cu edge results, it is important to consider conditions under which the difference edge technique could be in error. Extremely covalent Cu(II) complexes have features at ~8984 eV which resemble those found in Cu(I) edges [19]. As there is no apparent Cu(I) signal in the peroxide treated T2D sample, none of the Cu(II) sites in laccase appear to be sufficiently covalent to cause interference. A 2-coordinate Cu(I) complex is also known which exhibits an unusually intense 8984 eV transition [24]. While complications due to very low coordination number at the T3 site in T2D cannot be conclusively excluded, the presence of 2-coordinate Cu(I) would at most reduce the estimate of Cu(I) percentages to 70% of the calculated values [24]. Since edge structure does depend on absorber geometry [25], unusual geometries (e.g. tetrahedral Cu(II)) must be tested before applying this technique. In the absence of other information regarding coordination geometry, this problem can be addressed by examining the fully oxidized and fully reduced forms of a given sample in order to "fine-tune" $\Delta\varepsilon$ to that particular sample. The difference edge method is expected to achieve highset accuracy when the samples being compared have nearly identical structures, although this is not essential (cf. Fig. 1).

Difference edge analysis (Fig. 3A) of the reaction of 25X ferricyanide with the untreated T2D indicates 70±15 % Cu(I) and shows no significant change in Cu(I):Cu(II) composition relative to that of the untreated T2D. Thus, the binuclear cuprous site is inert to ferricyanide oxidation. Peroxide addition to this ferricyanide-treated T2D, however, does yield an exclusively cupric edge spectrum (Fig. 3C), difference analysis of which indicates <5% Cu(I), consistent with our results for peroxide on untreated T2D. Excess NO_2^- also oxidizes the untreated T3 site, but whereas no detectable Cu(I) was found in the peroxide oxidized samples, Fig. 3B represents 11±5 % Cu(I) which we attribute to ~40% of the binuclear sites being [Cu(I)Cu(II)] through one electron reduction subsequent to the two electron oxidation (vide infra).

Direct reduction of met T2D [T3:Cu(II)Cu(II); T1:Cu(II)] by nitrite [26] or by ferrocyanide [4] produces 45±5 % half-met T2D, as determined from

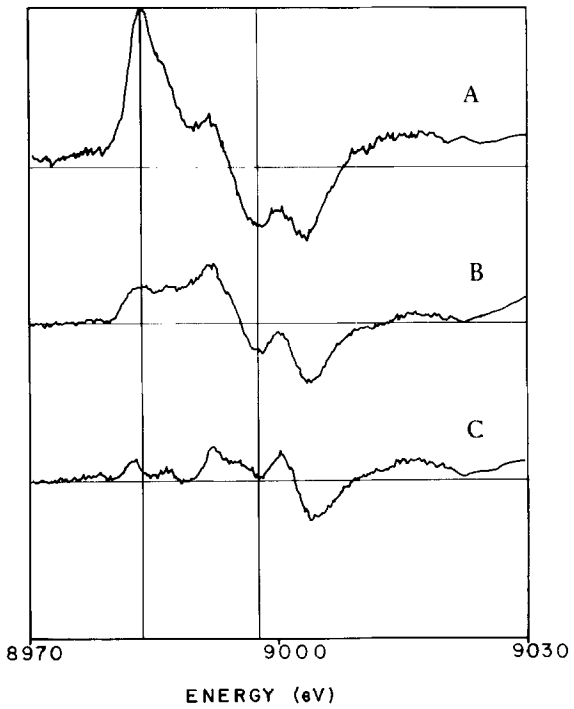

Fig. 3 Normalized difference edge spectra for laccase samples referenced to a Cu(II) (N_2,O_2,S coordination) model. A) Untreated T2D + 25 protein equivalents $K_3Fe(CN)_6$. B) Untreated T2D + 100 protein equivalents $NaNO_2$, 20hr. C) Sample A, in ferricyanide + 30 protein equivalents H_2O_2. Analysis at the marked energies indicates that these difference edges correspond to 70±15, 11±5 and 0±5 % Cu(I), respectively. [Protein] is ~1.7mM in 0.1M potassium phosphate, pH 6.0.

difference edge analyses (data not shown) and double integrated EPR intensities [4,26]. Furthermore, difference edge analyses of deoxy and met T2D derivatives treated with excess nitrite show identical Cu(I) compositions (Fig. 3B), which with our chemical and spectroscopic studies [26], indicate that NO_2^- reacts with deoxy T2D and met T2D to generate indistinguishable half met-NO_2^- protein derivatives.

In parallel with the reactivity of the coupled binuclear copper site in hemocyanin [5], $2e^-$ oxidation of T2D to form met T2D and the $1e^-$ reduction of met T2D to generate half met T2D presumably occur *via* the NO derived from aqueous nitrite at pH <7.0. This suggests that the lack of T3 reoxidation in the generally prepared T2D forms by O_2 is not due to neutral, small molecule inaccessibility [27]. Moreover, it is not clear how this NO_2^-/NO chemisty relates to earlier NO studies [28] which were on the T2D *Rhus* laccase prepared

under the conditions of [12] and which report no perturbation of the T3 site. The correlation of this work to other derivatives produced by variation in preparative conditions and to the products obtained via reaction of these derivatives requires analogous direct spectroscopic quantitation of the oxidation states of the copper sites, and a further systematic correlation of reaction conditions to those specified here.

ACKNOWLEDGMENTS

We gratefully acknowledge the National Institutes of Health (EIS Grant AM 31450) and National Science Foundation (KOH PCM-82-08115) for support of this research. Synchrotron radiation time was provided by the Stanford Synchrotron Radiation Laboratory, supported by the National Science Foundation (DMR 77-27489) and the National Institutes of Health (RR-01209).

REFERENCES

1. a)Reinhammar, B. and Malmström, B.G., Ch.3; b)Farver,O., and Pecht, I., Ch.4 in Copper Proteins, Academic Press, New York, 1981 (Spiro, T.G., ed.)
2. Graziani, M.T., Morpurgo, L., Rotilio, G., and Mondovi, B. (1976) FEBS Lett., 70, 87-90.
3. LuBien, C. D.; Winkler, M. E.; Thamann, T. J.; Scott, R. A.; Co, M. S.; Hodgson, K. O.; Solomon, E. I. (1981) J. Am. Chem. Soc. 103, 7014-7016.
4. Spira, D.J., Winkler, M.E., and Solomon, E.I. (1982) Biochem. Biophys. Res. Comm. 107, 721-726.
5. Solomon, E.I. (1981) Copper Proteins, Chapter 2, Academic Press, New York (Spiro, T.G., ed.).
6. Eickman, N.C., Himmelwright, R.S., and Solomon, E.I. (1979) Proc. Nat. Acad. Sci. USA 76, 2094-2098.
7. Winkler, M.E., Spira, D.J., Lubien, C.D., Thamann, T.J., and Solomon, E.I. (1982) Biochem. Biophys. Res. Comm. 107, 727-734.
8. Morpurgo, L., Desideri, A., Rotilio, G. and Mondovi, B. (1980) FEBS Lett. 113, 153-156.
9. Reinhammar, B. (1983) J. Inorg. Biochem., in press.
10. Morpurgo, L., Graziani, M.T., Finazzi-Argò, A., Rotilio, G. and Mondovi, B. (1980) Biochem. J. 187, 361-366, in 0.05 M NaOAc, pH 5.2 and 0.1 M phosphate, pH 6.0.
11. Morpurgo, L., Graziani, M.T., Desideri, A., and Rotilio, G. (1980) Biochem J. 187, 367-370.
12. Reinhammar, B., and Oda, Y. (1979) J. Inorg. Biochem. 11, 115-127. in 0.25 M potassium phosphate, pH 7.4.
13. Farver, O., Frank, P., and Pecht, I. (1982) Biochem. Biophys. Res. Comm. 108, 273-278 in 0.1 M HEPES, pH 7.4.
14. Essentially no absorption at 330 nm nor any N_3^- binding to T3 copper; $A_{\parallel}(T1)=38\times10^{-4}$ cm^{-1} and bleaching at 614 nm with increasing pH.
15. Scott, R. A.; Hahn, J. E.; Doniach, S.; Freeman, H. C.; Hodgson, K. O. (1982) J. Am. Chem. Soc. 104, 5364-5369.
16. Reinhammar, B. (1970) Biochim. Biophys. Acta 205, 35-47.
17. This transition has been assigned as 1s→4s with the corresponding (weak) 1s→4s transition in (approximately square planar) Cu(II) complexes being assigned as a shoulder at ~8987 eV [18]. Recent calculations [Kutzler, F. W. Ph. D. Thesis, 1981, Stanford University] demonstrate that such atomic descriptions are overly simplistic. To avoid future confusion, we will refer to this transition only as the "8984 eV transition".

18. a) Hu, V. W.; Chan, S. I.; Brown, G. S. (1977) Proc. Nat. Acad. Sci., USA, 74, 3821-3825. b) Hu, V. W.; Chan, S. I.; Brown, G. S. (1977) FEBS Letts., 84, 287-290.
19. a) Powers, L.; Blumberg, W. E.; Chance, B.; Barlow, C.; Leigh, J. S. Jr.; Smith, J. C.; Yonetani, T.; Vik, S.; Peisach, J. (1978), in "Frontiers of Biological Energetics, Vol. 2", Dutton, P. L., Leigh, J. S. and Scarpa, A. Eds., Academic Press, 863-871. b) Powers, L.; Blumberg, W. E.; Chance, B.; Barlow, C.; Leigh, J. S. Jr.; Smith, J. C.; Yonetani, T.; Vik, S.; Peisach, J. (1979) in "Cytochrome Oxidase", King, T. E. ; Orii, Y.; Chance, B.; Okunuki, K. Eds., Elsevier, 189-195. c) Powers, L.; Blumberg, W. E.; Chance, B.; Barlow, C.; Leigh, J. S. Jr.; Smith, J. C.; Yonetani, T.; Vik, S.; Peisach, J. (1979) Biochem. Biophys. Acta, 546, 520-538.
20. Blumberg, W. E.; Peisach, J.; Eisenberger, P.; Fee, J. A. (1978) Biochem. 17, 1842-1846.
21. Brown, J. M.; Powers, L.; Kincaid, B.; Larrabee, J. A.; Spiro, T. B. (1980) J. Am. Chem. Soc. 102, 4210-4216.
22. Tullius, T. D.; Gillum, W. O.; Carlson, R. M. K.; Hodgson, K. O. (1980) J. Am. Chem Soc. 102, 5670-5676.
23. Brudvig, G. W.; Bocian, D. F.; Gamble, R. C.; Chan, S. I. (1980) Biochim. Biophys. Acta, 624, 78-89.
24. Co, M. S.; Hodgson, K. O. Unpublished results.
25. Shulman, R. G.; Yafet, T.; Eisenberger, P.; Blumberg, W. E. (1976) Proc. Nat. Acad. Sci. USA, 73, 1384-1388.
26. Spira, D.J. and Solomon, E.I. (1983) Biochem. Biophys. Res. Comm., preceeding communication.
27. As further indicated by reversible CO binding studies to the reduced T3 site in untreated T2D; Spira, D.J. and Solomon, E.I., unpublished results.
28. Martin, C.T., Morse, R.H., Kanne, R.M., Gray, H.B., Malmstrom, B.G., and Chan, S.I. (1981) Biochem. 20, 5147-5155 in 0.025 M potassium phosphate, pH 7.0.