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Review Letter

Status of the cofactor identity in copper oxidative enzymes

J.P. Klinman[1], D.M. Dooley[2], J.A. Duine[3], P.F. Knowles[4], B. Mondovi[5] and J.J. Villafranca[6]

[1]*Department of Chemistry, University of California, Berkeley, CA 94720, USA,* [2]*Department of Chemistry, Amherst College, Amherst, MA 01002, USA,* [3]*Department of Microbiology and Enzymology, Delft University of Technology, Delft, The Netherlands,* [4]*Department of Biochemistry and Molecular Biology, The University of Leeds, Leeds, UK,* [5]*Department of Biochemical Sciences and C.N.R. Centre of Molecular Biology, University 'La Sapienza', Rome, Italy and* [6]*Department of Chemistry, Penn State University, College Park, PA, USA*

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Much conflicting data have appeared in the literature regarding the nature of the active site structures responsible for catalysis in three classes of copper enzymes: the copper amine oxidases, dopamine β-monooxygenase and galactose oxidase. Although pyrroloquinoline quinone has been proposed to be the active site cofactor in each instance, new findings indicate this is not the case. Instead, recently available data indicate a spectrum of strategies for substrate activation, which range from direct metal catalysis (dopamine β-monooxygenase) to the involvement of protein-derived radicals (galactose oxidase) and protein-derived quinones (copper amine oxidases).

Copper protein; Pyrroloquinoline quinone; Topa quinone

1. INTRODUCTION

A meeting on the subject of 'Copper in Biological Systems' was held on September 23–28, 1990 in Manziana, Italy. This meeting brought together a critical mass of investigators who have been pursuing cofactor structure in copper oxidative enzymes over the past five to ten years. During this period, much conflicting data have appeared in the literature regarding the true nature of the active site structures responsible for catalysis in three classes of copper enzymes: the copper amine oxidases, dopamine β-monooxygenase and galactose oxidase. At the present time considerable confusion exists among investigators not directly involved in studies of these enzymes. The goal of this paper is a clarification of the status of our knowledge regarding each system.

2. COPPER AMINE OXIDASES

Copper amine oxidases are widespread and have been studied from bacteria, plants and mammals. Historically, three different cofactor structures have been proposed for these enzymes.

2.1. *Pyridoxal phosphate*

A prominent feature of the copper amine oxidases is their reactivity toward carbonyl reagents such as phenylhydrazine, to yield chromophoric derivatives. In this context, early investigations focused on a role for pyridoxal phosphate as the reactive carbonyl [1]. However, the presence of this cofactor has never been directly demonstrated. Both NMR spectroscopy and resonance Raman studies have been carried out on several amine oxidases. ^{31}P-NMR studies of porcine serum amine oxidase failed to detect the expected signal for the phosphate group of pyridoxal phosphate [2], although such studies cannot rule out pyridoxal itself. Resonance Raman spectra of phenylhydrazine and nitrophenylhydrazine derivatives of amine oxidases from porcine and bovine sera, pea seedlings, porcine kidney and *Arthrobacter* P1 indicate different spectra from the analogous derivatives of pyridoxal phosphate [3]. Chemical evidence also argues strongly against a simple carbonyl cofactor. Amine substrates have been shown to be competitive with regard to hydrazine inhibitors of amine oxidases and, as such, are expected to form Schiff base intermediates. Yet no tritium transfer to enzyme could be demonstrated upon incubation of bovine serum amine oxidase with substrate and $NaBT_4$ [4]. More recently, it has been shown that $NaCNBH_3$ treatment of bovine serum amine oxidase with substrate leads to the incorporation of one mol of ^{14}C-labeled substrate per mol of enzyme subunit concomitant with enzyme inactivation. Once again, no transfer of tritium from $NaCNBT_3$ to enzyme could be detected, leading to the proposal of a dicarbonyl-type cofactor structure

Correspondence address: J.P. Klinman, Department of Chemistry, University of California, Berkeley, CA 94720, USA

which would release tritium from the initially reduced complex [5]. The numerous lines of investigation, outlined above, argue strongly against a role for pyridoxal phosphate in the copper amine oxidases. However, reports of this cofactor in serum amine oxidase have appeared in the recent literature [6,7].

2.2. *Pyrroloquinoline quinone*

Two independent reports, published in 1984 [8,9], focused attention on a newly discovered bacterial cofactor, pyrroloquinoline quinone, as the covalently bound cofactor in bovine serum amine oxidase. The approach developed by the Delft group [10] involved prolonged incubation of enzyme with dinitrophenylhydrazine in the presence of oxygen. Pronase digestion of derivatized enzyme was found to release a product which showed the same retention time on HPLC and ^{1}H-NMR spectrum as an authentic adduct of pyrroloquinoline quinone with dinitrophenylhydrazine. Extension of this methodology to a number of other amine oxidases, which include kidney amine oxidase [10], lysyl oxidase [11], methylamine oxidase from *Arthrobacter* P1 [12] and amine oxidase from pea seedlings [13], yielded similar results. Resonance Raman spectroscopy of phenylhydrazone derivatives of various amine oxidases was also pursued. While protein-dinitrophenylhydrazone spectra showed a much greater similarity to the dinitrophenylhydrazone derivative of pyrroloquinoline quinone than pyridoxal phosphate, significant differences in the intensity and position of peaks were apparent [14]. Subsequent comparison of resonance Raman spectra of 2-hydrazinopyridine and *p*-nitrophenylhydrazine derivatives has indicated that pyrroloquinoline is not the cofactor in amine oxidases [3]. It is now believed that the combination of prolonged treatment of enzyme with phenylhydrazine, followed by pronase digestion, leads to the formation of a *derivative* of a native structure and does not represent pyrroloquinoline quinone itself at the enzyme active site. The chemistry which produces pyrroloquinoline quinone is unknown and in need of further study. The requirement for oxygenation of reaction solutions to produce high yields of pyrroloquinoline quinone-like molecules [10] suggests the importance of redox reactions. Buechi et al. have already shown that tyrosine can be converted to pyrroloquinoline quinone in an oxidative manner [15].

2.3. *Topa quinone*

A recent study has focused on the proteolysis of a [^{14}C]phenylhydrazine derivative of bovine serum amine oxidase under mild conditions [16]. These studies used a highly purified preparation of enzyme, which shows a titration end point with phenylhydrazine close to one mol incorporated per enzyme subunit. Others authors had titrated only one mol of phenylhydrazine per dimer using similar enzyme preparations [17]; the relative reactivity of the two carbonyl groups will be discussed below. HPLC purification of a thermolytic digestion indicated one major radioactive peptide peak in ca. 40% yield. Micro-Edman sequencing of this peptide gave: **leu, asn, blank, asp, tyr.** Subsequent characterization of this pentapeptide by mass spectrometry and ^{1}H-NMR led to assignment of the blank at position 3 to the oxidized form of a 2,4,5-trihydroxyphenylalanine residue, referred to as topa quinone [16]. This assignment has been confirmed by chemical synthesis of a topa quinone model compound and by comparison of this model to the protein-derived peptide using ^{1}H-NMR [16] and resonance Raman spectroscopy [18]. A total of 75% of the radiolabel originally associated with protein could be recovered from the HPLC. A second radiolabeled peptide, obtained in ca. 10% yield, was sequenced and found to be comparable to the original pentapeptide (with the addition of two amino acids at its C-terminus). Thus, a total of ca. 50% of protein-associated radiolabel can be unambiguously assigned to the phenylhydrazone of topa quinone [19]. Since all of the protein-bound phenylhydrazine shows similar chromophoric properties, it seems unlikely that the remaining C-14 in derivatized enzyme is bound to a functional group different from topa quinone. In a recent study [18], resonance Raman spectra for the phenylhydrazine and nitrophenylhydrazine derivatives of bovine serum amine oxidase were compared to spectra for the respective, protein-derived peptides. The high degree of similarity observed between peptide and protein spectra indicates that the topa quinone in isolated peptide is not an artifact of the isolation procedure. Since the above described studies were carried out on a single amine oxidase (from bovine serum), it is important to question the generality of topa quinone as an active site structure in copper amine oxidases. At the current time, both resonance Raman spectroscopy [18] and sequencing of active site peptides [19] support a structure similar to topa quinone in the amine oxidases from porcine serum and kidney, pea seedling, yeast and bacteria. Lysyl oxidase may be an important exception to this generalization, since no sequence homology to active site peptides obtained from other amine oxidases can be discerned by examination of the lysyl amine oxidase c-DNA derived protein sequence [20].

One unresolved issue concerns the relative reactivity of the two prosthetic groups present in dimeric amine oxidase. While some of us find similar reactivity at both sites [21], others observe biphasic kinetics and/or variations in spectral properties following derivatization of bovine [17,22] and porcine serum amine oxidase [23]. The structural basis for the observation of differential reactivities at the two active site cofactors is in need of clarification. A comparative study of the ratio of copper to active site prosthetic groups, using enzyme preparations from different laboratories, may help to rationalize existing discrepancies (cf. [24]).

3. DOPAMINE β-MONOOXYGENASE

The kinetic mechanism of dopamine β-monooxygenase has been rigorously pursued through a combination of kinetics and spectroscopy [25]. Results from these studies show that redox cycling at the two copper atoms per subunit can account for the catalytic mechanism. A possible role for an organic cofactor was suggested through the use of phenylhydrazine derivatives as carbonyl reagents [26]. In these studies, Duine and co-workers [26] isolated a pyrroloquinoline quinone-like molecule which was shown to have the same retention time on HPLC as an authentic pyrroloquinoline quinone derivative. Phenylhydrazine had previously been shown to act as a stoichiometric, mechanism-based inhibitor, leading to covalent labeling of protein [27]. Subsequently, Villafranca and co-workers [28] focused on the isolation and characterization of labeled active site peptides. These studies demonstrated labeling of tyrosine and histidine by phenylhydrazine; no pyrroloquinoline quinone could be detected. Variances in experimental results between the laboratories of Duine and Villafranca are attributed to the very different experimental conditions used by the two laboratories for the derivatization of dopamine β-monooxygenase by phenylhydrazine. It is now believed that pyrroloquinoline quinone, as such, is absent, but that an analogous structure may be formed following treatment of enzyme under the prolonged conditions employed by Duine and co-workers.

4. GALACTOSE OXIDASE

Unlike dopamine β-monooxygenase, galactose oxidase is a monomeric enzyme containing a single copper atom per polypeptide chain [29,30]. In this context, it has been difficult to explain an enzyme-catalyzed, two electron oxidation of substrate. It has been known for some time that galactose oxidase can be oxidatively activated, through a process originally suggested to involve Cu(III) formation [31]. Because the redox potential for Cu(II) → Cu(III) is expected to be very high [32], the presence of such a species in a protein active site has been controversial. Independent reports in 1988 suggested the involvement of an additional redox cofactor, either pyrroloquinoline quinone (Duine and co-workers [33]) or a protein derived radical (Whittaker and co-workers [34]). A recent, high-resolution x-ray structure for galactose oxidase unambiguously eliminates the possibility of an active site quinone, revealing, instead, active site tyrosines [35]. This is consistent with a recent EPR characterization of the copper-free form of galactose oxidase, which implicates a tyrosyl radical [36]. Using ribonucleotide reductase as a model system for tyrosyl radical containing proteins, Duine and co-workers have been able to produce pyrroloquinoline quinone-like derivatives using their hexanol extraction procedure (B.W. Groen and J.A. Duine, unpublished results). Thus, all available evidence points toward the participation of a tyrosyl radical in the catalytic mechanism of galactose oxidase.

5. FINAL COMMENTS

The authors wish to point out the exciting new developments which have emerged concerning the use of amino acid derivatives as redox cofactors. With regard to copper containing enzymes, a spectrum of strategies exist for substrate activation: these range from direct metal catalysis (dopamine β-monooxygenase) to the involvement of protein-derived radicals (galactose oxidase) and protein-derived quinones (copper amine oxidases).

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