SPECTROSCOPIC ASPECTS OF COPPER BINDING SITE IN BOVINE SERUM AMINE OXIDASE

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1. Introduction

Amine oxidases are known to catalyze oxidative deaminations of amines by accepting two electrons from amines and transferring them to the molecular oxygen. They may be conveniently divided into two classes: copper-containing amine oxidases and FAD-containing ones. The copper-enzymes such as bovine [1–3] and pig [4–7] plasma amine oxidases, pig kidney [8–10] and pea seedling [11] diamine oxidases, and fungal amine oxidase [12–14] have been known to contain type-2 coppers (non-blue and ESR-active Cu(II)) [15].

In spite of many spectroscopic investigations on the copper binding site of these oxidases, ligating groups and function of the copper ion in the enzymes are still unknown; copper(II) was found to restore the activity of copper-depleted oxidase [1,11], but no change in the ESR spectrum has been observed even after anaerobic treatment of the enzyme with a substrate [2,8,14].

This paper describes the results of spectroscopic studies of bovine serum amine oxidase (SAO) as the first approach to the ligating groups around the copper ions.

2. Experimental

SAO was isolated from bovine serum and twice crystallized [16]. The SAO sample showed spec. act. 440 at 25°C by the method in [17], and disc gel electrophoresis of the sample showed a single band by protein staining. The pink colored protein in 0.2 M sodium phosphate buffer (pH 7.2) has molecular weight ~190 000 and contains 2.2 g atoms copper/mol protein; the protein concentration was determined by measuring the A_{280} using the value of $E^{1\,\%}_{1\,cm}$ = 17.4 and the concentration of copper was obtained according to the method of standard addition by use of a Nippon Jarrell-Ash atomic absorption spectrophotometer AA-1. Absorption spectra were taken with a Hitachi 323 spectrophotometer at room temperature. ESR spectra at 77 K were obtained with a JEOL JES-FE1X spectrometer with 100 kHz field modulation. CD spectra were recorded at room temperature with a JASCO J-500A spectropolarimeter and a JASCO MOE-1 spectropolarimeter for the spectral regions from 300–700 nm and from 700–1000 nm, respectively. The spectrum from 300–700 nm represented an average of 8 scans.

3. Results

The ultraviolet and visible absorption and the CD spectra of SAO are illustrated in fig.1. The pink color of the enzyme stems from the strong absorption band at ~460 nm, of which the extinction coefficient (ϵ_{Cu}) was estimated as 3300 $M^{-1} \cdot cm^{-1}$. Some other amine oxidases such as plasma [1,5] and fungal [12] amine oxidases, and kidney [10] and seedling [11] diamine oxidases, exhibit absorption $A_{460-480}$ maxima. The CD spectrum for SAO displayed a broad band at ~350 nm, a positive extremum at 660 nm, and negative extrema at 450, 540 and 810 mm. The CD curve from 300–600 nm resembles that of bovine plasma amine oxidase [3].

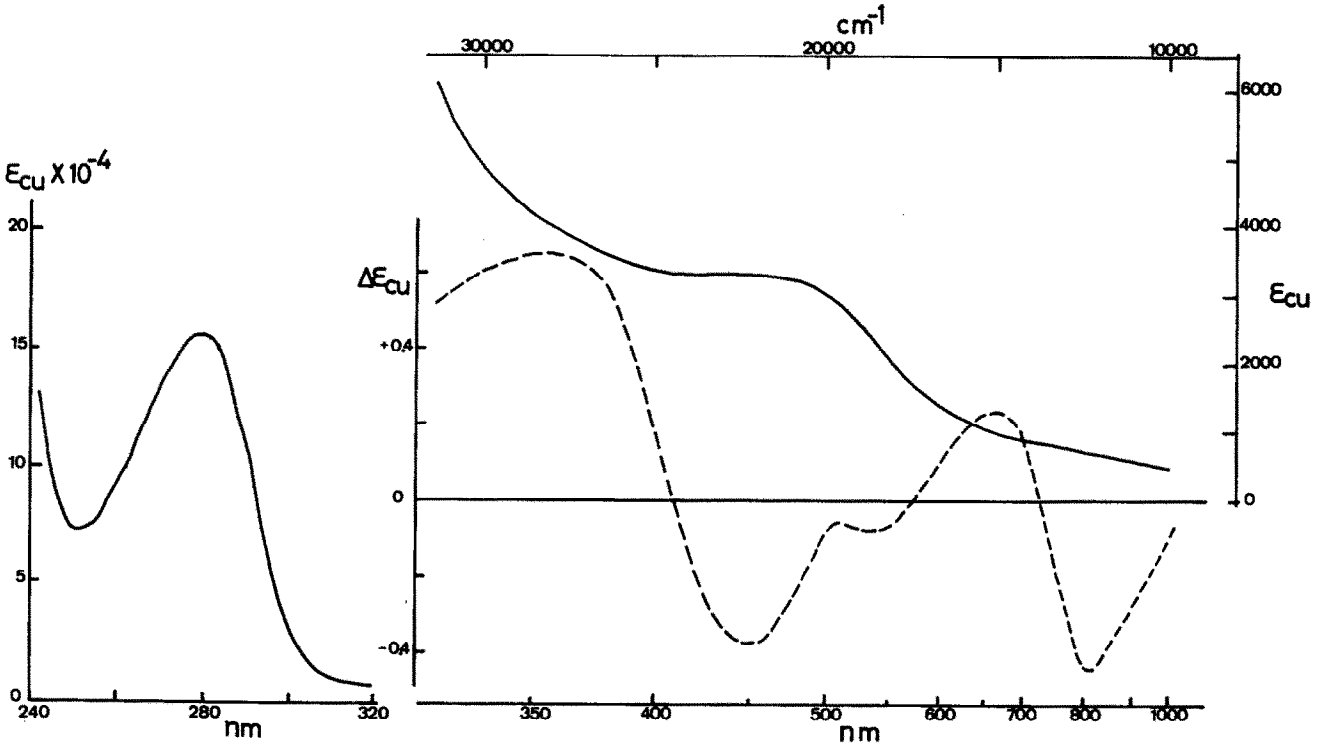

Fig.1. Optical absorption and CD spectra of SAO in 0.2 M sodium phosphate buffer (pH 7.2). Ultraviolet, visible, and near-infrared absorption spectra (——); CD spectrum (– – –). Protein conc. 0.38 mg/ml (UV spectrum) and 15 mg/ml (VIS and near-IR spectra).

The X-band ESR spectrum of SAO in 0.2 M phosphate buffer is illustrated in fig.2, which shows a signal at $g_{\perp} = 2.06$ and a 4-line hyperfine signal at $g_{\parallel} = 2.29$, with spacings ($A_{\parallel}$) between successive peaks of 16.1 mT. The ESR parameters are similar to those

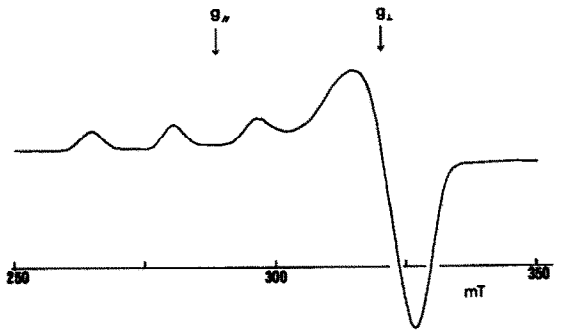

Fig.2. ESR spectrum of SAO in 0.2 M sodium phosphate buffer, (pH 7.2). Protein conc. 15 mg/ml, temp. 77 K. Microwave power 6.25 mW; frequency 9.27 GHz; modulation amplitude 0.63 mT. The ESR signal amplitude did not saturate in the range of microwave power, 0–8 mW.

generally found for simple Cu(II) complexes. The ESR-signal of SAO in 0.2 M phosphate buffer in the presence of ammonium sulfate (0.3 saturation), gave the spin Hamiltonian parameters of $g_{\parallel} = 2.28$, $g_{\perp} = 2.06$, and $A_{\parallel} = 16.2$ mT, exhibiting at least 5 nitrogen superhyperfine lines (spacing, $A_N = 1.4$ mT) at the perpendicular region arising from the interaction between the copper electron spin and the coordinated nitrogen nuclear spins (fig.3). The superhyperfine structures only became observable upon the addition of ammonium sulfate to SAO. Therefore, we attempted to find out the unambiguous answer to the question whether or not the above superhyperfine lines in the presence of excess ammonium ion are due to direct bindings of NH_3 to Cu(II). Since the nuclear spins of ^{14}N and ^{15}N are 1 and 1/2, respectively, the number of superhyperfine lines should decrease by the use of $(^{15}NH_4)_2SO_4$, if NH_3 is in coordination. The ESR spectrum obtained on addition of $(^{15}NH_4)_2SO_4$ (99 atom%) revealed no change in the pattern of superhyperfine lines compared to that obtained with natural $(^{14}NH_4)_2SO_4$, indicating that the NH_3-nitro-

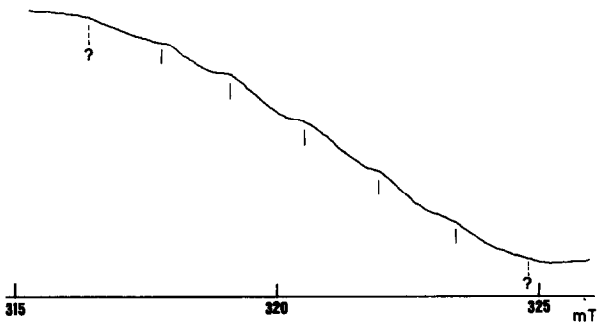

Fig.3. ESR spectrum (perpendicular region) of SAO in 0.2 M sodium phosphate buffer (pH 7.2) added ammonium sulfate to 0.3 saturation. Protein conc. 23 mg/ml, temp. 77 K. Microwave power 6.25 mW; frequency 9.26 GHz; modulation amplitude 0.5 mT.

gens are not bound by the copper(II). The spectrum represented in fig.3 can be interpreted by the coordination of at least two nitrogens around the copper(II).

4. Discussion

Out of the 5 CD extrema of SAO in the region of 350–1000 nm, the two peaks at 660 and 810 nm are considered to be due to the d–d transitions of Cu(II) because of the comparatively small ϵ (<1000) of the corresponding region in the optical spectrum. On the other hand, the ϵ of the optical absorption between 350–600 nm is considerably high (1000–5000), suggesting that the CD bands at 350, 450 and 540 nm are due to charge transfer from ligands to Cu(II) and/or some chromophore reported [1,4,5,9,18]. It is well known that the coordination of sulfur atoms (thiolate or thioether) around Cu(II) in low molecular weight complexes gives rise to charge transfer bands in the 330–400 nm region [91–21]. For this reason some of the CD peaks of SAO between 350–600 nm may be attributed to S → Cu(II) charge transfer transitions in a square-planar or a square-pyramidal Cu(II) system. The non-blue visible absorption band for SAO rules out the possiblity that the copper(II) in the oxidase has a distorted tetrahedral geometry, being bound by sulfur ligands as established in the type-1 coppers.

As is apparent from fig.2, the ESR signal of SAO is approximately axial with $g_{\parallel} > g_{\perp} > 2$. These g-values require that the Cu(II) ion has a tetragonal geometry. Further the number of superhyperfine lines and their spacings (A_N = 1.4 mT) led us to conclude that the Cu(II) in SAO is bound at least by two imidazole nitrogens of histidine residues [22].

It was suggested as the results of absorption, CD, and ESR studies that the copper(II)-binding site in SAO is in a tetragonal-like environment(square-planar, square-pyramidal, or their slightly distorted geometries), and the ligating groups of the copper(II) consist of a $N_{2-3}S_{1-2}X$ donor set (N, imidazole group; S, thiol and/or thioether group; X, oxygen of water [23] or carboxylate).

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