CHARACTERIZATION OF BOVINE CERULOPLASMIN

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1. Introduction

Ceruloplasmin (ferroxidase, iron II: oxygen oxidoreductase, EC 1.16.3.1) has been extensively studied, not only because it is a member of the blue copper oxidases, but also because of its clinical importance and possible role(s) in iron and copper metabolism and transport [1–3]. Human and porcine ceruloplasmins have been well characterized [1–3]. Both contain three spectroscopically distinguishable types of copper sites (types 1, 2 and 3) whose properties have been extensively discussed [1–5]. The copper stoichiometry of ceruloplasmin is still somewhat uncertain. Previous experiments indicated that porcine and human ceruloplasmin have identical copper contents [2]. Recent measurements of the molecular mass and copper content of the human protein are consistent with 6–7 intrinsic coppers [6]. One copper ion can be removed from a human ceruloplasmin preparation containing 8 coppers/enzyme molecule without affecting the catalytic activity [7]. In addition, human ceruloplasmin can bind up to 10 additional cupric ions [8]. Human, pig, horse, and rabbit ceruloplasmins are each composed of a single polypeptide chain with nearly the same molecular mass [6,9–11]. Further, the SDS gel electrophoretic patterns of multicomponent preparations of human ceruloplasmin have been qualitatively reproduced via limited proteolysis [12].

In comparison, bovine ceruloplasmin has been poorly characterized. We have purified it by a standard method and determined its enzymatic activity, copper content, and some of its spectroscopic properties.

2. Experimental

Fresh blood was obtained from a slaughterhouse and immediately fractionated (through step 4) as in [13]. Blue fractions from both DEAE-cellulose columns, in each case eluted by 0.1 M phosphate [Na_2HPO_4–KH_2PO_4] buffer (pH 7.0) were pooled and dialyzed *vs* 0.006 M phosphate buffer (pH 6.8). The solution was then applied to a 2.5 × 30 cm column of hydroxylapatite equilibrated with 0.006 M phosphate buffer (pH 6.8) and eluted with a 0.06–0.5 M phosphate buffer gradient (pH 6.8). Fractions with $A_{610}/A_{280} > 0.024$ from the form of the enzyme that eluted second (vide infra) were pooled and concentrated to ~3 ml by pressurized ultrafiltration. This concentrated solution was applied to a 2.5 × 40 cm Sephadex G-150 column equilibrated with 0.03 M phosphate buffer (pH 7.0) and eluted with the same buffer. Fractions with $A_{610}/A_{280} > 0.035$ were pooled and concentrated to 4 ml, then rechromatographed on the G-150 column to give pure ceruloplasmin with $A_{610}/A_{280} = 0.046$. We have also found that the scheme for purification of human ceruloplasmin in [14] can be used to purify bovine ceruloplasmin as well. Both of these methods permit bovine ceruloplasmin and amine oxidase to be purified from the same batch of plasma. Using the *N,N'*-dimethyl-*p*-phenylenediamine assay in [15], the average oxidase activity at 22°C of two preparations was $\Delta A_{550}^{\text{min}} = 9.2/\mu$mol ceruloplasmin (assuming $\epsilon_{610} = 10^4\ M^{-1}\ cm^{-1}$ [1–3]).

$A_{610}^{1\,cm} \simeq 0.5$ for visible CD samples, which were diluted 10-fold for use in the UV. Spectral band width was maintained at 4 nm in the visible region; UV CD spectra were recorded with a constant slit width of 1 mm (2 nm spectral band width at 250 nm). EPR measurements were made at 77 K using a Varian E-9 spectrometer. Ceruloplasmin was dialyzed extensively against Chelex-treated phosphate buffer (pH 7.0) prior to EPR or atomic absorption measurements. $Cu(EDTA)^{2-}$ (20% excess Na_2EDTA) in distilled, deionized water served as the standard for double integration. The copper content of ceruloplasmin was

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determined with a Perkin-Elmer 305A atomic absorption spectrometer.

3. Results and discussion

Two different forms of bovine ceruloplasmin, in approximately equal amounts, are clearly resolved during hydroxylapatite chromatography (fig.1). At least two forms had been observed for other mammalian ceruloplasmins [11,14,16]. Multiple forms of porcine and bovine ceruloplasmin were first detected by DEAE-cellulose chromatography [16]. However, in contrast to our results, the two components of bovine ceruloplasmin were present in a 9:1 ratio. This difference may indicate either variability in the relative amounts of the two forms present in bovine plasma or that more than two forms are present. It is also possible that the plasma fractionation procedures employed (ammonium sulfate *vs* alcohol and chloroform precipitations [16]) could affect the relative yields. It is likely that the two forms separated on hydroxylapatite differ in their carbohydrate composition [17].

Disk gel electrophoresis of the final solution under non-denaturing conditions showed only a single band. Analytical SDS gel electrophoresis separated the homogeneous ceruloplasmin into two major bands of about equal mobilities with $M_r \sim 100\,000$ and three very faint minor bands of lower molecular mass. These bands are probably proteolytic fragments of native bovine ceruloplasmin [10 12,14].

Atomic absorption determination of the total copper content and double integration of the EPR spectrum yields a ratio for EPR detectable Cu: total Cu equal to 0.50 ± 0.04. Further, the data are consistent with $\epsilon_{610} = 3.27 \times 10^3\ M^{-1}\ cm^{-1}$, referenced to the concentration of paramagnetic copper. Both porcine and human ceruloplasmin have three intrinsic paramagnetic coppers, one type 2 and two type 1, per enzyme molecule [18,19]. Taking $\epsilon_{610} = 10^4\ M^{-1}\ cm^{-1}$ [1–3], our data are quantitatively consistent with 3 paramagnetic and 6 total coppers/enzyme molecule, in

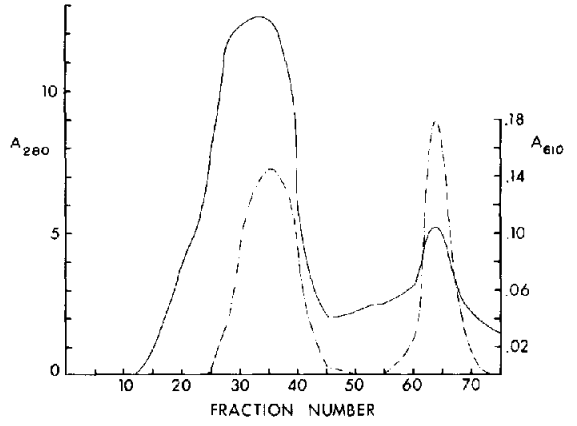

Fig.1. Elution of bovine ceruloplasmin from hydroxylapatite: (- —) A_{280}; (–·–·–) A_{610}/A_{280}.

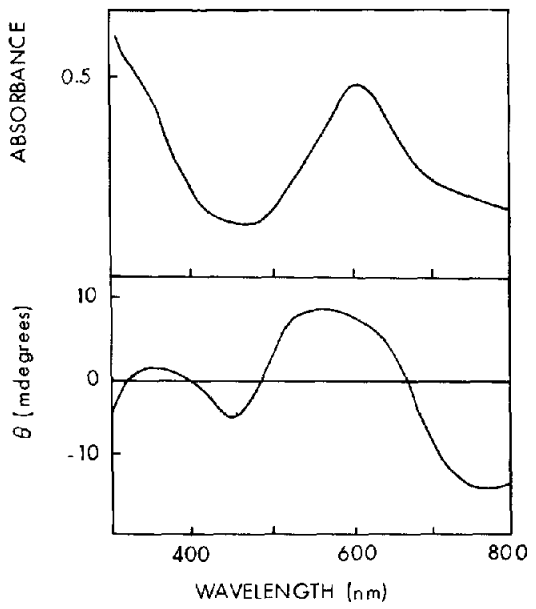

Fig.2. (A) Room temperature absorption spectrum of bovine ceruloplasmin in 0.03 M phosphate buffer (pH 7.0). The spectrum was recorded using a 1 cm pathlength quartz cell. (B) Room temperature CD spectrum of bovine ceruloplasmin under identical conditions.

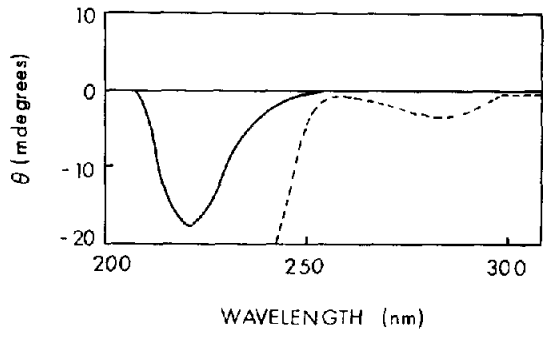

Fig.3. UV CD spectrum of bovine ceruloplasmin at room temperature in 0.03 M phosphate buffer (pH 7.0): (– –) sample in a 1 cm pathlength cell; (——) sample in a 1 mm pathlength cell.

agreement with recent results for the human enzyme [6].

Visible absorption and CD spectra of bovine enzyme are presented in fig.2. The intense peak at 610 nm is associated with the type 1 coppers and the shoulder at 330 nm from type 3 is also evident (fig.2A). All the CD bands (fig.2B) had been assigned to ligand-to-metal charge transfer transitions of the type 1 copper site [20]. These spectra are closely similar to those of porcine and human ceruloplasmin [20,22]. UV CD spectra are presented in fig.3. A weak negative feature is evident at 285 nm where aromatic amino acids display CD signals and an intense negative band is observed at 220 nm. UV CD spectra of porcine and human ceruloplasmin differ somewhat from that shown in fig.3; their CD curves become positive in the 270–275 nm region [21]. Interestingly, spectra of reduced and aporcine ceruloplasmin more closely resemble that of bovine ceruloplasmin. A detailed comparative study of the mammalian ceruloplasmins would be interesting.

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