PREPARATION OF PURE BOVINE APO-ERYTHROCUPREIN BY GEL FILTRATION

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1. Introduction

Studies on the bimetallic protein erythrocuprein have become of widespread interest [1–4]. Erythrocuprein can be isolated from erythrocytes by precipitation with organic solvents following gel and DEAE-chromatography. The metalloprotein contains two cupric and two zinc ions per mole and has an absorption ratio of $A_{259}/A_{680} = 31.5$. Almost no helical structure can be detected in the protein portion [1, 2]. Isolation of a pure metal free preparation would enable the nature of this copper and zinc binding ligand to be investigated.

The usual preparation of the apoprotein was carried out with excessive dialysis against chelating agents [2–4]. However, a labile apoprotein would not survive this process, so the native erythrocuprein was passed through a G-25 Sephadex column previously equilibrated with cyanide or EDTA buffer. A pure apoprotein is obtained free from smaller degradation products and low molecular weight Cu^{2+} and Zn^{2+} chelates. The purest apo-erythrocuprein was obtained using EDTA as a chelating agent.

2. Materials and methods

Pyrex glass columns (about 16 X 1400 mm) were packed with Sephadex G-25 (Pharmacia Co., Uppsala, Sweden) and equilibrated with buffer solutions, 10 mM EDTA, pH 3.8 adjusted with acetic acid, or 100 mM KCN + 5 mM potassium phosphate, pH 10.8. Reagent grade chemicals and deionized water distilled over quartz were used. Erythrocuprein was prepared from fresh bovine blood as described in [1] and stored under liquid nitrogen. Dilute erythrocuprein solutions were concentrated by covering the erythrocuprein containing dialysis bag with Sephadex-G-150 powder for 12 hr at 4°. Usually 2 ml samples of the concentrated metalloprotein (A_{259} = 2.5 ± 0.2, approx. 8 mg/ml) were applied on top of a G-25-column at 25°. The apoprotein was monitored using a LKB Uvicord at 253 nm. Copper and zinc were measured by means of an atomic absorption spectrophotometer (Zeiss M4 QIII combined with PMQ II and a Linecomp recorder) [5]. Copper was determined at 324.75 nm and zinc at 213.85 nm. Excessive chelating agents were removed by passing the apoprotein through a Sephadex G-25 column (14 X 170 mm).

3. Results

Cyanide was thought to be a convenient chelator to remove copper and zinc from the native erythrocuprein. At pH 7.9 only negligible amounts were removed while at pH 9.5, 90% of the metal ions dissociated from the protein. However, after a pH rise to 10.8 virtually no ($> 1\%$) copper and zinc could be detected in the first peak containing most of the apo-protein (fig. 1). Furthermore, the slightly elevated base line suggested some denaturation of the erythrocuprein. This denaturation may be attributable to the cyanide treatment and not to the pH rise. This was demonstrated by subjecting native erythrocuprein to alkaline treatment up to pH 11.8. No detectable denaturation was observed after the pH

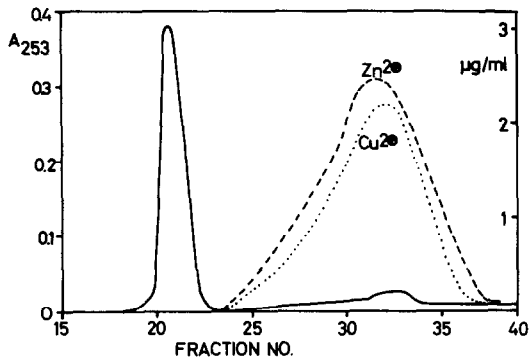

Fig. 1. Elution profile of bovine apo-erythrocuprein. 20.5 mg freshly prepared erythrocuprein dissolved in 2.5 ml 100 mM phosphate buffer pH 7.2 were applied on a Sephadex G-25 fine grade column (15 × 1500 mm) previously equilibrated with 100 mM KCN–5 mM potassium phosphate buffer, pH 10.8, and eluted with the same buffer. The temperature was 25°. Elution time 7 hr to fraction No. 22, 13 hr to fraction No. 40; fraction volume 5.5 ml. (——) A_{253} nm, (. . . .) Cu^{2+}, (- - - -) Zn^{2+}.

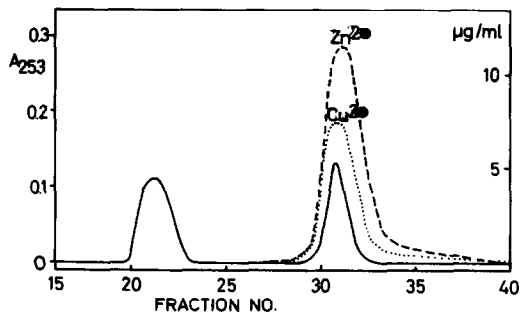

Fig. 2. Elution profile of bovine apo-erythrocuprein. 40.1 mg erythrocuprein dissolved in 5 ml 100 mM phosphate buffer, pH 7.2, were applied on a Sephadex G-25 medium grade column (16 × 1400 mm) previously equilibrated with 10 mM EDTA buffer, pH 3.8, and eluted with the same buffer. Temperature 25°. Elution time 10.5 hr to fraction No. 22, 16 hr to fraction No. 32; fraction volume 6.5 ml. (——) A_{253} nm, (. . . .) Cu^{2+}, (- - - -) Zn^{2+}.

was readjusted to pH 6.8. The second peak contained a mixed Cu–Zn–cyanide complex as shown by separate experiments using Cu–Zn–cyanide, which absorbed at 253 nm. If native erythrocuprein was passed through a G-25 column previously equilibrated with 5 mM potassium phosphate buffer, pH 10.8, without added cyanide, no copper or zinc was removed from the metalloprotein.

The alternative use of EDTA as a chelating agent yielded a highly purified apo-erythrocuprein (fig. 2). It was interesting to note that the absorption at 253 nm of this apoprotein was much lower compared to the above apoprotein where cyanide was employed. No copper and zinc were detected in the apoprotein or in fractions following the main peak. The second peak proved identical with mixed Cu–Zn–EDTA chelates, as confirmed by control experiments.

The molar coefficient of absorption was determined using the method described in [1] and was substantially lower A_{259} = 3675 compared to A_{259} = 9840 of the native erythrocuprein. Fully spectral and enzymic properties were obtained again after incubating the apoprotein overnight at 4° in the presence of Cu^{2+} and Zn^{2+}.

4. Discussion

The gel filtration technique for preparation of apoproteins has several important advantages. It is economical of time since, for example, according to Carrico and Deutsch [4] 72 hr of dialysis is necessary for complete removal of copper and zinc using human erythrocuprein. In contrast, only 8–10 hr is required in the present procedure employing chelator equilibrated Sephadex columns. Less stable metalloproteins such as thermolysin or neutral proteases, can be converted into the apoproteins within 10–20 min [6]. In those studies short columns (30 × 100 nm) proved sufficient. Longer columns as used in our laboratory allowed the convenient separation of possible degradation products and low molecular chelates within one step. The dialysis procedure, however, requires additional gel filtration for the separation of these products [4]. The gel filtration technique offers the further advantage that in preparing apoproteins from oxygen sensitive metalloproteins, anaerobic conditions can be maintained without elaborate precautions.

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