ERYTHROCYTE $Cu_2(HAEM_b)_2$ PROTEIN

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1. Introduction

The copper concentration in red blood cells is ~10 μM [1]. In 1938 a protein called erythrocuprein was successfully isolated [2] and found to bind 40% of the total copper [3–5]. Furthermore, 2 mol zinc were also coordinated. The superoxide dismutase activity of this Cu_2Zn_2 protein [6] led to conflicting conclusions regarding its biological function [7–9].

A second copper-containing protein called 'pink-copper protein' was claimed to be present in erythrocytes [10]. Unfortunately, no supporting evidence for the existence of such a protein was obtained [11] (H. Deutsch et al. and G. Rotilio et al., unpublished). Thus, it seemed very challenging to search for Cu proteins responsible for the remaining high copper content in the red blood cells.

Bovine erythrocyte lysate was chromatographed using 5 different techniques including gel filtration, ion-exchange and adsorption chromatography. A homogeneous protein as deduced from discontinous polyacrylamide electrophoresis and gel filtration (M_r 400 000 ± 40 000) was obtained. The protein contained 2 mol Fe and Cu each. The copper was of the type II and fully EPR detectable ($g_\perp = 2.0309, g_\parallel = 2.2122, A_\parallel = 150$ G). All iron was present as $haem_b$ ($g = 5.702$). At present, the functional side of this protein remains obscure. The catalase activity of the haem iron was only 0.1%. Many other enzymic assays including cytochrome *c* oxidase, dopamine-β-hydroxylase, galactose oxidase, monoamine oxidase, peroxidase, tyrosinase and uricase they all proved to be negative. However, the intracellular concentration of this haem copper protein is considered to be close to that of erythrocuprein.

2. Experimental

2.1. *Reagents*

DEAE-cellulose (DE-23) was purchased from Whatman, Maidstone; Sephadex G-25, G-75 and G-200 were from Pharmacia, Uppsala; hydroxylapatite was from LKB, Stockholm. Dialysis tubing (Visking) was from Serva, Heidelberg and treated with 1 mM EDTA prior to use. Cytochrome *c* oxidase was a gift of Professor Dr G. Buse, Aachen. All other employed enzymes were obtained through Boehringer, Mannheim; Miles, Frankfurt and Sigma, München. They were used without further pretreatment. Cu_2Zn_2 superoxide dismutase was prepared as in [5].

2.2. *Analytical procedures*

Metals were quantified on a Perkin Elmer atomic absorption unit (400 S) equipped with a graphite furnace (HGA 76 B). EPR spectra were run on a Varian E-109 spectrometer. Circular dichroism was measured using a Jasco J-20A and electronic absorption was recorded on a Beckman 25 spectrometer. Protein was determined using the biuret microassay [12]. Amino acid residues were quantitated after 24–72 h hydrolysis at 110°C [13] employing a Kontron Liquimat III amino acid analyzer. Analytical polyacrylamide gel electrophoresis was carried out as in [14]. The relative molecular mass (M_r) was estimated using the gel filtration technique (Sephadex G-200) [15]. Ferritin, xanthine oxidase, catalase, mushroom tyrosinase and haemoglobin served as reference compounds Haem was determined after conversion into pyridine haemochromes [16]. Orcinol/H_2SO_4 was used to measure the carbohydrate content [17].

2.3. *Preparation*

Citrated whole bovine blood was washed 3 times

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with 0.9% NaCl. Lysis of packed cells was accomplished by exhaustive dialysis against 10 mM Tris–HCl buffer (pH 6.9). This buffer was used throughout if not otherwise stated. The haemolysate was passed through DE-23. The bound non-haemoglobin proteins were eluted with 0.5 M NaCl and concentrated (1:10) using membrane filtration under nitrogen pressure. Gel filtration on Sephadex G-75 yielded fractions containing Cu-proteins of both high and low M_r. The latter species was Cu_2Zn_2 superoxide dismutase. The fractions of the high M_r Cu-proteins were collected on hydroxylapatite and eluted with a linear phosphate gradient (0–500 mM) (pH 6.9). The copper containing fractions were concentrated to 2 ml using membrane filtration. Further purification was successful employing Sephadex G-200 chromatography. Upon repeated performance of the last two chromatographic steps no further separation was seen.

3. Results

The treatment of erythrocyte lysate with chloroform/ethanol similar to that used for the isolation of erythrocuprein (Cu_2Zn_2 superoxide dismutase) was not advisable. Apart from erythrocuprein virtually all other copper containing fractions co-precipitated with the haemoglobin. No further separation was possible. Thus, the aqueous technique employing dialysis of the blood cell lysate against 10 mM Tris–HCl buffer (pH 6.9) reduced the osmotic pressure by removal of low M_r components. The DE-23 cellulose chromatography may be considered a haemoglobin filtration as virtually only the non-haemoglobin proteins were bound. Hydroxylapatite chromatography as used in [2] still proved a powerful means to remove firmly bound catalase. This was further improved by the last Sephadex G-200 gel filtration step. Hydrophobic chromatography did not affect the properties of the isolated copper haem protein. The copper haem protein migrated as one single band on polyacrylamide gel electrophoresis (fig.1). M_r determinations employing comparative gel filtration on Sephadex G-200 corresponded to 400 000 ± 40 000. The copper and iron concentration of crude and purified material was monitored by atomic absorption spectroscopy (table 1).

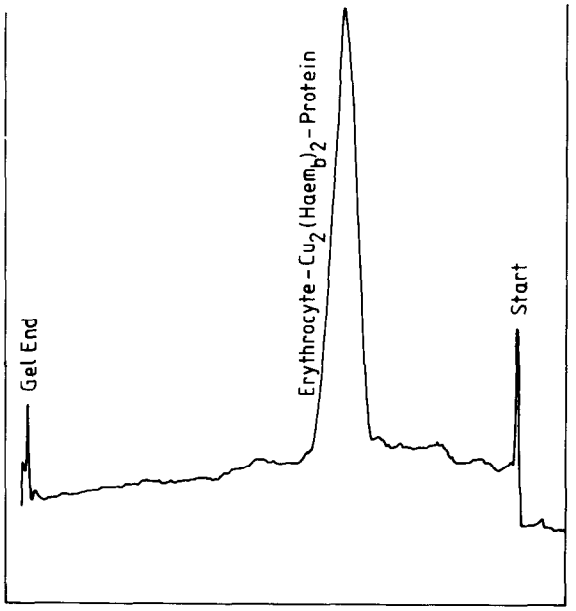

Fig.1. Electronic absorption of $Cu_2(haem_b)_2$ protein in 10 mM Tris–HCl (pH 6.9) at 22°C and of the same material treated with excessive $Na_2S_2O_4$ (dotted).

Table 1
$Cu_2(haem_b)_2$ protein isolation, copper and iron content of different purification steps

Fraction	Volume (ml)	Copper (μg)	% of total Cu	Iron (μg)
Haemolysate	1000	600	100	1 000 000
DE-23-eluate	480	450	75	5500
Ultrafiltration	50	435	72.5	5500
Sephadex G-75 eluate (high M_r-fraction)	350	105	17.5	800
Hydroxylapatite-eluate	50	90	15	180
Ultrafiltration	2	85	14	170
Sephadex G-200 eluate	25	57	9.5	61

The data depicted here are the results of >10 different preparations. The standard error was ±10% indicating the constancy of the Cu/Fe ratio

Three orders of magnitude of the erythrocyte iron was removed in the first chromatographic step. The second efficient iron-removal succeeded during chromatography on hydroxylapatite. Approximately equimolar Cu and Fe concentrations were found in the final fraction containing the homogeneous protein. Zn, Ni, Mn, Mo and Cd were below the detection limit. Assuming the above M_r, 2 mol Cu and Fe each are bound in this protein.

The amino acid composition was (in % of residues): Asx 10.3, Ser 4.3, Glx 15.9, Ala 5.4, Val 6.8, Met 2.4, Leu 10.4, Ile 4.7, Tyr 4.3, Phe 5.2, Lys 7.6, His 2.4, Thr 4.3, Gly 3.9, Arg 6.3, Pro 4.3, Cys 1.5. Cysteine was determined as cysteic acid. The carbohydrate content was <1%.

Electronic absorption spectroscopy resulted in a strong ultraviolet absorption at 280 nm and a band at 403 nm of remarkable intensity. Four more absorption peaks appeared at 495, 535, 570 and 625 nm. Throughout they were assigned to the contribution of the iron chromophore (fig.2). Upon reduction with dithionite, 4 absorption bands were levelled off and 2 new bands appeared at 412 and 550 nm. Due to this absorption profile the presence of a haem chromophore was deduced.

Acetone/HCl treatment [16] resulted in the removal of the iron chromophore. The supernatant was suspended in pyridine/KOH and measured in the oxidized and reduced state at 556 nm. According to this technique 2 haem residues were calculated/400 000 M_r. The haem species was further characterized using dual wavelength spectrometry following the direct addition of KOH/pyridine [18–20]. In the difference spectrum between the reduced and oxidized protein 3 bands appeared at 556, 524 and 485 nm, respectively. The pronounced Soret band was seen at 418 nm. Attributable to the position of the 556 and 418 bands the haem chromophore was assigned to a protohaemochrome as usually found in cytochromes of the *b*-type. The dichroic properties of the protein were rather limited. Apart from the Cotton band at 221 nm (θ = 880 000 grad . cm^2 . dmol^{-1}), indicative for some α-helical portion no further significant extrema were seen.

Electronic absorption spectroscopy was unsuitable to detect the nature of the copper chromophore. The high absorption of the protohaemochrome overlapped the contribution of the coordinated copper. According to magnetic measurements all copper was fully EPR detectable ($g_\perp$ = 2.0309, $g_\parallel$ = 2.2122 and $A_\parallel$ = 150 G)

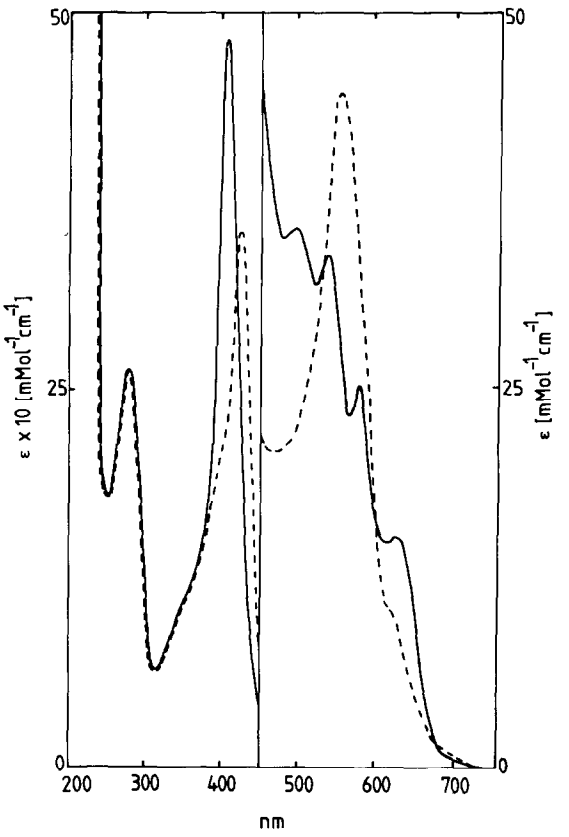

Fig.2. Polyacrylamide disc electrophoresis of $Cu_2(haem_b)_2$ protein. Acrylamide was 4% in the concentrating gel and 7% in the separating gel. Electrophoresis was performed at 50 V and 1 mA/gel stick for 2 h. Staining was with Coomassie brilliant blue. The scanning speed was 0.2 mm/s; chart speed 0.2 mm/s; slit openings were 0.2 mm perpendicular and 3 mm parallel to the gel. Scanning was performed at 560 nm.

and was assigned to the non blue or type II copper [21].

The occurrence of two redox active transition metals suggested that this protein might be involved in some electron-transport reactions. Unfortunately, enzymic activities including cytochrome *c* oxidase, dopamine-β-hydroxylase, galactose oxidase, monoamine oxidase, peroxidase, tyrosinase and uricase were not detected. Catalase activity referred to 0.1% of the haem iron.

4. Discussion

The main problem was to cope with the huge mass of iron proteins including haemoglobin and catalase.

Thus, any precipitation step was carefully omitted to avoid trapping of the intracellular copper proteins. The Cu/Fe ratio in the haemolysate was 1:1700 which eventually ended in an equimolar ratio in the purified protein.

From immunological studies it is known that 40% of the erythrocyte copper is bound in erythrocuprein [3,4]. At present no such studies were performed on the haem-copper-protein. However, the initial concentration in the haemolysate should be considered >10% of the total copper attributable to unavoidable losses in the course of the preparation.

The haem moiety is definitely not attributable to unspecifically bound catalase or catalase fractions. The absorption band at 570 nm is only seen on recording the copper-haem-protein. Throughout the absorption coefficients were 4–5-times higher compared to those of the Fe(III) catalase. In contrast to the unsuccessful reduction of catalase using dithionite distinct spectral changes were monitored when the haem-copper-protein was reduced. Less than 0.1% catalase activity calculated on the basis of total haem content was measured. The non-blue copper chromophore is supposedly deeply buried in the protein. In general, copper chelates having free coordination sites react with superoxide catalytically [6,7]. No such reactivity was detectable.

Due to the occurrence of two haem and two coppers, the biological role would be spontaneously sought in a kind of cytochrome oxidase activity. Unfortunately this function was not seen, neither the enzymic actions of many more redox enzymes proved to be positive. The search for the functional side remains open. Unlike in the case of erythrocuprein it is hoped that no lag phase of 30 years has to be overcome until a biochemical action other than iron and copper binding will be described.

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