Erythrocuprein (Cu_2Zn_2 superoxide dismutase) is the major copper protein of the red blood cell

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The copper balance in the red blood cell deserved special attention as the exact amount of copper being bound in erythrocuprein (Cu_2Zn_2 superoxide dismutase) is poorly understood. An improved aqueous isolation of erythrocuprein revealed that essentially all erythrocyte copper is found in this protein. This fact is supported by both superoxide dismutase activity measurements of the haemolysate and EPR-quantification studies throughout the course of the isolation.

Copper *Erythrocyte* *Erythrocuprein* *Cu_2Zn_2 superoxide dismutase* *Red blood cell*

1. INTRODUCTION

Numerous studies on the biochemistry of copper proteins isolated from different tissues have been published within the last 20 years. Surprisingly, only limited data on copper proteins of erythrocytes are available. The 1700-fold excess of haem iron in relation to copper may be one possible reason for this phenomenon.

In 1938 Mann and Keilin isolated a copper protein from bovine erythrocytes [1]. It is presently termed Cu_2Zn_2 superoxide dismutase [2]. Of the total copper concentration in red blood cells, 37% is present in this protein as determined immunochemically [3].

Another copper-containing protein fraction was separated from human erythrocytes in 1961 [4]. Eight years later a pink copper protein was found in human erythrocytes [5]. No further reports dealing with the last two copper proteins were published. A fourth copper protein called Cu_2haem_b-protein was isolated from bovine and human erythrocytes [6–8].

Attributable to the many different reports on the copper content of the red blood cell, it appears that the exact amount of copper being coordinated in erythrocuprein is still not fully understood. Thus a re-examination of the assignment of copper bound to the above 3 copper proteins was of high interest.

2. EXPERIMENTAL

2.1. *Reagents*

DEAE–Sephacel, Sephadex G-75 and CM-Sephadex were from Pharmacia (Uppsala). Visking dialysis tubing, gelatine and xanthine were obtained from Serva (Heidelberg). Nitroblue tetrazolium was purchased from Calbiochem (San Diego CA). Membrane filters were obtained from Amicon Corp. (Oosterhout). All other employed reagents were from Merck (Darmstadt). As analytical grade potassium phosphate is known to contain up to 10^{-5} M copper, suprapure potassium phosphate was used. [Copper] in the buffer solutions was < 1 nM. Deionized water was additionally distilled over quartz and had a conductivity of $< 0.5\ \mu$S.

2.2. *Analytical procedures*

Metal analyses, protein determination and polyacrylamide gel electrophoresis were done as in [7]. Superoxide dismutase activity was assayed using the tetrazolium blue method [9].

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Table 1

Aqueous isolation of erythrocuprein – specific activity, copper and iron content of different purification steps

Fraction	Vol. (ml)	Copper (μg)	% of Cu	Iron (μg)	SOD[a] (U/mg)
Haemolysate	2000	1020	100	1800000	0.94
DEAE–Sephacel eluate	470	870	85	1600	160
G-75 (low M_r)	350	850	83	520	600
Second DEAE-eluate	240	600	58	140	1200
CM-Sephadex	670	635	62	30	1300
Third DEAE-eluate	280	530	50	–	3200

[a] Superoxide dismutase

2.3. *Isolation procedure*

Haemolysate from bovine blood was passed through DEAE-Sephacel. The bound copper proteins were eluted with NaCl. Gel filtration on Sephadex G-75 resulted in 2 fractions containing copper proteins of both high and low M_r. The first copper protein was the previously described M_r 400000 $Cu_2(haem_b)_2$-protein. It was purified as in [6]. The latter species was Cu_2Zn_2 superoxide dismutase which was further isolated as in [3].

3. RESULTS AND DISCUSSION

Unlike the copper concentration in red blood cells obtained from lactating cows the copper content in male bovine cells is usually 50% higher (~10 μM). Regardless of sex, [copper] in human erythrocytes is 5–10 μM. Up to twice this amount of copper was reported in [3,4]. This may be attributed to less accurate quantification methods.

The general approach for the isolation of copper proteins from erythrocytes includes the treatment of haemolysate with organic solvents. Using this technique, only 30–40% of the intracellular copper is recovered. Due to co-precipitation and/or denaturation of possible other copper proteins, the major portion is lost. A strictly aqueous preparation method allows the recovery of 85% of the erythrocyte copper, in the first and 50% after the final step. The aqueous isolation of erythrocuprein as in [3] includes discontinuous absorption of erythrocyte copper proteins to DEAE–cellulose. Contrary to this method, 85% of the copper proteins of the erythrocyte are recovered when haemolysate was continuously passed through DEAE–Sephacel. A typical improved aqueous isolation of erythrocuprein is summarized in table 1.

One striking result of the new preparation is that virtually all of the intracellular copper is found in erythrocuprein. This fact is supported by EPR-spectra of the copper protein fractions. The copper concentrations were high enough to allow direct EPR measurements after each DEAE chromatographic step. Quantification of the EPR-signals was accomplished by adding defined amounts of purified superoxide dismutase which was isolated by the usual solvent treatment of haemolysate [10]. In comparing the EPR properties of the eluate of the very first DEAE chromatography with those of later ion-exchange chromatographic steps no significant improvement of the EPR properties was seen (fig.1).

All copper of the erythrocyte is of type (II) ($g_\perp$ = 2.09; $g_\parallel$ = 2.23; $A_\parallel$ = 0.013 cm^{-1}). In contrast to erythrocuprein initially treated with organic solvents the EPR-signal of the aqueous protein showed a significant line-broadening in the $g_\perp$-region and a sharpening in the $g_\parallel$-region. Electrophoresis of purified aqueous erythrocuprein showed the usual two-banded pattern similar to those described for the solvent-treated protein [11–13].

Further evidence that erythrocuprein is the sole copper protein of erythrocytes is supplied by M_r determination (32000), copper and zinc analyses and determination of superoxide dismutase activity throughout the preparation. Starting from haemolysate the EDTA resistant superoxide dismutase activity was monitored in the course of

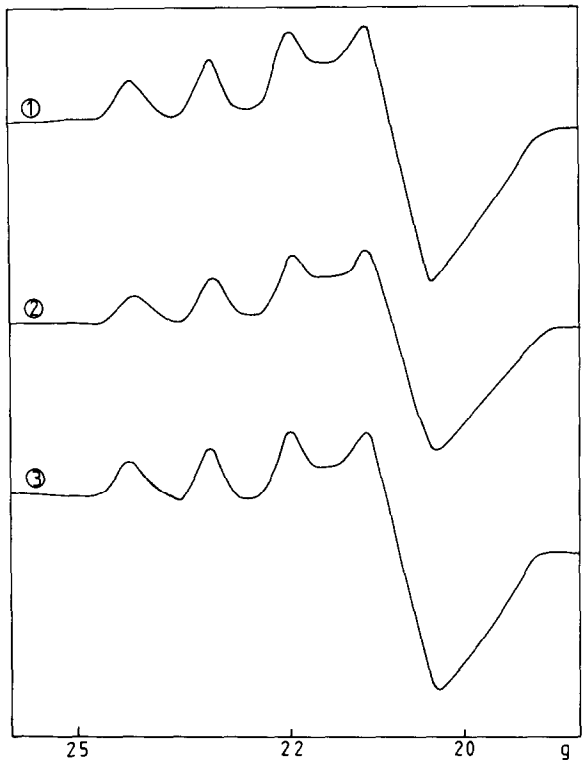

Fig.1. EPR spectra of fractions of erythrocuprein throughout the isolation course. Spectra were recorded after each DEAE-chromatographic step: (1) DEAE–Sephacel eluate; (2) second DEAE eluate; (3) third DEAE eluate (homogeneous erythrocuprein); scan range, 1000 G; field set, 3000 G; modulation amplitude, 10 G; modulation frequency, 100 kHz; temp., liquid N_2; microwave power, 12.5 mW; microwave frequency, 9.24 GHz.

the isolation procedure. It could be demonstrated that in all preparation steps the specific activity related to copper is attributable to erythrocuprein.

Erythrocuprein, isolated by the aqueous procedure, carries 1.9 mol copper and 2.0 mol zinc/mol protein and has a specific superoxide dismutase activity of 3200 U/mg protein. From 2 l of packed cells, 140 mg homogeneous erythrocuprein were isolated within 2 weeks omitting any organic solvent.

If erythrocuprein is indeed the sole copper protein of the red blood cell, which portion of erythrocyte copper is bound in the other 3 reported copper proteins including the $Cu_2(haem_b)_2$-protein? Separate gel filtration runs of crude haemolysate on Sephadex G-75 revealed 95% of intracellular copper and superoxide dismutase activity migrating in the region of M_r 32000. The amount of high M_r proteins including $Cu_2(haem_b)_2$-protein is only 5%. This indicates that initially an extremely low concentration of $Cu_2(haem_b)_2$-protein is present. It cannot be excluded that this high M_r protein is formed at a later stage of the isolation procedure. This conclusion is supported by electronic absorption properties of mixtures containing 10 μM oxyhaemoglobin and 30 μM methaemoglobin. Alternatively, 10 μM oxyhaemoglobin and 30 μM catalase give rise to the same absorption profile. However, the absorption coefficients are only half to those of the $Cu_2(haem_b)_2$-protein. As no superoxide dismutase activity is observed, the EPR signal indicative for the type (II) copper may be attributed to trapped erythrocuprein by the polymerized haem proteins. Taking into account that 95% of red blood cell copper is bound in erythrocuprein, it is concluded that this Cu_2Zn_2-protein is the major intracellular copper binding protein, leaving the other 3 copper proteins in a dubious state. Many authors have evaluated the amount of Cu_2Zn_2 superoxide dismutase immunochemically [11–15]. This technique was deliberately not employed because of the strongly deviating erythrocuprein concentrations.

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