Aqueous Isolation of Erythrocyte Copper Proteins: Formation of Bis[(Heme b)Copper] Protein

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Abstract

Bis[(heme b)copper] protein a protein other than Cu_2Zn_2 superoxide dismutase was isolated from both bovine and human erythrocytes (U. Weser, A. Gärtner and K.-H. Sellinger, *Biochemistry, 21,* 6133 (1982)). This protein was believed to contain 20–50% of the total cellular copper. A separate detailed study on the distribution of copper in the red blood cell revealed that essentially all copper was bound in the Cu_2Zn_2 superoxide dismutase. EPR-measurements and determination of the specific activity of superoxide dismutase expressed per copper in the hemolysate as well as in the course of the aqueous isolation support the latter statement. Gel filtration of crude hemolysate yields less than 5% of the bis[(heme b) copper] protein. The yield increased markedly when copper was added to the hemolysate prior to isolation. The electronic absorption properties of the bis[(heme b)copper] protein can be simulated by mixtures of either oxyhemoglobin and methemoglobin or alternatively oxyhemoglobin and catalase. Thus, it is concluded that the bis[(heme b)-copper] protein must have been formed during the long lasting aqueous preparation.

Introduction

The occurrence of copper in the red blood cell was demonstrated in 1832 by Sarzeau. One hundred years later Mann and Keilin succeeded in isolating erythrocuprein [1] which is currently named Cu_2Zn_2 superoxide dismutase (EC 1.15.1.1.). Many reports dealing with the isolation of this protein followed [2–4]. Furthermore, some other copper proteins were found in erythrocytes [5, 6], however, their existence could never be confirmed. Moreover, quantification studies on the amount of copper coordinated to the different proteins of the red blood cell were missing.

In 1981 a bis[(heme b)copper] protein of M_r = 400 000 was prepared from bovine and human erythrocytes [7, 8]. 20–50% of the total erythrocyte copper was suggested to be present in this protein.

Recently it was demonstrated that nearly all of the erythrocyte copper is coordinated in erythrocuprein [9]. Thus, it was of considerable interest to examine the relationship between the bis[(heme b) copper] protein and Cu_2Zn_2 superoxide dismutase. Emphasis was placed on the possible formation of the former protein during the course of the isolation.

Experimental

Chemicals

Ion exchange and other gels were the same as earlier described [8]. Suprapure as well as analytical grade potassium phosphate was from Merck (Darmstadt). All other chemicals employed were as in Gärtner and Weser [9]. Apart from potassium phosphate all buffer solutions had a copper concentration of less than 1 nM. Quartz distilled water with a conductivity of less than 1.0 μS was used throughout.

Spectrometry

EPR-spectra were run on a Varian E-109 spectrometer at 77 K. Ultraviolet and visible absorption was recorded on a Beckmann 25 spectrometer.

Isolation

The bis[(heme b)copper] protein was isolated using the sequential aqueous column chromatography [7]. The isolation was performed avoiding any metal contamination.

Results

The initial step for isolating bis[(heme b)copper] protein is ion exchange chromatography of the hemolysate on DEAE-Sephacel. This was followed by gel filtration on Sephadex G-75. Special emphasis was placed on these two steps as they turned out to

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TABLE I. Copper Concentrations after Gel Filtration of Crude Hemolysate on Sephadex G-75.[a]

	$M_r > 70\,000$	$M_r \sim 60\,000$	$M_r \sim 30\,000$	$M_r \sim 1\,000$
Cu [ng]	15.0 ± 3.0	11.4 ± 2.3	259.5 ± 13.0	14.1 ± 2.8
[%]	5.0 ± 1.0	3.8 ± 0.8	86.5 ± 4.3	4.7 ± 1.2

[a]The data depicted here are the results of 4 different gel filtration runs. The standard error was ±10% in the constancy of copper determination below 20 ng/ml and ±2.5% above 20 ng/ml. One ml hemolysate was applied to Sephadex G-75 (1 × 100 cm) in 10 mM Tris/HCl pH 7.0.

be crucial for the formation of bis[(heme b)copper] protein.

During the course of the ion exchange chromatography the non-hemoglobin proteins are almost completely separated from hemoglobin. More than 80% of the erythrocyte copper was recovered, whereas 99.9% of iron was removed. Nevertheless, the remaining 0.1% of iron in the DEAE-eluate is, by means of metal concentration, in the same ratio as the copper, namely 30 μM copper and 60 μM iron, which is essentially bound by catalase, hemoglobin and the bis[(heme b)copper] protein.

Gel chromatography on Sephadex G-75, the second purification step, yielded one fraction of high and two fractions of medium relative molecular mass, respectively. The proteins of the first fraction ($M_r >$ 70 000) were essentially assigned to catalase and the bis[(heme b)copper] protein. The second $M_r \sim$ 60 000 fraction was crude hemoglobin, whereas the last fraction ($M_r \sim 30\,000$) contained erythrocuprein (Cu_2Zn_2 superoxide dismutase). Approximately 25% of the eluted copper was found in the high M_r-fraction as reported by Sellinger and Weser [7]. The remaining copper was coordinated in erythrocuprein.

Upon repeating these two inital preparation steps for the bis[(heme b) copper] protein using suprapure phosphate buffer, less than 5% of the erythrocyte copper was found in the high M_r-fraction. Essentially all of the eluted copper from Sephadex G-75 was erythrocuprein-copper.

It was interesting to note the absence of any low molecular weight species both in the DEAE-Sephacel and in the Sephadex G-75 eluate.

The different concentrations of bis[(heme b)-copper] protein obtained in former experiments encouraged a more detailed study. Separate gel filtration runs of crude hemolysate were carried out to shed some light on this problem. Although hemolysate which had passed the DEAE-Sephacel-column contained no detectable copper, the possible loss of high molecular weight Cu-proteins during the first DEAE-chromatography had to be excluded.

91% of both copper and superoxide dismutase activity were detected in the region of M_r = 32 000. High M_r Cu-proteins including bis[(heme b)copper] protein totalled less than 5%.

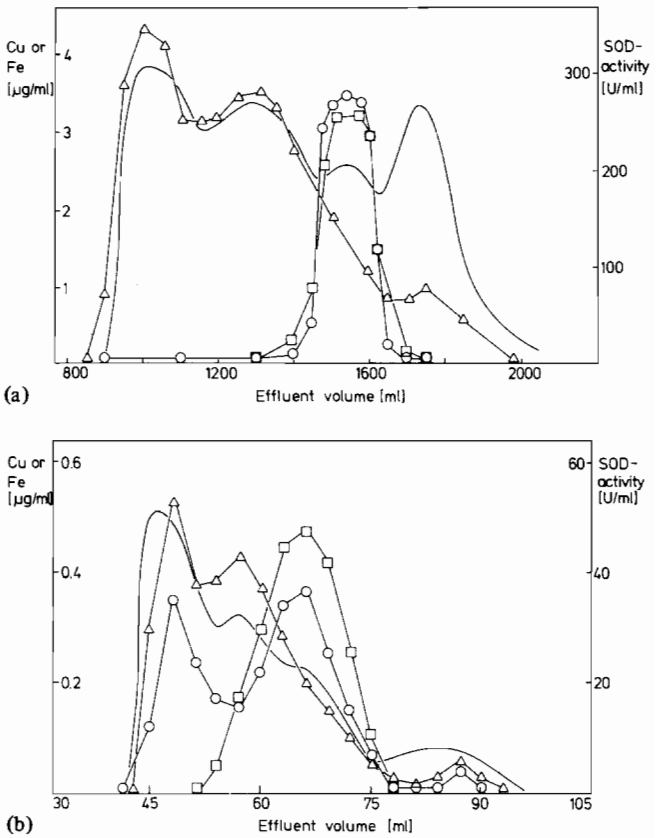

Fig. 1. Gel filtration of the DEAE-eluate on Sephadex G-75 with and without addition of copper to the hemolysate prior to isolation. Fractions were assayed for copper (○), iron (△), superoxide dismutase (□) (NBT assay) and A_{254} (——). (a) Hemolysate without addition of copper. Column dimensions: 4 × 180 cm. (b) Hemolysate supplemented with the same amount of copper sulfate originally present. Column dimensions: 1 × 45 cm.

These results are supported by enzymic assays of SOD in the hemolysate expressed per copper. The Cu_2Zn_2-form of superoxide dismutase is sensitive to cyanide but resistant to EDTA. The superoxide dismutase activity of the hemolysate remains unchanged after the addition of 1 mM EDTA. In the presence of 1 mM cyanide the activity was totally inhibited. Quenching effects in the test can be excluded, as the Cu-deficient hemolysate after the first

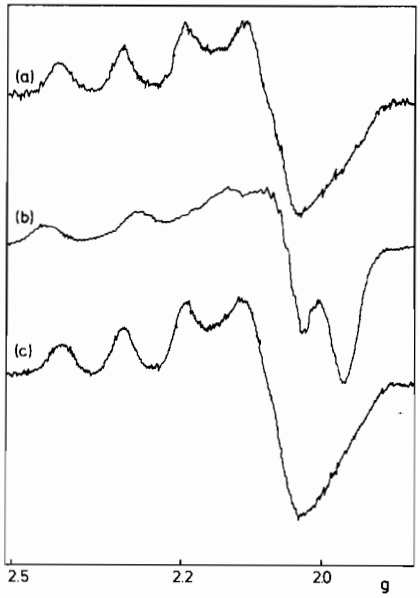

Fig. 2. ESR properties of fractions of Cu-proteins throughout the aqueous isolation. (a) Eluate of the first DEAE-chromatography. (b) Purified bis[(heme b)copper] protein. (c) Aqueously isolated Cu_2Zn_2 superoxide dismutase. Scan range, 1000 G; field set, 3000 G; modulation frequency, 100 KHz; temp., liquid N_2; microwave power, 12.5 mW; microwave frequency, 9.24 GHz.

DEAE-chromatography completely lacks SOD-activity.

The specific activity (3.0×10^{-8} M Cu for 50% inhibition) does not allow the assignment of copper to Cu-proteins other than erythrocuprein in the hemolysate. In other words, the bis[(heme b)copper] protein initially does not exist in significantly high concentrations.

The EPR spectrum of the bis[(heme b)copper] protein at g = 2 is characteristic for type II copper. In the $g_{\perp}$-region a minor contribution is seen which was attributed to unspecifically bound copper of the biuret type. By way of contrast, no extraneously bound copper was detected in the eluate of the first DEAE-chromatography.

Provided that in the hemolysate no specific high molecular copper proteins can be detected, the question arose as to how the bis[(heme b)copper] protein was formed during the isolation procedure. For this purpose the preparation of this protein was repeated starting from hemolysate which was supplemented with the same and twice the amount of copper originally present. Upon chromatography on DEAE-Sephacel followed by gel filtration on Sephadex G-75, the yield of the high M_r-copper fraction increased substantially leaving the erythrocuprein fraction unchanged. Addition of equimolar copper concentrations to hemolysate resulted in a high-M_r copper fraction with a similar copper content as in the erythrocuprein fraction.

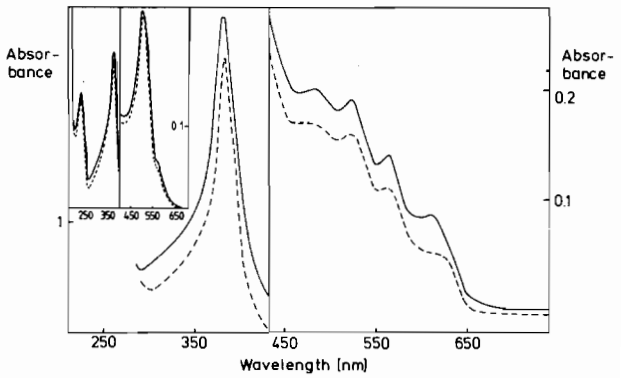

Fig. 3. Simulation of electron absorption properties of bis-[(heme b)copper] protein in 10 mM Tris-HCl (pH 7.0). Solid line: bis[(heme b)copper] protein. Dotted line: 1 oxyhemoglobin + 3 methemoglobin or 1 oxyhemoglobin + 3 catalase. The inset refers to the reduced protein. Solid $Na_2S_2O_4$ was added to the solution until no optical changes were recorded. Temperature, 22 °C; 1 cm quartz-cuvettes.

When a twofold excess of copper was added to the hemolysate, the yield of the eluted high-M_r copper proteins was twice that of erythrocuprein.

The electronic absorption profile of the high M_r-fractions after the addition of copper resembled the spectrum of the bis[(heme b)copper] protein. Alternatively, this spectrum can be simulated using molar mixtures of 1 oxyhemoglobin and 3 methemoglobin or 1 oxyhemoglobin and 3 catalase. The absorption bands of the oxidized bis[(heme b)-copper] protein at 625, 570, 535 and 495 nm as well as the Soret peak at 304 nm reflect the sum spectra of the preceding hemoproteins.

This explains why the difference spectrum oxidized *versus* reduced heme of the bis[(heme b)-copper] protein heme of the b-type was detected [8]. Unlike with catalse the bis[(heme b)-copper] protein can be reduced by dithionite or ascorbate. The mixture of catalase and hemoglobin was subjected to the isolation procedures described by Weser *et al.* [8]. Although the electronic absorption profile of the isolated catalase was changed, no polymerisation of these hemeproteins was seen under these conditions.

However, it is by no means clear which of the initial steps of the aqueous isolation causes the binding of copper to the different hemolysate proteins. The very first step is an exhaustive dialysis of hemolysate against Tris-buffer. Upon repeating the dialysis experiments with Tris-buffer, bidistilled water and even tap water, no changes in the copper concentration of the hemolysate were seen. A possible source of copper may be analytical grade potassium phosphate as it contains up to 10^{-5} M Cu. Of course, catabolic fragments of Cu_2Zn_2 superoxide dismutase must also be considered as an internal copper contaminating source.

Discussion

When isolating copper proteins from the red blood cell three principal difficulties have to be overcome. The first is the enormous amount of heme iron in relation to copper (2000:1). Secondly, the ubiquitous occurrence of copper even in analytical grade chemicals has to be considered and thirdly, the extremely high binding affinity of proteins for copper, especially hemoglobin and catalase must be seen. Actually, catalase was characterized as a Cu-heme-protein in the early days of inorganic biochemistry [10]. Hemoglobin has a specific binding site for copper [11]. Binding of one copper leads to oxidation to methemoglobin. In addition, the ion exchange chromatography of hemolysate requires very low ionic strength. In general, hemoproteins are very sensitive to these conditions. Both copper binding from buffers and low ionic strength may cause a denaturation of hemeproteins resulting in species which can act as a possible source of radical reactions.

The aqueous preparation procedure does not readily remove these heme species in the first isolation steps. Thus, there is enough time for the formation of the bis[(heme b)copper] protein by polymerisation reactions.

From this standpoint the isolation method for superoxide dismutase, *i.e.* treating hemolysate with organic solvents, is an excellent technique, as it removes hemoproteins very rapidly from nonhemoglobin proteins. Unfortunately, only 30% of the intracellular copper is recovered by this technique and conformational changes in the isolated proteins cannot be fully excluded.

The aqueous isolation of the bis[(heme b)copper] protein has in terms of the final yield of this protein another delicate feature. One of the last purification steps includes chromatography on hydroxyapatite. Hydroxyapatite is known as a very potent Cu-chelator, accumulating copper from buffers. This copper is successively eluted, applying the crude bis[(heme b)copper] protein to the column. EPR measurements support the former conclusion. The EPR spectrum in the g = 2 region indicative for type II copper was superimposed by a signal of the biuret type copper, which was not detected in the eluate of the first DEAE-chromatography.

In critically reviewing the other copper proteins from erythrocytes, including the copper fraction of Shields *et al.* [5] and the pink copper protein [6], our results are well placed in the present knowledge on erythrocyte copper proteins.

Shields *et al.* isolated a copper fraction from hemolysate after treatment of erythrocytes with radioactive copper. This fraction was the precursor of the bis[(heme b)copper] protein.

Apart from some different spectral data, the pink copper protein had similar molecular properties to the Cu_2Zn_2 superoxide dismutase. Although the latter is one of the most stable enzymes, it does not completely survive chromatography on strong acidic ion exchangers originally applied for isolating the pink copper protein. Even repeated chromatography of aqueous isolated superoxide dismutase on weak acidic ion exchangers (DEAE) led to a considerable loss of both copper and protein. This lost fragment might have been converted into a pink copper protein under the purification described by Reed *et al.* [6].

Unlike erythrocuprein, the bis[(heme b)copper] protein appears to suffer the fate of the other copper proteins reported to be present in the red blood cells. The transient formation of bis[(heme b)copper] protein should be kept in mind when searching for new hemoproteins. This copper-heme protein was not only found in blood, but also in bovine liver (unpublished). Attributable to the relatively long life span of erythrocytes nearly all vertebrate tissues contain hemoglobin of variable age. Even in homogenates of washed livers hemoglobin can be detected in significant amounts. Thus, it is of the utmost importance to examine the existence of any new heme b protein found in vertebrate tissues.

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References

1 T. Mann and D. Keilin, *Proc. Roy. Soc. London Ser. B, 126,* 303 (1939).
2 H. Markowitz, G. E. Cartwright and M. M. Wintrobe, *J. Biol. Chem., 234,* 40 (1959).
3 J. W. Hartz and H. F. Deutsch, *J. Biol. Chem., 244,* 4565 (1969).
4 J. M. McCord and I. Fridovich, *J. Biol. Chem., 244,* 6049 (1969).
5 G. S. Shields, H. Markowitz, W. H. Klassen, G. E. Cartwright and M. M. Wintrobe, *J. Clin. Invest., 40,* 2007 (1961).
6 D. W. Reed, P. G. Passon and D. E. Hultquist, *J. Biol. Chem., 245,* 2954 (1970).
7 K.-H. Sellinger and U. Weser, *FEBS Lett., 133,* 51 (1981).
8 U. Weser, A. Gärtner and K.-H. Sellinger, *Biochemistry, 21,* 6133 (1982).
9 A. Gärtner and U. Weser, *FEBS Lett., 155,* 15 (1983).
10 K. Anger, *Biochem. J., 32,* 1702 (1938).
11 J. M. Rifkind in H. Sigel (ed.), 'Metal Ions in Biological Systems', Marcel Dekker, New York and Basle, 1981, pp. 191–232.