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ESR INVESTIGATIONS AT LOW TEMPERATURES OF OXYGEN RADICAL INTERACTION WITH RAT CHROMOCHELATIN AND METALLOTHIONEINS

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SUMMARY: Radiolysis of aqueous solutions of renal and liver low molecular weight metal binding copper proteins was studied by the ESR method at low temperatures. Renal bismuth-copper chromochelatin and liver cadmium-zinc metallothionein like zinc-copper superoxide dismutase dismutate the superoxide radicals. Renal mercury-copper metallothionein traps effectively the hydroxyl radical.

The low molecular weight metalloproteins isolated from kidneys and livers of rats following administration of the inductor metals, such as bismuth, cadmium and mercury (1,2), contain considerably amounts of cysteine and, beside the inductor metals, the endogenous copper and zinc. Because of that they were classified as metallothioneins with exception of bismuth-binding protein isolated from rat kidney which because of the high content of copper, presence of histidine, and low cysteine contact was classified as chromochelatin (3).

The metabolic role and function of chromochelatins and metallothioneins is as yet not fully established. It has been postulated (4,5) that they play some role in detoxification of free heavy metals (6).

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In the present paper the ability of renal chromochelatin and renal or liver metallothioneins to act as oxygen radical scavengers is studied with application of low-temperature γ-radiolysis and ESR technique. These methods (7) allow to investigate the fate of radiolytically generated oxygen radicals in presence of metalloproteins, to determine the paramagnetic intermediate products, to follow its decay upon the thermal annealing and to observe changes of oxidation states of the metals. Renal chromochelatin and liver metallothionein seem to scavenge the radiation produced hydroperoxy radicals while renal metallothionein traps very effectively the hydroxyl radicals.

MATERIALS AND METHODS

Low-molecular weight renal or liver proteins containing bismuth, cadmium or mercury were isolated as described previously (3,5,8-10) and for the present studies the pure isoforms obtained by preparative electrophoresis were used. Metal content in these isoforms is presented in TAB. 1.

High content of cysteine (29-34 mol%) was characteristic for amino acid composition of all investigated metallothioneins. Metal free protein (thionein) was obtained according to the method of Weser et al. (11) from renal chromochelatin.

The superoxide dismutation activity of metalloproteins was determined according to the method of Marklund and Marklund (12), and compared with zinc-copper superoxide dismutase supplied by Miles Laboratories (PTY) Ltd. (12 300 units/mg).

To prepare the samples for radiolytical generation of hydroxyl and superoxide radicals, 200 µl aliquots of the buffered solutions (0.005 M phosphate buffer, pH = 6.88, 10 mg protein/ml) containing metalloproteins, were dropped into the liquid nitrogen to form solid spheres of diameter equal to about 3 mm. Frozen samples were γ-irradiated at 77 K in Co-60 source at a dose rate of 6 kGy/hr to the total dose of 10 kGy. The γ-irradiated samples were transferred into the liquid nitrogen ESR dewar for measurements at 77 K, or into a cold nitrogen gas flow systems for annealing at higher temperatures. A copper-constantan thermocouple placed in contact with the samples was used to measure the temperature controlled by the flow rate of cold nitrogen gas. In that way the constant annealing temperature was obtained. Before and after each heat treatment the ESR spectra were recorded at 100 kHz magnetic field modulation with X-band microwave spectrometer SE-X/20 (Poland) provided with TE_{104} cavity. 1,1'-Diphenyl-2-picrylhydrazyl radical and Mn^{2+} were used as standards.

RESULTS AND DISCUSSION

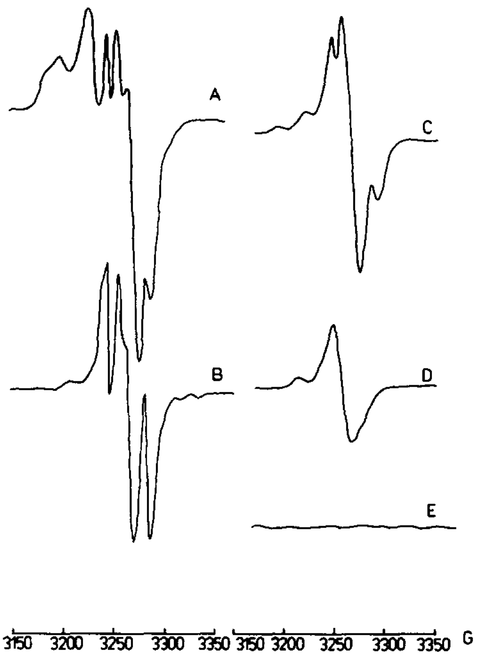
Renal and liver metallothioneins and renal chromochelatin used in the present investigations are diamagnetic.

Table 1. Metal content in metalloproteins used in the present studies

Metalloprotein	Bi	Cd	Hg	Cu	Zn
	mg of metal/g of protein				
renal chromochelatin					
(Bi,Cu)BP3	90	-	-	23	3.6
renal metallothioneins					
(Cd,Cu)MT1	-	83	-	7.5	12
(Hg,Cu)MT3	-	-	56	44	1.8
liver metallothionein					
(Cd,Zn)MT2	-	76	-	0.4	20.9

The ESR spectrum of the γ-irradiated frozen solution of thionein is shown in Fig. 1A. It consists of the doublet of OH radicals (doublet split by about 40 G at g = 2.009 with a broad hump at g = 2.048 (13)) superimposed onto the signals due mainly to the carbon-based radicals, which are clearly seen after the removal of OH radicals upon thermal annealing at 150 K, cf. Fig. 1B. These radicals start to react with oxygen at about 200 K, cf. Fig. 1C, and at 230 K, Fig. 1D, there appears the signal of peroxy radicals, $RO_2^{\bullet}$, similar to that observed in γ-irradiated frozen solutions of carbohydrates (14), in polymers (15) and proteins (16), cf. Tab. 2 for g-factors. The peroxy radicals decay upon further thermal annealing, cf. Fig. 1E.

The presence of the metals in protein molecules changes dramatically the above picture. The ESR spectra recorded at 77 K for the γ-irradiated frozen solutions of renal chromochelatin (Bi,Cu)BP3, renal metallothioneins (Cd,Cu)BP1, (Hg,Cu)MT3, liver metallothionein (Cd,Zn)MT2, and zinc-copper superoxide dismutase SOD for comparison, are presented in Fig. 2A. All these spectra consist mainly of the doublet of OH radicals with only minor contribution of other radia-



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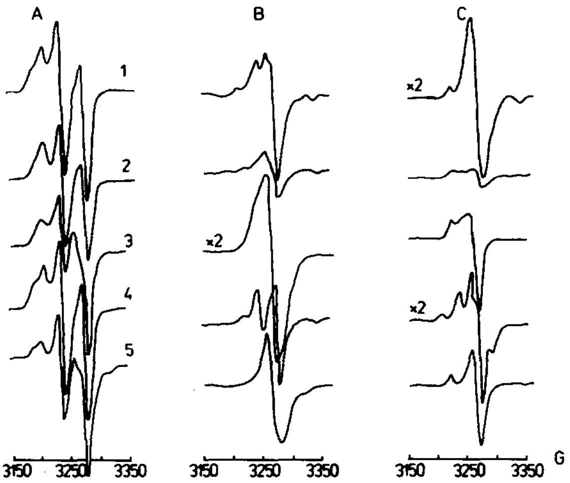

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Fig. 1. ESR spectra recorded at 77 K (frequency 9.13 GHz, microwave power 1 mW, modulation amplitude 10 G) for the frozen solution of thionein in 0.005 M phosphate buffer (pH = 6.88, protein content 10 g/l) γ-irradiated at 77 K (10 kGy, 6 kGy/hr) before (A) and after thermal annealing for 10 min. at 150 (B), 210 (C), 230 (D), and 250 K (E).

Fig. 2. ESR spectra recorded at 77 K (frequency 9.13 GHz, microwave power 1 mW, modulation amplitude 10 G) for the frozen solutions of (Bi,Cu)BP3, (Cd,Cu)BP1, (Cd,Zn)MT2, (Hg,Cu)MT3, and SOD in 0.005 M phosphate buffer (pH = 6.88, protein content 10 g/l) before (A) and after thermal annealing for 10 min. at 150 (B) and 190 K (C).

tion produced radicals and do transform upon thermal annealing to the spectra, Fig. 2C, quite different from that for thionein, Fig. 1D.

In the end spectra recorded for the γ-irradiated frozen solutions of (Bi,Cu)BP3, (Cd,Zn)MT2, and to a lesser extent for (Cd,Cu)BP1, Fig. 2C (1,3 and 2), one recognizes the characteristic features of HO_2 radicals in the loose complex $[E\text{-}Cu^{2+}\ldots HO_2]$ specific for SOD (18), Fig. 2C (5), cf. Tab. 2 for g-factors. Decay of these species upon further thermal annealing proceeds without changes in the oxidation states of metals bound to the proteins; (Cd,Cu)BP1, (Bi,Cu)BP3 and

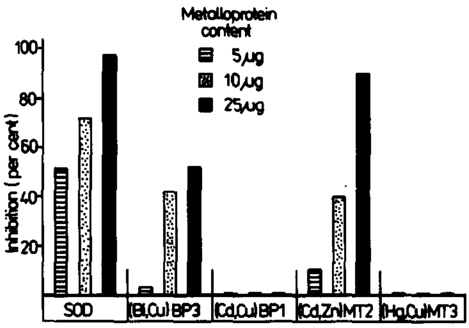

Fig. 3. Determination of pyrogallol autooxidation inhibition by metalloproteins according to the method of Marklund and Marklund (12).

(Cd,Zn)MT2 remain diamagnetic. All this implies that interaction of HO_2 radicals with these metalloproteins proceeds according to the dismutation mechanism developed recently for superoxide dismutase (20). Checking the inhibition of pirogallol autooxidation we have found, Fig. 3, that (Bi,Cu)BP3 and (Cd,Zn)MT2 inhibit in concentration dependent fashion this O_2^- mediated reaction.

In the end spectrum recorded for γ-irradiated frozen solution of (Hg,Cu)MT3, Fig. 2C (4), the first derivative maxima at g equal to about 2.027 and 2.015 and minimum at

Table 2. g-Factors of hydroperoxide and peroxy radicals

Radical		$g_{\perp}$	$g_{\parallel}$	Ref.
HO_2	trapped in ice	2.0044	2.039	17
	in loose complex with SOD	2.008	2.039	18
	Cp	2.01	2.04	19
	(Bi,Cu)BP3	2.004	2.036	this work
	(Cd,Cu)BP1	2.004	2.035	"
	(Cd,Zn)MT2	2.009	2.038	"
RO_2	from thionein	2.0031	2.034	"
	PMMA	2.0024	2.032	15
	carbohydrates	2.0055	2.031	14

Cp - human ceruloplasmin

g equal to about 2.000 evidence the presence of sulfurperoxy radicals (21). (Hg,Cu)MT3 shows no superoxide dismutase activity but in its frozen solutions the OH radicals are hard to remove upon thermal annealing, cf. Fig. 2B (4). In polycrystalline ice the OH radicals decay quickly at about 115 ± 5 K (22). In γ-irradiated frozen solution of (Hg,Cu)MT3 they are present at about 150 K which evidences efficient trapping.

Dismutation of O_2^- radicals by (Bi,Cu)BP3 and (Cd,Zn)MT2 or trapping of OH radicals by (Hg,Cu)MT3 reverting in net effect the production of oxygen radicals may prove to be pertinent for the metabolic role of the metalloproteins under studies.

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