Antioxidant Effects of Albumin-bound Sulfur in Lipid Peroxidation of Rat Liver Microsomes

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The antioxidant potential of albumin-bound sulfur (SBA) was investigated in rat liver microsomes using lipid peroxidation systems in vitro. Sulfur bound to protein is a reduced metabolite which is produced from cysteine by γ-cystathionase. Lipid peroxidation was induced either chemically by ferrous ions and ascorbate or enzymatically by carbon tetrachloride or tert-butyl hydroperoxide as indicated by the increase in thiobarbituric acid reactive substances (TBA-RS) and oxygen consumption. Although the antioxidant effect of SBA was weak on the non-enzymatic lipid peroxidation system, the addition of SBA significantly inhibited TBS-RS formation and oxygen consumption compared with non-treated bovine serum albumin (BSA) in a microsomal lipid peroxidation system induced enzymatically.

The sulfur bound to albumin disappeared during incubation with liver microsomes. However, slight differences in the disappearance were observed depending on whether or not lipid peroxidation was induced in the enzymatic systems. In the CCl₄-induced lipid peroxidation system, the cytochrome P-450 level was significantly decreased by the addition of BSA. Therefore, in cytochrome P-450 dependent lipid peroxidation system, the potential effects of sulfur bound to albumin are due to an inhibition of cytochrome P-450 rather than by the oxidation itself caused by radical trapping.

Key words: lipid peroxidation; rat liver microsome; bound sulfur; albumin; antioxidant

An endogenous sulfur atom, having a reduced oxidation state with a valence of 0 or −1, can be produced enzymatically from sulfur-containing amino acids. γ-Cystathionase (E.C. 4.4.1.1) enriched in liver cytosol catalyses the β-elimination reaction of cysteine to generate the endogenous reduced sulfur. Recent evidence indicates that nearly 50% of cystine catabolism occurs via the desulfuration pathway in intact rat hepatocytes. Stipanuk has suggested that the reduced sulfur released from cysteine in vivo is followed by incorporation of the sulfur into some pools of active reduced sulfur (sulfane sulfur), which has a relatively long half-life prior to its oxidation to a sulfate.

In previous studies, we have proved that a reduced sulfur species commonly exists in mammalian sera and tissues. This sulfur species is referred to as “bound sulfur,” which is defined as a divalent sulfur that is easily liberated as sulfide by reduction with excess thiols. The physiological function of these sulfur species is not well known; however, it is estimated that biological sulfur having reduced oxidation states plays several roles in vivo. In mammalian serum, the following two types are assumed to be forms of bound sulfur in the high molecular weight fraction: elemental sulfur bound to hydrophobic sites on albumin (albumin-sulfur complex, albumin-Sₓ), and protein persulfide (protein-S–S–). It is known that serum albumin binds sulfur derived physiologically from thiosulfate. Our previous study also suggests that bound sulfur in mammalian serum is in a certain equilibrium with the charged and uncharged states, protein-S– + S²⁻ → protein-S–S–. Furthermore, we recently suggested that bound sulfur may affect redox regulation by the modification of active thiol residues in some liver enzymes.

On the other hand, Everett et al. indicated that the structurally related disulfur analogs or perthiols (persulfide, RSSH) have significantly different free radical-scavenging and acid/base properties compared to thiol because of the relative differences in S-H bond energies. The outer sulfur atom in persulfide residue is a typical bound sulfur; therefore, we noted the antioxidant properties of albumin-bound sulfur in biological systems.

In this paper, we prepared albumin-bound sulfur (SBA) as a model of high molecule bound sulfur components, tested its inhibitory effects on lipid peroxidation in rat liver microsomes, and evaluated the action of bound sulfur on this process.

MATERIALS AND METHODS

Chemicals 2-Thiobarbituric acid, HPLC grade ethanol and CCl₄ were supplied by E. Merck (Darmstadt, Germany). tert-Butyl hydroperoxide (t-BuOOH), cysteine, cystine, α-tocopherol, β-NADPH, and butylated hydroxytoluene were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ferrous sulfate, sodium sulfide nonahydrate and dithiothreitol were from Wako Pure Chemical Co. (Osaka, Japan). Standard solutions of analytes and reagents for bound sulfur determination were prepared according to our previous report. All other reagents were commercial products of the highest available grade of purity.

Animals Male Wistar rats weighing between 260 g and 280 g were used in all the experiments. Rats were housed in a temperature-controlled (23 °C) and light-controlled (12-h cycle) animal room. The animals had ad libitum access to water and standard laboratory chow CE-2 (CLEA Japan) before the experiment.

Preparation of Albumin-bound Sulfur SBA was prepared as previously described with minor modifications. A solution of crystalline bovine serum albumin (BSA) (Sigma, St. Louis, MO, U.S.A.) containing 200 mg protein/10 ml phosphate buffered saline (PBS, pH 7.4) was prepared with 0.01 M sodium sulfide and incubated for 8 h at 37 °C. The reaction mixture was chromatographed on a column of

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Sephadex G-25 (Pharmacia, 1.5×25 cm) to remove the excess sulfide. The bound sulfur contents and forms in the SBA solution were determined by a method we described previously. The concentration of SBA was expressed as its bound sulfur content. The same BSA was used as a non-treated reference sample after purification by Sephadex G-25 column chromatography.

**Preparation of Rat Liver Microsome** Rats were anesthetized intraperitoneally with pentobarbital according to institutional guidelines. Livers were removed after perfusion in situ through a portal vein with ice-cold phosphate buffered saline (PBS, pH 7.4). 10% (W/V) liver homogenate in 1.15% KCl was prepared using a Potter-Elvehjem homogenizer with a teflon pestle. The homogenate was centrifuged once at 10000 g, at 4°C for 20 min, and the supernatant was recentrifuged at 105000 g for 60 min. The microsomal pellet thus obtained was washed twice with 1.15% KCl, and final microsomal precipitates were suspended in 50 mM Tris–HCl buffer (pH 7.4). The protein content of the microsomal suspension was determined by the method of Lowry et al. and adjusted to 5 mg protein/ml of suspension. Microsomes were prepared fresh before each batch of experiments.

**Measurement of TBA-RS and Oxygen Consumption** Thiobarbituric acid-reactive substances (TBA-RS) were estimated by a conventional method after the reaction was stopped by the addition of trichloroacetic acid.

The oxygen concentration of the incubation mixture was monitored in a closed glass vessel protected from light, thermostated at 37°C, and provided with a stirrer, using a Clark-type oxygen electrode (YSI 5331 model, Yellow Spring Inst.). Reactions were started by the addition of Fe²⁺, t-BuOOH and CCl₄ to each mixture, respectively, and changes in the oxygen tension were recorded.

**Lipid Peroxidation Systems** SBA was tested as an antioxidant using the three model systems of rat liver microsomes indicated below, in comparison with reference test samples, non-treated BSA and α-tocopherol.

SBA and reference test samples were preincubated at 37°C for 5 min in a microsomal suspension. Peroxidation induced by the Fe²⁺-ascorbic acid (Asc) was carried out by incubating the microsomal suspension (1 mg protein/ml) at 37°C for 10 min in 0.1 M phosphate buffer (pH 7.4) containing 0.5 mM Asc and 20 μM ferrous sulfate. Peroxidation induced by t-BuOOH was carried out by incubating the microsomal suspension (0.8 mg protein/ml) at 37°C for 30 min in 0.1 M phosphate buffer (pH 7.4) containing 1 mM t-BuOOH. Peroxidation induced by CCl₄ was carried out by incubating the microsomal suspension (1 mg protein/ml) at 37°C for 30 min in 0.15 M phosphate buffer (pH 7.5) containing 0.25 mM NADPH, 9 mM CCl₄ and 1 mM EDTA.

In all systems, the amount of lipid peroxidation was determined via the measurement of the concentration of TBA-RS, and the rate of oxidation was followed by monitoring the oxygen consumption.

**Measurement of Bound Sulfur** The consumption of bound sulfur in the lipid peroxidation system was monitored using a HPLC method originally developed for the analysis of biological reduced sulfur. At specific time intervals, 500 μl aliquots were taken from the incubation mixture and transferred to a snap-cap tube in an ice-cold bath. After the addition of 500 μl of 20 mM dithiothreitol (DTT) to the sample, the mixture was incubated at 37°C for 15 min. The reduced reaction mixture was subjected to gas flow dialysis followed by derivitization reaction and chromatographic separation with fluorometric detection according to the method previously reported.

**Effect of SBA on Microsomal Enzymes** Microsomal suspensions (1 mg protein/ml) were incubated at 37°C in the presence or absence (control) of SBA (2.3 mg albumin/ml, 100 μM as bound sulfur) and BSA (2.3 mg albumin/ml). At 30 min after the addition of a test sample or buffer solution for the control, a portion of the mixture was withdrawn to assay for cytochrome P-450 content and NAD(P)H cytochrome P-450 reductase activity.

NAD(P)H-cytochrome P-450 reductase activity was measured spectrophotometrically as described by Masters et al., then the content of cytochrome P-450 was determined using the method of Omura and Sato.

**RESULTS**

To clarify the ability of bound sulfur in protein to act as an antioxidant in biological systems, we prepared and characterized SBA containing bound sulfur atoms and compared it with non-treated BSA. Lipid peroxidation was induced in rat liver microsomes by three different systems. Table 1 shows the extent of inhibition of TBA-RS formation by SBA, BSA and α-tocopherol as a positive control. The antioxidant action of α-tocopherol (50 μM) was confirmed against CCl₄ and t-BuOOH-induced systems, although no significant difference from the value of BSA was observed in the Fe-Asc system. In Fe-Asc, CCl₄ and t-BuOOH-induced systems, lipid peroxidation was inhibited by approximately 14%, 22% and 18% by SBA, respectively. The inhibitory effects of TBA-RS formation by SBA were significantly different from those observed by the same concentration of BSA in the CCl₄ and t-BuOOH-induced systems.

Figure 1 shows the effects of varying concentrations of SBA on TBA-RS formation induced by the t-BuOOH, Fe-Asc and CCl₄-induced systems. In all systems, SBA suppressed peroxidation in a concentration-dependent manner.

The effects of SBA on the rates of oxygen consumption in the peroxidation system induced by t-BuOOH (A) and Fe-Asc (B) are shown in Fig. 2. A microsomal suspension was initially saturated with air, and preincubated with a test sample. After the addition of t-BuOOH (A) or ferrous sulfate (B), the oxygen concentration in the mixture was traced im-

| Table 1. Inhibitory Effects of SBA on Lipid Peroxidation Induced by Various Systems |
|-----------------|----------|----------|
|                 | α-Tocopherol | BSA⁴     | BSA⁶     |
| Fe²⁺-Asc        | 8.1±5.46   | 13.8±7.80| 7.9±4.78 |
| CCl₄            | 7.7±3.59   | 22.1±6.11| 8.5±0.98 |
| t-BuOOH         | 29.6±1.93  | 18.2±4.64| 4.2±2.48 |

Induced systems were carried out as described in Materials and Methods. Lipid peroxidation was determined by TBA-RS test. Values as means±S.D. from three independent experiments. a) Inhibition (%)= (control-sample/control-blank×100) control: without sample, blank: without inducer and sample. b) 50 μM, c) 2.3 mg albumin/ml (100 μM as bound sulfur), d) 2.3 mg albumin/ml. e) Significantly different from the value of BSA, p<0.01.
mediately using an oxygen electrode. While the SBA decreased the rate of lipid peroxidation in both systems, BSA increased the rate of lipid peroxidation in the t-BuOOH-induced system, but was almost ineffective in the Fe-Asc system.

To examine the antioxidant mechanism of bound sulfur in SBA, we evaluated the oxidative consumption of sulfur during the phases of lipid peroxidation. The bound sulfur contained in SBA was decreased during incubations on the Fe-Asc and CCl₄-induced lipid peroxidation systems. The decrease in bound sulfur was accelerated with peroxidation inducers on the Fe-Asc system (Fig. 3A); however, there were slight differences in the extent of its decrease depending on whether there was an inducer on the CCl₄ system (Fig. 3B).

Following this evaluation, the effects of SBA on the enzymatic systems related to free radical generation from CCl₄ or t-BuOOH were investigated. As shown in Table 2, the content of cytochrome P-450 rapidly decreased by the incubation with SBA. When the same concentration of BSA was added to the microsomes, no significant decrease in cytochrome P-450 level was observed.

DISCUSSION

In the previous study, we found that reduced sulfur species are abundant in mammalian tissues and these are referred to as "bound sulfur."⁴,⁵ Under physiological conditions, bound sulfur concentrations in human serum range from 0.5 to 3 μM, all of which is recovered from a high molecular weight fraction of the serum.⁴ Bound sulfur in the serum does not appear to play a significant role as an antioxidant because of its low concentration. However, with fairly high contents of bound sulfur in the liver and kidney,⁵ we noted a redox potency of bound sulfur in the tissues. Although it is evident that bound sulfur is enriched in the kidney and liver as a high molecular form,⁵ the physiological carrier of bound sulfur is still unclear. Therefore, we prepared SBA as a model of high molecular weight components including bound sulfur, because it was proposed that serum albumin might act as a specific carrier of sulfur formed in the liver for transport to other tissues.²² A molecule of SBA obtained in this work was bound to three atoms of bound sulfur. Two atoms were unchanged, and another existed as a charged persulfide residue, which was allowed to be masked by N-ethylmaleimide. As indicated by Westley et al., it was considered that two unchanged sulfur atoms bind to the hydrophobic site on the serum albumin subdomain 3-AB.²²,²³ On the other hand, it is known that Cys-34, the only free thiol of albumin, is the location of bound cysteine and glutathione.²² Thus, one charged type of sulfur is possibly the persulfide formed (Alb[S-S⁻]) by a thiol exchange on Cys-34 of the albumin molecule.

In this paper, we evaluated the antioxidant effects of SBA in microsomal lipid peroxidation. SBA significantly inhibited
the lipid peroxidation rather than the non-treated albumin used as a reference in the CCl₄ and t-BuOOH systems. These systems use the one-electron enzymatic reduction of carbon tetrachloride⁵⁰ or t-BuOOH²⁷,²⁸ by cytochrome P-450 present in the liver microsomes. When the consumption of bound sulfur during lipid peroxidation systems was determined, significant differences in the residual level of bound sulfur were not observed on the CCl₄-induced system after incubation for 30 min. From the results that 20—30% of bound sulfur rapidly decreased in the microsomal incubation mixture without peroxidation inducers, it appeared that parts of the bound sulfur in SBA were converted to another form by reacting with microsomal components rather than by oxidative alteration. Thus, we noted the interactions of bound sulfur in SBA against the microsomal enzymatic systems to generate radicals. To clarify the effect of SBA on the enzymatic lipid peroxidation, changes in the cytochrome P-450 level were examined in the incubation mixtures after the addition of SBA. Comparison to the non-treated BSA indicated that bound sulfur in SBA degraded liver microsomal cytochrome P-450 (Table 2), which plays an essential role in the lipid peroxidation induced by CCl₄ and t-BuOOH.

A persulfide (RSSH), one of the typical bound sulfur species, has been suggested to inhibit rat liver cytochrome P-450.²⁷ This inhibition by benzyl hydrodisulfide (BzSSH) has been examined as a model system for the inactivation of cytochrome P-450 during the microsomal metabolism of thiono sulfur-containing compounds (e.g., carbon disulfide, parathion). When thiono compounds are metabolized by mixed-function oxidase, the sulfur is released from the substrate and becomes covalently bound to cytochrome P-450.²⁸ It is regarded that the persulfide-mediated alteration of the structure of the heme moiety in cytochrome P-450 plays an important role in the inactivation of the enzyme. On the other hand, our data indicated that the bound sulfur atom in SBA gradually converted to another form in the microsomal incubation mixture, regardless of the induction of lipid peroxidation (Fig. 2). If the bound sulfur added to the microsomal incubation mixture covalently binds to the microsomal protein, this occurs because the sulfur which covalently bound to the carbon atom is not recovered as a sulfide by the DTT reduction. Although details of the interaction between cytochrome P-450 and bound sulfur are still unclear, it is certain that bound sulfur rapidly reacts with cytochrome P-450 and then causes the degradation.

The direct action between protein persulfide and various free radical species are also notable in vivo, because a physiological protein persulfide has been demonstrated in rhodanese (EC 4.8.1.1).²⁹,³¹ Rhodanese is widely distributed in mammalian tissues and designed to stabilize a persulfide involving the sulfhydryl group of Cys-247 in the form RSSH. Interestingly, it has been indicated that this enzyme is sensitive to oxygen radicals, which results in inactivation of the enzyme with the oxidation of a persulfide.³²,³³ It seems that the inhibitory effect of bound sulfur in SBA was caused mainly by the inhibition against microsomal enzymes on the lipid peroxidation induced by CCl₄ or t-
BuOOH; however, the protective effect of bound sulfur on the Fe-Asc system was accompanied by the oxidative loss of the bound sulfur atom throughout the incubation process. Determination of which type of protein-bound sulfur contributes to the inhibitory effect of the non-enzymatic lipid peroxidation system is currently under investigation.

REFERENCES