Synthesis of a Cytochrome c Derivative with Prolonged in vivo Half-life and Determination of Ascorbyl Radicals in the Circulation of the Rat*

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Since cytochrome c and acetylated cytochrome c disappear from the circulation with a half-life of 4 min, these proteins cannot be used for in vivo detection of superoxide radicals and related metabolites. To determine superoxide and other radicals in vivo, a cytochrome c derivative (SMAC) was synthesized by linking 1 mol of poly(styrene-co-maleic acid) butyl ester (SM) to cytochrome c, followed by acetylation of its lysyl amino groups. SMAC retained 8 and 80% of cytochrome c activity to react with ascorbyl and superoxide radicals, respectively. However, SMAC did not serve as a substrate for cytochrome c reductase and cytochrome c oxidase. When injected intravenously to the rat, SMAC circulated bound to albumin with a half-life of 130 min. SMAC was rapidly reduced in the circulation of intact animals. Treatment of animals with paraquat markedly enhanced the reduction of the circulating SMAC. We have synthesized an SM-conjugated superoxide dismutase (SOD) derivative (SM-SOD) that circulates bound to albumin with a half-life of 6 h. Kinetic analysis revealed that SM-SOD effectively inhibited the superoxide-dependent reduction of SMAC either in the presence or absence of 0.5 mM albumin. However, the reduction of the circulating SMAC was not inhibited by SM-SOD both in normal and paraquat-treated animals. Plasma samples from both animal groups also reduced cytochrome c and SMAC by an SOD-insensitive mechanism. However, after treatment with ascorbate oxidase, both plasma samples lost their activity to reduce cytochrome c and SMAC. These and other results suggest that ascorbyl radical might principally be responsible for the reduction of circulating SMAC and that plasma levels of ascorbyl radical might increase in paraquat-treated animals.

Reactive oxygen species, such as superoxide radicals, have been postulated to play important roles in the pathogenesis of various diseases (1−3). In fact, various types of tissue injury caused by circulatory disturbance, such as reperfusion injury of heart (4), brain (5), and liver (6) and gastric mucosal injury (7), were successfully inhibited by long acting superoxide dismutase (SOD) derivatives and scavengers for active oxygen species (8, 9). Superoxide radicals and other active oxygen species can be detected in vitro by several methods (10−14). However, quantitative analysis of these species in vivo is particularly difficult, predominantly because of short half-lives of reactive oxygen species. Using ultrasensitive chemiluminescence methods, Suematsu et al. (15) and Takahashi et al. (16) showed the occurrence of active oxygen species in vivo. However, these methods require expensive equipment, and chemiluminescence of tissues can be measured only in animals by laparotomy or thoracotomy.

Cytochrome c has been used to quantitate superoxide radicals in vitro. However, this protein also reacts with various compounds with reducing activity and serves as a substrate for cytochrome c oxidase and cytochrome c reductase. In contrast, acetylated cytochrome c (AC) does not react with the two enzymes but retains reactivity with superoxide radicals (17, 18). Thus, AC has been used for determining superoxide radicals in some experiments with a complex biological sample, such as neutrophils, mitochondria, and cell membranes. However, the half-lives of both cytochrome c and AC in the circulation are short (4 min) and, hence, they cannot be used for in vivo detection of superoxide radicals and related compounds.

We previously synthesized an SOD derivative (SM-SOD) by covalently linking poly(styrene-co-maleic acid) butyl ester (SM, Mf = 1600) to the enzyme (9, 19). SM-SOD circulates bound to albumin with a half-life of 6 h and accumulates on cell surface membranes of injured tissues whose extracellular pH is decreased (4, 9). Thus, SM-SOD efficiently prevented tissue injury caused by ischemia and reperfusion of the heart (4), brain (5), and liver (6). These observations suggested that superoxide radicals might play important roles in the pathogenesis of tissue injury caused by circulatory disturbance. Since SM tightly binds to the warfarin site on albumin (9), the ligand can be used for increasing half-lives of proteins and peptides in the circulation. To determine superoxide radicals and related metabolites in the circulation, we synthesized a cytochrome c derivative (SM-cytochrome c) by linking 1 mol of SM to 1 mol of cytochrome c via amido linkage. To minimize enzymic reduction and oxidation, the lysyl amino groups of SM-cytochrome c were acetylated. The acetylated SM-cytochrome c (SMAC) also reacted with ascorbyl and superoxide radicals but failed to serve as a substrate for the two enzymes. The present work reports synthesis, biochemical properties, and the fate of SMAC in the circulation. The

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results suggest that significant amounts of ascorbyl radicals occur in the circulation of normal and paraquat-treated rats.

**EXPERIMENTAL PROCEDURES**

**Materials—**Cytochrome c, xanthine, xanthine oxidase, L-ascorbic acid, uric acid, bilirubin, bovine serum albumin, ascorbate oxidase, ascorbate reductase, and bovine Cu/Zn-SOD were purchased from Sigma. GSH, acetic anhydride, 2,4,6-trinitrobenzene sulfonic acid (TNBS), and paraquat were obtained from Wako Pure Chemical Co. (Osaka). SM-SOD was synthesized by using Cu/Zn-SOD and SM as described previously (19); the specific activity of SM-SOD was 2700 units/mg of protein, as determined by the cytochrome c method (10).

Both free and albumin bound SM-SOD efficiently dismutate superoxide radicals.

**Synthesis of SMAC—**SM-conjugated cytochrome c was synthesized essentially as described for the synthesis of SM-SOD (9, 19). The incubation mixture contained, in a final volume of 10 ml, 0.1 m borate buffer, pH 8.0, 5 mM of cytochrome c, and 6 mM SM. The reaction was started by adding SM dissolved in dimethyl sulfoxide; the final concentration of dimethyl sulfoxide was 5%. After incubation at 25 °C for 3 h, the incubated mixture was dialyzed against 5 liters of distilled water. Under these conditions, 1 mol of SM covalently bound to 1 mol of cytochrome c as determined spectrophotometrically. SM-conjugated cytochrome c was acetylated as described for the synthesis of AC (18). The dialyzed SM-cytochrome c solution (50 mg/ml) was diluted with the same volume of saturated sodium acetate solution. Under vigorous stirring at 0 °C, acetic anhydride was added in 10 portions to give a final concentration of 200 mM. After 30 min, the reaction mixture was dialyzed at 4 °C for 20 h against 5 liters of 10 mM phosphate buffer, pH 7.4, containing 0.9% NaCl (PBS) with two changes of the buffer solution. The SMAC solution thus obtained was concentrated by an Amicon ultrafiltration membrane (PM-10) and was stored at -20 °C until use. Storage at this temperature did not affect the biochemical properties of SMAC for at least 3 months.

**Protein Chemical Analysis—**One mg of either cytochrome c or SMAC was dissolved in 1 ml of 0.2 M borate buffer, pH 8.0, containing 2% sodium deoxycholate and boiled for 10 min. Then, TNBS was added to give a final concentration of 15 mM. After incubation at 37 °C for 30 min, 0.1 ml of 90% formic acid was added. The number of TNBS-titrable amino groups of cytochrome c and SMAC was determined from absorbance at 420 nm (20) and protein concentrations. After reduction by either dithionite or ascorbic acid, total concentrations of cytochrome c and SMAC were determined spectrophotometrically at 550 nm, using the molar extinction coefficient of cytochrome c and SMAC were eluted with degassed distilled water. The activities, 50 units/kg of ascorbate oxidase was injected with 2 pmol/kg of SMAC. The rate of their reduction was determined spectrophotometrically as described above. Ascorbate oxidase was also added to plasma samples during the incubation with cytochrome c and SMAC.

**Statistical Analysis—**All data are expressed as mean ± S.D. derived from four to six animals. Statistical analysis was carried out according to Student’s t test.

**RESULTS**

**Physicochemical Properties of SMAC—**Previous studies in this laboratory revealed that SM-conjugated proteins, such as SOD, bound to the warfarin site on albumin and circulated for a fairly long time without being filtered by the kidney (9, 19, 22). To increase the in vivo half-life of cytochrome c, SM-cytochrome c was synthesized. Under the present experimental conditions, 1 mol of SM was incorporated to 1 mol of cytochrome c via an amide linkage. Incorporation of 1 mol of SM to 1 mol of cytochrome c had no appreciable effect on the absorbance of the reduced form of this protein at 550 nm. Fig. 1 shows the absorption spectra for 30 μM SM, AC, and SMAC. Cytochrome c and SMAC were incubated with 2 units/ml of ascorbate oxidase at 25 °C for 30 min. After adding 20 μM cytochrome c or SMAC, the rate of their reduction was determined spectrophotometrically as described above. Ascorbate oxidase was also added to plasma samples during the incubation with cytochrome c and SMAC.

**Fate of Cytochrome c and SMAC in Vivo—**After fasting for 16 h, male Wistar rats (200 g) were used for experiments. In vivo experiments were performed under pentobarbital anesthesia (50 mg/kg of body weight). Heparinized rats (800 units/kg) were intravenously injected with 2 μmol/kg of either cytochrome c or SMAC. At indicated times after administration, 0.2 ml of blood samples were withdrawn from the left femoral vein, diluted with 0.9 ml of PBS, and immediately centrifuged at 15,000 rpm for 1 min in an Eppendorf centrifuge. Levels of total and the reduced form of cytochrome c and SMAC in the supernatant fractions were determined spectrophotometrically at 550 nm (17, 18).

**Chemical Reduction of SMAC in the Circulation—**Five min after administration of either 0.1 ml of saline or SM-SOD (10 mg/kg) to the left femoral vein of the heparinized rat, 2 μmol/kg of SMAC was administered intravenously with 0.1 ml of saline or 50 μg/kg of paraquat. At indicated times after injection, 0.2 ml of blood samples were withdrawn, diluted with 0.9 ml of PBS, and immediately centrifuged at 15,000 rpm for 1 min. Since paraquat is reduced by dithionite, and the reduced form of paraquat interferes with the spectrophotometric measurement of cytochrome c and SMAC, their levels in the diluted plasma were determined before and after reducing the samples with 20 mM ascorbic acid. This concentration of ascorbic acid rapidly reduced cytochrome c and SMAC but not paraquat. In some experiments, 50 units/kg of ascorbate oxidase was injected with 2 μmol/kg of SMAC, and the rate of SMAC reduction was determined as described above. All experiments in vivo were carried out with four to six animals with similar results.

**Chemical Reduction of SMAC in Fresh Plasma—**Heparinized blood was withdrawn from the left femoral vein of normal rats. Blood samples were also obtained from animals 0 and 60 min after administration of paraquat (50 mg/kg). After centrifugation at 4 °C and 15,000 rpm for 1 min, plasma samples were obtained. To these samples were added potassium phosphate buffer to give a final concentration of 20 mM and pH 7.4. In some experiments, plasma samples were incubated with 2 units/ml of ascorbate oxidase at 25 °C for 30 min. After adding 20 μM cytochrome c or SMAC, the rate of their reduction was determined spectrophotometrically as described above. Ascorbate oxidase was also added to plasma samples during the incubation with cytochrome c and SMAC.

**Fig. 1.** Absorption spectra for cytochrome c and its derivatives. Absorption spectra for SM, AC, and SMAC were obtained in 20 mM phosphate buffer, pH 7.4, containing 0.15 M saline. 1, 30 μM SM; 2, 30 μM AC; 3, 30 μM SMAC. The inset shows the spectrum for 30 μM SMAC.
Each sample (10 pg in protein) was subjected to electrophoresis on an agarose film using 50 mM barbital buffer, pH 8.6. Cytochrome c and SMAC were also electrophoresed in the presence or absence of equimolar albumin. After electrophoresis for 15 min at room temperature, the film was stained with 0.5% Amido Black for 10 min and then destained in 5% acetic acid. Cyt. C, cytochrome c; SMAC/Alb., SMAC + albumin; Cyt. C/Alb., cytochrome c + albumin; Alb., albumin.

Fig. 2. Electrophoresis of cytochrome c and its derivatives. Each sample (10 μg in protein) was subjected to electrophoresis on an agarose film using 50 mM barbital buffer, pH 8.6. Cytochrome c and SMAC were also electrophoresed in the presence or absence of equimolar albumin. After electrophoresis for 15 min at room temperature, the film was stained with 0.5% Amido Black for 10 min and then destained in 5% acetic acid. Cyt. C, cytochrome c; SMAC/Alb., SMAC + albumin; Cyt. C/Alb., cytochrome c + albumin; Alb., albumin.

Fig. 3. Chemical reduction of cytochrome c and SMAC by superoxide radicals. Reaction mixtures contained, in a final volume of 1 ml, 0.1 M phosphate buffer, pH 7.4, 200 μM xanthine, 0.04 unit/ml of xanthine oxidase, and varying concentrations of either cytochrome c (open circles), AC (squares), or SMAC (closed circles). The reaction was started by adding xanthine oxidase at 25 °C. During the incubation, time-dependent changes in absorbance at 550 nm were monitored.

and electrophoresed as a single band. These observations suggest that SMAC might also bind to albumin, as did SM-SOD.

Chemical Reduction of SMAC by Superoxide Radicals—Superoxide radicals generated by xanthine oxidase reduce cytochrome c. As shown in Fig. 3, the rate of reduction increased with the increased concentration of cytochrome c until a maximum was reached at 60 μM. Under identical conditions, SMAC was also reduced by superoxide radicals; a maximum was reached at 80 μM of SMAC. The maximum rate of superoxide-dependent reduction of SMAC was lower by 20% than that of cytochrome c. This observation indicates that cytochrome c significantly retained the reactivity with superoxide radicals even after modification by SM and acetic anhydride. Since SM binds to the warfarin site on albumin (9), interaction of SMAC with this plasma protein may affect the reactivity of SMAC. To test this possibility, 60 μM of cytochrome c and SMAC were reduced by superoxide radicals generated by 0.03 unit/ml of xanthine oxidase and 200 μM xanthine in the presence or absence of a physiological concentration of albumin. The presence of 0.5 mM albumin decreased the rate of reduction of cytochrome c and SMAC by 40 and 60%, respectively (data not shown). Since albumin binds a wide variety of amphipathic organic acids, the inhibitory effect of this plasma protein may reflect the decrease in free concentrations of substrate xanthine. To test this possibility, binding of xanthine to albumin was determined by the ultrafiltration method, as described previously (23). As expected, the presence of 0.5 mM albumin decreased the free concentration of xanthine from 200 to 180 μM. However, superoxide-dependent reduction of cytochrome c occurred similarly with the reaction mixtures containing 180 and 200 μM xanthine. Thus, the decrease in free concentration of xanthine might not account for the inhibitory action of albumin. It should be noted that some chelates of heavy metals have SOD-like activity (24–26). Since various heavy metals, such as Cu++, bind to the N-terminal aspartic acid and histidine residues of albumin, the protein-bound metal(s) may have SOD-like activity. To test this possibility, the inhibitory effect of albumin was also observed in the presence of EDTA. Though 1 mM EDTA had no appreciable effect on the rate of superoxide-dependent reduction of cytochrome c, the chelating agent decreased the inhibitory action of albumin by about 60%.

Though both SM-SOD and SMAC tightly bound to albumin, superoxide-dependent reduction of SMAC was completely inhibited by 30 units/ml of SM-SOD, irrespective of the presence of 0.5 mM albumin. Thus, SM-SOD can be used for dismutating superoxide radicals in plasma that contains a high concentration of albumin.

Enzymatic Reduction and Oxidation of Cytochrome c and SMAC—Table I shows the reactivity of cytochrome c and SMAC with cytochrome c oxidase and cytochrome c reductase. Though cytochrome c was oxidized by cytochrome c oxidase, no appreciable oxidation occurred with the same concentration of SMAC. In the presence of NADH, cytochrome c was reduced by cytochrome c reductase. Under identical conditions, however, no appreciable reduction occurred with SMAC. Thus, the redox state of SMAC but not cytochrome c might not be affected by these enzymes.

Chemical Reduction of Cytochrome c and SMAC by Low Molecular Weight Compounds—Cytochrome c is reduced by naturally occurring low molecular weight compounds. Among various metabolites tested, ascorbic acid was the major component in plasma that reduced cytochrome c. As shown in Table II, cytochrome c was reduced rapidly with a physiological concentration of ascorbic acid (60 μM). Though the reactivity of SMAC was lower than that of cytochrome c, the modified protein was also reduced by ascorbic acid. It should be noted that no appreciable reduction of cytochrome c and SMAC occurred with physiological concentrations of other.

Table I: Reactivity of cytochrome c and SMAC with enzymes

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<tr>
<td>Cytochrome c reductase</td>
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*Charge in absorbance at 550 nm/min. nd, below detectable levels (<0.001).
SMAC can be used for quantitative analysis of the circulating metabolites with reducing activity.

Chemical Reduction of SMAC—The observation that SM-SOD did not inhibit the reduction of circulating SMAC drove us to speculate that some metabolite(s) of paraquat might increase in paraquat-treated animals. To test whether superoxide radicals accounted for the reduction of circulating SMAC, the rate of reduction was determined in animals that were injected with long acting SM-SOD. However, SM-SOD did not decrease the rate of reduction of the circulating SMAC in both normal and paraquat-treated animals.

Effect of Ascorbate Oxidase on the Reduction of SMAC—Interestingly, no appreciable difference in the rate of SMAC reduction was found between plasma samples from paraquat-treated animals and those from normal animals. The rate of reduction was increased markedly by adding ascorbate oxidase to the circulation mixture. In contrast, the rate of reduction markedly decreased if plasma samples were pretreated with ascorbate oxidase. Since the rate of reduction of the circulating SMAC was increased by treating animals with paraquat, SMAC was also incubated with plasma samples from paraquat-treated animals (Fig. 7). Interestingly, no appreciable difference in the rate of SMAC reduction was found between plasma samples from normal and paraquat-treated animals. SMAC was also administered with 50 mg/kg of paraquat (squares).

Antioxidants

<table>
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<td></td>
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<td>Bilirubin + albumin</td>
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* Change in absorbance at 550 nm/min. nd, below detectable levels (<0.001).

**TABLE II**

Reactivity of cytochrome c and SMAC with various compounds.

Incubation mixtures contained, in a final volume of 1 ml, 0.1 M phosphate buffer, pH 7.4, varying concentrations of low molecular weight compounds, and SMAC (open circles) or SMAC (closed circles) into the tail vein. At the indicated times after administration, 0.2-ml blood samples were collected from the left femoral vein, diluted with 0.9 ml of ice-cold PBS, and immediately centrifuged at 15,000 rpm for 1 min. The plasma levels of cytochrome c and SMAC were determined as described in the text. Data shows mean ± S.D. derived from four animals.

metabolites, such as GSH, uric acid, and bilirubin.

**Fate of Cytochrome c and SMAC in the circulation**—Fig. 4 shows the fate of cytochrome c and SMAC in the circulation of intact rats. When injected intravenously, cytochrome c disappeared from the circulation with a half-life of 4 min. After 30 min, more than 70% of the injected cytochrome c appeared in the urine in its intact form, suggesting that renal glomerular filtration was responsible for the rapid removal of the circulating cytochrome c. In contrast, SMAC circulated with a half-life of 130 min without being filtered by the kidney. Thus, SMAC can be used for quantitative analysis of the circulating metabolites with reducing activity.

**Chemical Reduction of SMAC in the circulation**—Fig. 5 shows the reduction of SMAC in the circulation. Within 10 min after injection, 44% of the injected SMAC was reduced in the circulation of intact animals. Administration of paraquat markedly increased the rate of reduction of the circulating SMAC. The rate of SMAC reduction remained increased even 60 min after administration of paraquat (data not shown). These observations suggested that some metabolite(s) with potent reducing activity might occur in the circulation of normal rats and that plasma levels of this metabolite(s) might increase in paraquat-treated animals. To test whether superoxide radicals accounted for the reduction of the circulating SMAC, the rate of reduction was determined in animals that were injected with long acting SM-SOD. However, SM-SOD did not decrease the rate of reduction of the circulating SMAC in both normal and paraquat-treated animals.

**Effect of Ascorbate Oxidase on the Reduction of SMAC**—The observation that SM-SOD did not inhibit the reduction of circulating SMAC drove us to speculate that some metabolite(s) other than superoxide radicals might principally be responsible for the reduction of SMAC. To know the chemical nature of this metabolite(s), SMAC was incubated with freshly isolated plasma samples. During the incubation with fresh plasma from normal rats, SMAC was also reduced time dependently (Fig. 6). Since ascorbic acid was the major compound in plasma that reduced cytochrome c and SMAC (see Table II), we tested the effect of ascorbate oxidase on the reduction of SMAC in plasma. The rate of SMAC reduction was increased markedly by adding ascorbate oxidase to the incubation mixture. In contrast, the rate of reduction markedly decreased if plasma samples were pretreated with ascorbate oxidase. Since the rate of reduction of the circulating SMAC was increased by treating animals with paraquat, SMAC was also incubated with plasma samples from paraquat-treated animals (Fig. 7). Interestingly, no appreciable difference in the rate of SMAC reduction was found between plasma samples from normal and paraquat-treated animals. SMAC was reduced at the same rate with plasma samples obtained from animals 10, 30, and 60 min after administration of paraquat (data not shown).

SMAC was also incubated with plasma samples from paraquat-treated animals (Fig. 7). Interestingly, no appreciable difference in the rate of SMAC reduction was found between plasma samples from normal and paraquat-treated animals. SMAC was reduced at the same rate with plasma samples obtained from animals 10, 30, and 60 min after administration of paraquat (data not shown). Again, ascorbate oxidase added in the plasma from paraquat-treated animals markedly increased the rate of SMAC reduction, whereas preincubation of plasma with the oxidase abolished the reducing activity. Paraquat added in normal plasma (10 mg/ml) did not affect the rate of reduction of SMAC (data not shown).

To know whether ascorbate oxidase also affects the rate of reduction of the circulating SMAC, SMAC was injected intra-
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Fig. 6. Chemical reduction of SMAC in plasma from intact rats. To freshly isolated rat plasma was added 1 m potassium phosphate buffer to give a final concentration of 20 mM and pH 7.4. The plasma sample was incubated with 20 μM SMAC at 25 °C, and time-dependent changes in absorbance at 550 nm were determined (1). At the indicated times, 2 units/ml of ascorbate oxidase (AO) was added to the incubation mixture (2). Incubation was also carried out with plasma samples that were pretreated with plasma samples were pretreated with 0.1 μl of saline (open symbols) or 10 mg/kg of SM-SOD (closed symbols). After 5 min, 2 μmol/kg of SMAC was administered with (squares) or without (circles) 50 units/kg of ascorbate oxidase. Then, time-dependent reduction of the circulating SMAC was determined as described in the text.

Fig. 7. Chemical reduction of SMAC in plasma samples from paraquat-treated rats. Animals were intravenously injected with 0.1 ml of saline (1) or 50 mg/kg paraquat (2–4). Sixty min after administration, blood samples were withdrawn from the left femoral vein and immediately centrifuged at 4 °C for 1 min. Then, the rate of SMAC reduction by plasma samples was determined (2). During the incubation, 2 units/ml of ascorbate oxidase (AO) was added to the reaction mixture (3). The rate of reduction was also determined with plasma samples that were pretreated with 2 units/ml of the oxidase at 25 °C for 30 min (4).

Fig. 8. Reduction of SMAC in ascorbate oxidase-administered rats. Animals were intravenously injected with either 0.1 ml of saline (open symbols) or 10 mg/kg of SM-SOD (closed symbols). After 5 min, 2 μmol/kg of SMAC was administered with (squares) or without (circles) 50 units/kg of ascorbate oxidase. Then, time-dependent reduction of the circulating SMAC was determined as described in the text.

Discussion

Though cytochrome c and AC have been used for determining superoxide radicals in vitro, they cannot be used for in vivo detection predominantly due to rapid removal by glomerular filtration. To overcome such frustrating situations, we synthesized SMAC, an AC derivative with a prolonged in vivo half-life, and determined the occurrence of ascorbyl radicals in plasma and the circulation of normal and paraquat-treated rats.

Kinetic analysis revealed that SMAC did not serve as a substrate for cytochrome c reductase and cytochrome c oxidase but retained significant activity to react with ascorbyl and superoxide radicals. Though incorporation of 1 mol of SM to cytochrome c had no appreciable effect on the rate of superoxide-dependent reduction, acetylation of lysine residues of SM-cytochrome c decreased the reactivity by about 20%. These results are consistent with the observation that acetylation of 60% of the lysine residues of cytochrome c decreased the rate of superoxide-dependent reduction by 10% (18).

Xanthine oxidase-dependent reduction of cytochrome c and SMAC was decreased significantly by the presence of a physiological concentration of albumin. Kinetic analysis revealed that the decrease in free form xanthine by albumin had no appreciable effect on the rate of superoxide production by the oxidase. This is consistent with the fact that the concentration of xanthine used for superoxide generation (200 μM) was 118-fold higher than the K_m value (1.7 × 10^{-6} M) of the oxidase (27). The inhibitory action of albumin was decreased significantly by the presence of EDTA. Thus, some metal(s) associated with the albumin sample might be responsible for the inhibition of cytochrome c reduction. Consistent with this notion is the fact that some protein-bound metals, such as Cu^{2+}, exhibit SOD-like activity (28). To determine the chemical nature of albumin-associated compound(s), the effect of chelating agents other than EDTA was also tested. However, the presence of 1 mM of desferrioxamine and bathocuproinedisulfonate, specific chelators for Fe^{2+} and Cu^{2+}, respectively, had no appreciable effect on the inhibitory action of albumin. Thus, metal(s) other than iron and Cu^{2+} may possibly be responsible for the inhibitory action of albumin. However, it should be noted that these agents chelate free forms of metals but not protein-bound forms. Thus, EDTA, but not desferrioxamine and bathocuproinedisulfonate, may form a complex with an albumin-bound metal, thereby inhibiting its SOD-like activity. In fact, incubation of albumin samples with 1 mM EDTA followed by extensive dialysis against PBS abolished its inhibitory activity (data not shown). The fact that dithiocarbamate forms a ternary complex with the active site Cu^{2+} of Cu/Zn-SOD, thereby inhibiting its catalytic activity.
of the enzyme (29), is consistent with this hypothesis. The mechanism by which albumin inhibited the superoxide-dependent reduction of cytochrome c should be studied further.

It should be noted that albumin decreased the rate of reduction more potently with SMAC than with cytochrome c. Since motional freedom of a protein is decreased by increasing its molecular size, binding to the warfarin site on albumin might decrease the collisional interaction of SMAC with superoxide radicals. Alternatively, steric hindrance and/or conformational change caused by binding to albumin may decrease the reactivity of SMAC with superoxide radicals. The mode and the effect of albumin binding on the reactivity of SMAC should be studied further. Though SM-SOD also binds to the warfarin site on albumin, the enzyme completely inhibited the superoxide-dependent reduction of SMAC, irrespective of the presence of a physiological concentration of albumin.

The circulating SMAC was rapidly reduced by some SM-SOD-insensitive mechanism. In the present experiments, 10 mg/kg of SM-SOD was injected intravenously. Assuming 10 ml for the plasma volume of a 200-g rat (30), 0.2 mg/ml of SM-SOD might circulate during the early time after injection. This concentration of the enzyme corresponds to 400 units/ml of dismutating activity. Under the present experimental conditions, about 15 and 63% of the circulating SMAC was reduced within 1 min after injection in intact and paraquat-treated animals, respectively (see Fig. 5). Thus, the initial rate of SMAC reduction was 6 and 25 nmol/min/ml plasma in intact and paraquat-treated animals, respectively. Since both free and albumin-bound SM-SOD efficiently dismutated superoxide radicals, 400 units/ml of the enzyme would be sufficient for complete inhibition of superoxide-dependent reduction of the circulating SMAC. Thus, metabolite(s) other than superoxide radicals might predominantly be responsible for the reduction of SMAC in the circulation of both intact and paraquat-treated animals.

Kinetic analysis using ascorbate oxidase suggested that ascorbic acid-related metabolite(s) is the major component that reduced SMAC in plasma and in the circulation. During the oxidation of ascorbic acid to dehydroascorbic acid either enzymatically or nonenzymatically, monodehydroascorbate radical is generated (31–34). Since monodehydroascorbic acid, but not dehydroascorbic acid, has a potent activity to reduce cytochrome c, ascorbly radicals might principally be responsible for superoxide-dependent reduction of SMAC. In fact, the rate of SMAC reduction by plasma markedly increased if ascorbate oxidase was added during the incubation, whereas pretreatment of plasma by the oxidase completely abolished the activity to reduce SMAC. This is consistent with the notion that ascorbly radical might be the major component that reduced SMAC in plasma and in the circulation.

It should be noted that paraquat generates superoxide radicals intracellularly through its redox cycling and induces lipid peroxidation in cell membranes (35). \( \alpha \)-Tocopherol is the major antioxidant that protects membrane/lipid bilayers from hazardous chain reactions of free radicals (36). Through inhibition of radical chain reactions within membrane/lipid bilayers, \( \alpha \)-tocopherol is converted to tocopheroxyl radical. The tocopheroxyl radical thus formed reacts with ascorbic acid in aqueous phase, thereby generating ascorbyl radicals (37, 38). Since ascorbic acid is localized intracellularly and extracellularly, ascorbly radical would be generated on both sides of plasma membranes. Thus, the circulating SMAC may possibly be reduced by extracellularly generated ascorbly radicals. These sequential reactions may well explain the reason why paraquat increased the rate of reduction of the circulating SMAC by an SM-SOD-insensitive mechanism.

It should be noted that, predominantly due to the amphiphatic nature of the SM moiety of SM-SOD, the enzyme binds to the outer surface of cell membrane/lipid bilayers, particularly those of injured tissues whose environmental pH is decreased (4, 9). Because of such preferential accumulation in an injured site of tissues, SM-SOD inhibited oxygen toxicity more effectively than did native SOD (4–7). We recently developed a fusion protein (HB-SOD) consisting of human Cu/Zn-SOD and C-terminal heparin-binding peptide (39, 40). When injected intravenously to rats, HB-SOD disappeared rapidly from the circulation, specifically bound to heparan sulfates on vascular endothelial cells, and underwent transcellular movement from luminal to extraluminal compartments (41). Because of such specific localization in vivo, HB-SOD efficiently inhibited cold-induced brain edema, stress ulcer of the stomach, and postischemic reperfusion arrhythmias of the rat (39, 42). These results suggest that pathogenic superoxide might appear at or quite close to plasma membrane/lipid bilayers of vascular endothelial cells (such as in an unstirred water layer on the cell surface) and/or parenchymal cells, rather than in the central stream of blood circulation. The finding that the reduction of SMAC within the circulation was not inhibited by site-directed SM-SOD is consistent with the notion described above. During the preparation of this paper, we came across a report describing that the paraquat-induced injury of cultured hepatocytes was inhibited by liposomal SOD, but not free SOD (43). This observation also supports the hypothesis that superoxide radicals in and around plasma membrane/lipid bilayers might play critical roles in the pathogenesis of paraquat-induced tissue injury.

Apart from the mechanism for paraquat-induced tissue injury and the sites for the generation of active oxygen species, combined use of SMAC and SM-SOD might permit quantitative studies on the occurrence of superoxide and ascorbly radicals in the circulation of animals that were challenged with oxidative stress.

REFERENCES

Synthesis of a Cytochrome c Derivative