Synthesis of a Superoxide Dismutase Derivative That Circulates Bound to Albumin and Accumulates in Tissues Whose pH Is Decreased

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ABSTRACT: Protection of tissues from oxidative stress is one of the major prerequisites for aerobic life. Since intravenously injected Cu^{2+}/Zn^{2+}-type superoxide dismutase (SOD) disappears from the circulation with a short half-life of 5 min, its clinical use as a scavenger for superoxide radical is limited. We synthesized a human erythrocyte type SOD derivative (SM–SOD) by linking 2 mol of hydrophobic organic anion, α-4-{[6-(*N*-maleimido)hexanoyloxymethyl]cumyl}half-butyl-esterified poly(styrene-*co*-maleic acid) (SM), to the cysteinyl residues of the dimeric enzyme without decreasing enzymic activity. SM–SOD, but not SOD, bound to an albumin–Sepharose column; the bound SM–SOD was eluted by a buffer solution containing 0.5% sodium dodecyl sulfate or 10 mM warfarin, suggesting that SM–SOD reversibly binds to the warfarin site on albumin. Due to the amphipathic nature of the SMI moiety, SM–SOD bound also to cell membranes particularly when the pH was decreased. In vivo analysis in the rat revealed that intravenously injected SM–SOD circulated bound to albumin with a half-life of 6 h. Postischemic reperfusion arrhythmias were almost completely prevented by a single dose of SM–SOD, but not SOD. Thus, the prolonged half-life of SM–SOD in the circulation and its preferential accumulation in an injured site with decreased pH appeared to be responsible for preventing myocardial injury. These results suggest that superoxide radical and/or its metabolite(s) would play an important role in the pathogenesis of postischemic reperfusion arrhythmias and that SM–SOD may be useful for decreasing tissue injury in ischemic heart disease.

Reactive oxygen species, such as superoxide radicals, play an important role in cellular defense mechanisms including bactericidal action of leukocytes (Baldridge & Gerard, 1933). However, these oxygen radicals also produce oxidative damage to tissues in various inflammatory diseases (Balentine, 1982; Sies, 1985) and ischemic myocardial injury (Burton et al., 1984). The hazardous effects of reactive oxygens can be decreased by SOD[1] and catalase (Fee & Teitelbaum, 1972; Huber & Saifer, 1977). However, because of their intracellular localization, oxidative damage of plasma membrane surface constituents cannot be prevented by these intracellular enzymes. Bovine erythrocyte type SOD has been used to suppress inflammatory damage in a patient with rheumatoid arthritis (Rister et al., 1978). However, clinical use of this dimeric enzyme (M_r = 32000) is limited because of potential immunogenicity and rapid renal filtration and disappearance from the circulation (Petkau et al., 1976). To overcome these problems, masking of the protein surface by synthetic polymers, such as poly(ethylene glycol), has been considered for several enzymes, including SOD (Pyatak et al., 1980). However, this approach to control in vivo behavior of an enzyme is still premature.

Binding of a hydrophobic organic anion to albumin is an important factor that determines the fate of a ligand in the circulation (Inoue, 1985). In a preliminary study on the biological role of ligand–albumin interaction, we found that reversible binding of macromolecules to a plasma protein is also important for determining the in vivo fate of the bound molecules in the circulation (Inoue, 1987). The present work describes chemical modification of human erythrocyte type SOD by SMI, a hydrophobic organic anion that specifically reacts with thiol groups, physicochemical properties of the derivatized enzyme (SM–SOD), and SM–SOD's effect on the postischemic reperfusion arrhythmias in the rat.

Materials and Methods

Materials. Xanthine, nitroblue tetrazolium, xanthine oxidase, cytochrome *c*, bovine SOD, and human and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis). Ethylenediaminetetraacetic acid (EDTA), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and cyanogen bromide were obtained from Wako Pure Chemical Co. (Osaka). α-4-{[6-(*N*-Maleimido)hexanoyloxymethyl]cumyl}half-butyl-esterified poly(styrene-*co*-maleic acid) (SM, M_r = 2170) was obtained from Kuraray Co. (Kurashiki). Poly(styrene-*co*-maleic acid)butyl ester, a precursor of SM, has been used for chemical modification of neocarcinostatin, an anticancer agent (Maeda et al., 1979). SOD was purified from human erythrocytes as described previously (Gaertner et al., 1984). The specific activity of the purified enzyme was 3000 units/mg of protein as described previously (Beauchamp & Fridovich, 1971). Although human erythrocyte type SOD has 4 free cysteinyl residues/mol of dimeric enzyme, only two (Cys^{111}) are located on the enzyme surface and react readily with various thiol reagents (Hartz & Deutsch, 1972; Tainer et al., 1982). The enzyme preparation contained 1.8 mol of sulfhydryl groups that were titratable with DTNB per mole of the enzyme under nondenaturing condition. The protein concentration of enzyme samples was determined by the method of Lowry et al (1951), using bovine SOD as the standard.

Conjugation of SOD with SM. The incubation medium contained, in a final volume of 1 mL, 0.1 M Tris-HCl buffer,

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[1] Abbreviations: SOD, superoxide dismutase; SM, α-4-{[6-(*N*-maleimido)hexanoyloxymethyl]cumyl}half-butyl-esterified poly(styrene-*co*-maleic acid); SM–SOD, SM-conjugated SOD; EDTA, ethylenediaminetetraacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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pH 8.0, 0.1 mM EDTA, 30 mg of SOD, and 10 mM SM. The reaction was started by adding SM dissolved in dimethyl sulfoxide; the final concentration of dimethyl sulfoxide was less than 10%. During incubation at 37 °C, aliquots of 20 μL were withdrawn, and the enzyme activity and the number of DTNB-titratable free thiol groups of the enzyme were determined as described (Ellman & Lysko, 1979). Incubation was performed until the DTNB-titratable thiol group became undetectable (about 6 h). The incubation mixture was passed over a Sephadex G-100 column (2 × 30 cm) that was equilibrated with 20 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS). Protein fractions eluted from the column were combined. SOD samples were labeled with ^{125}I-labeled Bolton–Hunter reagent, as described previously (Bolton & Hunter, 1973). Specific radioactivity of the labeled samples was 5×10^6 counts min^{-1} (mg of protein)$^{-1}$. Radioactive samples were used after extensive dialysis against PBS and appropriate dilution with unlabeled SOD preparations.

Chromatographic analysis of SOD samples was performed by using an albumin–Sepharose (0.8 × 4 cm), an aminohexyl-Sepharose (1 × 13 cm), and a Sephacryl S-200 column (2 × 30 cm) as described in each experiment.

Binding of SM-SOD to Erythrocyte Membranes. Heparinized blood samples were obtained from rats and washed by centrifugation at 3000 rpm three times with a 10-fold excess of ice-cold PBS. The washed erythrocytes were resuspended in PBS (2×10^9 cells/mL). Binding of radioactive SOD samples to erythrocyte membrane surface was determined in 0.15 M NaCl containing 20 mM HEPES buffer.

In Vivo Experiments. Male Sprague-Dawley rats (200 g of body weight) and male ddY mice (15 g) were fed laboratory chow and water ad libitum and used for experiments after fasting for 16 h. In vivo experiments were performed between 9:00 a.m. and 12:00 noon under light ether or pentobarbital anesthesia (50 mg/kg of body weight, ip). The fate of intravenously injected radioactive SOD and SM–SOD was determined in rats and mice as described previously (Inoue et al., 1983).

The chest was opened by using a left thoracotomy, followed by sectioning of ribs 4 and 5, approximately 2 mm to the left of the sternum. Positive pressure artificial respiration was started immediately with room air, using a volume of 1.5 mL/100 g and a rate of 55 strokes/min, to maintain normal PCO_2, PO_2, and pH. After incision of the pericardium, the left anterior descending artery was occluded by suction for 15 min as described previously (Manning et al., 1984). Blood was then reflowed by releasing the occlusion. Under the experimental conditions, postischemic reperfusion arrhythmias were elicited within 10 s and occurred intermittently for 2–3 min after reperfusion. A standard lead I electrocardiogram was recorded throughout the experiments.

Results

Physicochemical Properties of SM-Modified SOD. During the study of ligand–albumin interaction (Inoue, 1987), we observed that SM, a hydrophobic organic acid, reversibly attached to the warfarin-binding sites on albumin and escaped from renal glomerular filtration. To test whether reversible binding of SOD to albumin increases its half-life, SM was linked to the enzyme via its free thiol group. The radiolabeled sample of SM-linked SOD (SM–SOD) was subjected to albumin–Sepharose column chromatography (Figure 1). Although SOD appeared in an unbound fraction (Figure 1A), a significant part of SM–SOD bound to the column; less than 20% of radioactivity was recovered from an unbound fraction (Figure 1B). When the column was washed with buffer solution containing 10 mM warfarin, the remaining radioactivity eluted from the column. The radiolabeled sample bound to the column could also be eluted by 0.5% sodium dodecyl sulfate; however, it was eluted in a fraction significantly later than that eluted by warfarin (Figure 1C). The enzyme could not be eluted by buffer solution containing 0.5% caproic acid which bound to a different site (domain 3) on albumin from that for warfarin (data not shown). These observations suggested that SM–SOD bound to the warfarin site on albumin with high affinity.

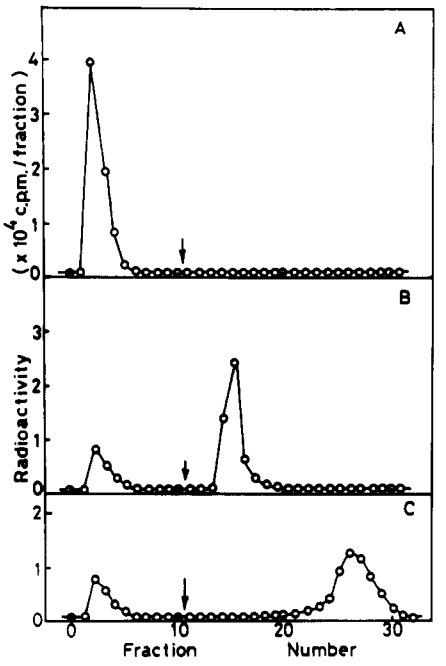

FIGURE 1: Affinity chromatography of SOD samples on albumin–Sepharose column. ^{125}I-Labeled enzyme samples (100 000 cpm) were subjected to affinity chromatography in an albumin–Sepharose column (0.8 × 4 cm) that was equilibrated with PBS. The column was washed with PBS at 25 °C, and fractions of 1 mL were collected. At the indicated point (arrow), 10 mM warfarin (B) or 0.5% sodium dodecyl sulfate (C) was added to the elution buffer. (A) SOD; (B and C) SOD samples reacted with SMI.

To study the physicochemical properties of the SOD derivative eluted from the albumin–Sepharose column by warfarin, the enzyme was subjected to aminohexyl-Sepharose column chromatography. SM–SOD which bound to the albumin–Sepharose column also bound to the aminohexyl-Sepharose and was eluted by increasing NaCl concentration (Figure 2). Under identical conditions, intact SOD did not bind to the column. Since SM is an organic anion with seven carboxyl groups, incorporation of this ligand to SOD would increase the negative charge of the enzyme. To test this possibility, SOD samples isolated by using these columns were subjected to electrophoresis. As expected, SM–SOD electrophoresed significantly faster than did SOD (Figure 3). Figure 4 shows the absorption spectra for SM, SOD, and SM–SOD. Comparison of these spectra suggested that SM–SOD is largely accounted for as a 2:1 admixture of SM and SOD.

To know the specificity of protein binding of SM–SOD, gel permeation chromatographic analysis was performed by using a Sephacryl S-200 column and radioactive SM–SOD with albumin or rat serum (Figure 5). In the absence of albumin or serum, SM–SOD eluted in the same fraction as did intact SOD (arrow). In the presence of albumin, most SM–SOD eluted in a fraction that was slightly earlier than that for albumin. When chromatographed with rat serum, most SM–SOD eluted in a similar fraction to that expected for the

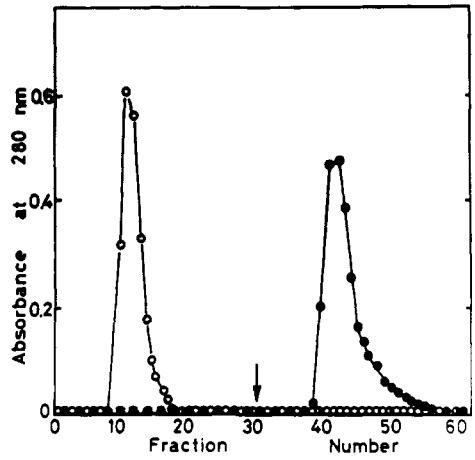

FIGURE 2: Aminohexyl-Sepharose column chromatography of SOD samples. SM-SOD, which was eluted from the albumin-Sepharose column by warfarin, was dialyzed against 5 L of 20 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl. SOD and SM-SOD (4 mg) were subjected to chromatography on an aminohexyl-Sepharose column (1 × 13 cm) equilibrated with the same buffer solution. The column was washed with the same buffer solution at 25 °C; fractions of 1 mL were collected, and the absorbance at 280 nm was determined. At the indicated point (arrow), 1 M NaCl was added to the elution buffer. (Open circles) Unmodified SOD; (solid circles) SM-SOD purified by albumin-Sepharose column.

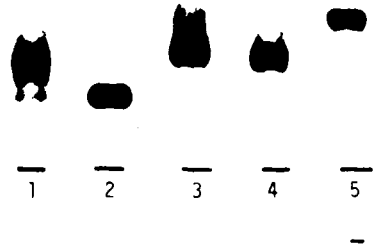

FIGURE 3: Electrophoresis of SOD preparations. After extensive dialysis of the enzyme preparations against 5 L of PBS, each sample (about 20 μg in protein) was subjected to electrophoresis on an agarose film using 50 mM barbital buffer, pH 8.6. After electrophoresis for 30 min at room temperature, the film was stained by 0.5% Amido Black for 10 min and then destained by 5% acetic acid. (Lane 1) SOD sample reacted with SM; (lane 2) SOD; (lane 3) SOD sample eluted from albumin-Sepharose column by warfarin; (lane 4) SOD sample eluted from aminohexyl-Sepharose column by 1 M NaCL solution; (lane 5) albumin.

enzyme complexed with albumin. No peak other than the shoulder at the position for SOD (arrow) was seen. Thus, among various plasma proteins, SM-SOD bound preferentially to albumin.

Stability of SM-SOD. SOD is heat stable (Forman & Fridovich, 1973). To know whether chemical modification of SOD by SM affects its physicochemical properties, the heat stabilities of SOD and SM-SOD were compared. After incubation of the two samples at 70 °C for 10 min, more than 90% of catalytic activity remained (data not shown). Table I compares the effect of various proteases on the catalytic activity of SOD and SM-SOD. No significant decrease in catalytic activity of either SOD or SM-SOD was observed during incubation for 24 h with papain, trypsin, or chymotrypsin. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that the electrophoretic mobility of SOD and SM-SOD remained unaffected under the present experimental conditions (data not shown). Thus, chemical modification by SM had no appreciable effect on SOD stability against heat and protease treatment.

Affinity of SM-SOD to Plasma Membrane Surface. Preliminary experiments revealed that when the carboxyl group

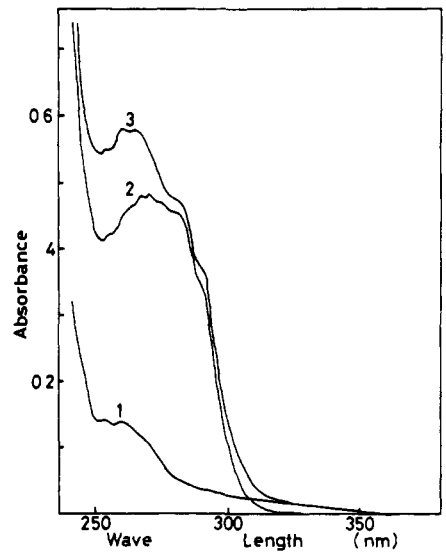

FIGURE 4: Absorption spectra for SOD preparations. SM-SOD purified by albumin- and aminohexyl-Sepharose column chromatography was dialyzed extensively against 5 L of PBS. Absorption spectra for SM, SOD, and SM-SOD were obtained in the same buffer solution. (1) 63 μM SM; (2) 1 mg of SOD/mL; (3) SM-SOD (1 mg of protein/mL).

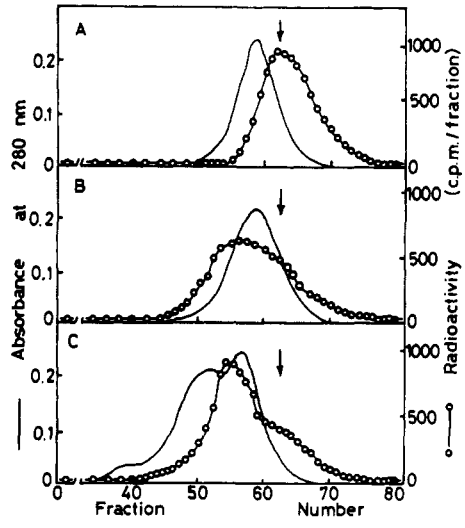

FIGURE 5: Gel permeation chromatography of SOD preparaitons. Radioactive SM-SOD (20000 cpm) was subjected to gel permeation chromatography in a Sephacryl S-200 column (2 × 30 cm) with or without 3 mg of human albumin or 0.07 mL of rat serum. Chromatography was carried out with PBS at 25 °C. Fractions of 1 mL were collected, and the absorbance at 280 nm and radioactivity (open circles) were determined. Arrows show the elution position for SOD. (A) SM-SOD and albumin were chromatographed separately; (B) SM-SOD ± albumin; (C) SM-SOD ± rat serum.

Table I: Stability of SOD and SM-SOD against Proteases[a]

	remaining activity (%)					
	papain		trypsin		chymotrypsin	
time (h)	SOD	SM-SOD	SOD	SM-SOD	SOD	SM-SOD
0	100	100	100	100	100	100
1	90	100	92	100	91	100
3	92	87	92	92	97	100
6	89	74	88	86	89	86
24	90	87	91	91	88	90

[a] The incubation mixtures contained, in a final volume of 1 mL, 50 mM HEPES buffer, pH 7.4, 100 units of SOD or SM-SOD, and 0.1 mg of trypsin, chymotrypsin, or papain. At indicated times after incubation at 37 °C, aliquots of 10 μL were removed from the incubation mixtures and determined for the remaining SOD activity.

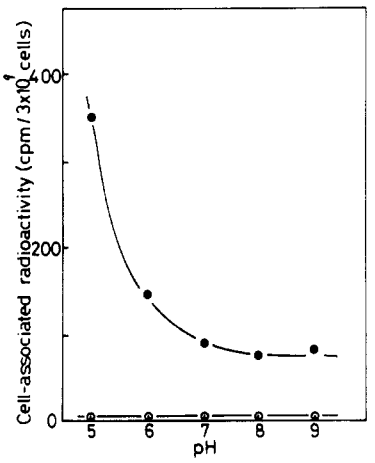

FIGURE 6: Binding of SOD preparations to erythrocyte membrane. Incubation mixtures contained in a final volume of 1.5 mL 0.15 M NaCl, 10^9 rat erythrocytes, 20 mM HEPES buffer, and 0.65 μg of radioactive SOD or SM–SOD preparations (1700 cpm). After incubation at 37 °C for 10 min, erythrocytes were washed three times by centrifugation at 3000 rpm for 3 min with 5 mL of the ice-cold buffer solution used for incubation except for radioactive enzymes. Then radioactivity associated with erythrocytes was determined. All values are corrected for radioactivity (25–30 cpm) found in the absence of erythrocytes. (Open circles) SOD; (solid circles) SM–SOD.

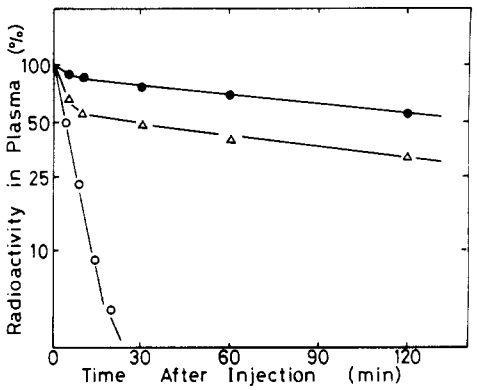

FIGURE 7: Fate of SOD and SM–SOD in the circulation. Under pentobarbital anesthesia (50 mg/kg of body weight), animals were intravenously administered with 1 mg of radioactive SOD or SM–SOD (200 000 cpm/rat) into the tail vein. At indicated times after administration, 0.1-mL blood samples were collected from the left femoral vein, and their radioactivity was determined. Data show one typical experiment derived from 10 animals. (Open circles) SOD; (solid circles) SM–SOD; (triangles) SM–SOD injected with warfarin (15 mg/kg).

of SM was protonated, the lipophilic nature of the ligand increased significantly (Inoue, 1987). To test whether protonation of the SM moiety of SM–SOD also affected the lipophilic nature of the enzyme, interaction of radioactive SM–SOD with erythrocyte plasma membranes was studied. When incubated with rat erythrocytes at physiological pH, a small amount of SM–SOD was bound to the membrane surface. Binding markedly increased when the pH of the incubation medium was decreased below 7 (Figure 6). In contrast, SOD did not bind to erythrocytes at any pH tested.

Fate of SOD and SM–SOD in Vivo. Figure 7 shows the fate of SOD and SM–SOD in the circulation of intact rats. When injected intravenously, SOD disappeared from the circulation with a half-life of 5 min. Under identical conditions, 60–70% of the injected dose was recovered intact in urine within 30 min after administration. In contrast, the rate of disappearance of SM–SOD was markedly reduced; the semilogarithmic plots of radioactivity in plasma revealed a half-life of 6 h. When chromatographed on a Sephacryl S-200 column, most radioactivity in the plasma samples, which were collected 1 h after administration of radioactive SM–SOD, eluted in a fraction that was slightly earlier than that for albumin. Administration of SM–SOD with a loading dose (15 mg/kg) of warfarin significantly decreased plasma levels of SM–SOD during the first 10 min. Thereafter, it decreased with a half-life of 6 h. These observations sugggest that SM–SOD circulates bound to the warfarin site on albumin. Table II shows the tissue distribution of radioactivity 3 h after intravenous administration of SOD and SM–SOD. Although a major part of SM–SOD remained in plasma, liver and kidney accumulated higher radioactivity than did other tissues. In contrast, most radioactivity derived from SOD was in urine, and only a small fraction was in plasma and various tissues. Only a small fraction (4.5%) of radioactivity from SM–SOD was excreted in urine.

Table II: Distribution of Radioactivity Derived from SOD and SM–SOD Samples[a]

location	SOD (cpm/organ) (% of dose)	SM-SOD (cpm/organ) (% of dose)
brain	27 ± 23 (0.03)	93 ± 19 (0.1)
heart	63 ± 24 (0.06)	301 ± 45 (0.3)
lung	95 ± 32 (0.09)	880 ± 97 (0.9)
liver	132 ± 34 (0.1)	10663 ± 1632 (10.7)
kidney	8011 ± 390 (8.0)	15669 ± 1177 (15.7)
urine	64863 ± 19136 (64.9)	4549 ± 556 (4.5)
spleen	40 ± 27 (0.04)	527 ± 51 (0.5)
small intestine[b]	44 ± 26 (0.04)	4683 ± 1459 (5.7)
testis	38 ± 36 (0.04)	469 ± 231 (0.5)
muscle[b]	33 ± 25 (0.03)	304 ± 240 (0.4)

[a] Under light ether anesthesia, animals were injected with 1 mg of radioactive SOD or SM–SOD (100 000 count/min) into the tall vein. Three hours after injection, they were exsanguinated by bleeding from the left femoral artery, and the tissue-associated radioactivity was determined. Data show mean ± SD derived from three animals. [b] Radioactivity was determined for 1 g of tissue.

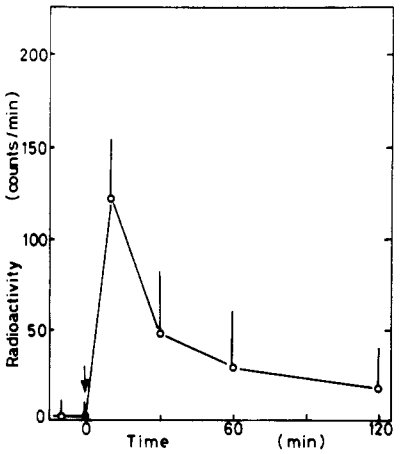

FIGURE 8: Accumulation of SM–SOD to a tissue whose local pH is decreased. Under light ether anesthesia, mice were intravenously injected with 7.6 μg of radioactive SM–SOD (20 000 cpm/animal). After 10 min (arrow), they were injected with 50 μL of 0.15 M NaCl or HEPES buffer, pH 6.0, to the right or the left femoral muscle, respectively. At the indicated times, animals were exsanguinated by bleeding from the abdominal artery, and each side of the thigh muscles was removed for determining radioactivity. Radioactivity found in the right thigh muscles (about 230–250 cpm) was subtracted from that found in the left thigh muscles. Data show mean ± SD derived from three animals.

To test whether pH-dependent binding of SM–SOD to plasma membranes occurred in vivo, mice were injected intramuscularly with saline or HEPES buffer (pH 6.0) into the right or left femoral muscle, respectively. Radioactive SM–SOD was then injected intravenously, and time-dependent

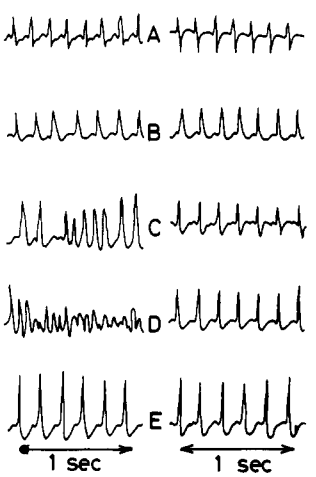

FIGURE 9: Effect of SM–SOD on postischemic reperfusion arrhythmias. Under pentobarbital anesthesia (50 mg/kg), animals were intravenously administered with 1 mg of SOD or SM–SOD into the tail vein. After 15 min of injection, the left anterior descending artery was occluded for 15 min. Then the blood was reflowed by releasing the occlusion. During the experiments, a standard lead I electrocardiogram was recorded. Data show one typical experiment derived from 30 animals. (Left) SOD-injected animal; (right) SM–SOD injected animal. (A) Before occlusion; (B) during occlusion; (C) 10 s after reflow; (D) 40 s after reflow; (E) 3 min after reflow.

radioactivity changes in each side of the thighs were compared (Figure 8). SM–SOD accumulated more markedly in the acidic (Hepes-injected) thigh than in the control side. Radioactivity in the left side of the thigh was highest 10 min after injection of the acidic buffer and decreased thereafter.

Effect of SM–SOD on Postischemic Reperfusion Arrhythmias. Since oxygen-free radicals are postulated to be important in the pathogenesis of ischemic myocardial injury (Burton et al., 1984), we tested the effect of SM–SOD on postischemic reperfusion arrhythmias of the rat. Even if the left anterior descending artery was occluded for 15 min, no significant arrhythmias occurred (Figure 9). However, immediately after reperfusion of the coronary branch, a marked ventricular tachycardia, fibrillation, and other arrhythmias occurred. Postischemic reperfusion arrythmias were prevented almost completely (more than 95%) by intravenous administration of SM–SOD. Administration of an equimolar amount of SM, SOD, or heat-inactivated SM–SOD had no such inhibitory action.

Discussion

The present work demonstrates that SM–SOD, but not SOD, reversibly binds to the warfarin site on albumin, circulates with a half-life of 6 h, and prevents postischemic reperfusion arrhythmias in the rat.

Erythrocyte-type SOD (homodimer) from human, but not from other species, has two surface Cys^{111} residues that are fully exposed to solvent (Hartz & Deutsch, 1972; Tanier et al., 1982). After incubation with SM, the derivatized enzyme failed to react with DTNB, while SOD that was concurrently incubated in the absence of SM fully retained the reactivity with DTNB; even after SM–SOD was treated with 1 mM dithiothreitol and EDTA at 25 °C for 1 h under nondenaturing condition, the enzyme failed to react with the reagent. Thus, the two Cys^{111} residues presumably reacted with the maleimide group of SM. This assumption is also supported by the finding that SM did not react with bovine erythrocyte type SOD with a serine residue at position 111, or a recombinant human erythrocyte type SOD in which Cys^{111} residues are substituted by serine by site-directed mutagenesis (unpublished observation).

In contrast to SOD, which was rapidly excreted in urine, SM–SOD escaped renal extraction and circulated with a half-life of 6 h. A similarly long half-life of SM–SOD was also observed with mice (6 h) and dogs (about 4–6 h) (data not shown). Since the molecular weight of SM is about 2170, the molecular size of SM–SOD is larger by only 13%, which is still below the threshold molecular weight for glomerular filtration (M_r = 50000) (Levine, 1981). However, if SM–SOD were bound to the circulating albumin, the apparent molecular weight of the complex would be greater than 100000, which is sufficiently larger than the glomerular threshold. Masking of the xenobiotic SM moiety by an autologous albumin in the circulation may also favor SM–SOD's escape from removal by reticuloendothelial systems.

Protonation of organic acids increases their lipophilicity (Roberts & Caserio, 1964). Such a property of amphipathic SM may account for the pH-dependent binding of SM–SOD to erythrocyte membranes and, at least in part, for its transient accumulation in the acidic area of the muscle. The local pH of an ischemic heart decreases significantly due to enhanced anaerobic glycolysis (Abiko & Sakai, 1980). SM–SOD binds to cell membrane surfaces when the local pH is decreased. Therefore, SM–SOD may accumulate on the plasma membrane of the ischemic heart, particularly following reperfusion. In fact, preliminary experiments reveal that intravenously administered SM–SOD rapidly accumulates in the heart during reflow of the left anterior descending artery (Inoue, 1988). Presumably due to efficient dismutation of superoxide radicals in the circulation and at the injured myocardial site, postischemic reperfusion arrhythmias were prevented by a single dose of SM–SOD. These results suggest that reactive oxygen species play an important role in the pathogenesis of postischemic reperfusion arrhythmias and that SM–SOD may be useful in decreasing tissue injury in ischemic heart disease. Immunological properties of a protein drug are important for its in vivo use. Preliminary experiments revealed that, upon double-immunodiffusion analysis, SM–SOD failed to form precipitine lines with plasma obtained from rats that received SM–SOD (1 mg/kg per day for 1 week) 2 weeks before experiments. Further studies on immunochemical properties of SM–SOD are under our current investigation.

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Novel Adenosine 3′,5′-Cyclic Monophosphate Dependent Protein Kinases in a Marine Diatom†

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ABSTRACT: Two novel adenosine 3′,5′-cyclic monophosphate (cAMP) dependent protein kinases have been isolated from the diatom *Cylindrotheca fusiformis*. The kinases, designated I and II, are eluted from DEAE-Sephacel at 0.10 and 0.15 M NaCl. They have a high affinity for cAMP and are activated by micromolar cAMP. They exhibit maximal activity at 5 mM Mg^{2+} and pH 8 with the preferred phosphate donor ATP (K_m = 0.1 mM) and phosphate acceptor histone H1 (K_m = 0.2 μM). They phosphorylate sea urchin sperm histone H1 on a single serine site in the sequence Arg-Lys-Gly-Ser(^{32}P)-Ser-Asn-Ala-Arg and have an apparent M_r of 75 000 as determined by gel filtration and sucrose density sedimentation. In the kinase I preparation a single protein band with an apparent M_r of about 78 000 is photolabeled with 8-azido[^{32}P]cAMP and is also phosphorylated with [γ-^{32}P]ATP in a cAMP-dependent manner, after autoradiography following sodium dodecyl sulfate gel electrophoresis. The rate of phosphorylation of the 78 000-dalton band is independent of the enzyme concentration. The photoaffinity labeling and cAMP-dependent phosphorylation also occur on a 78 000-dalton protein in the kinase II preparation. The catalytic activities of the two kinases cannot be freed by cAMP affinity column chromatography. The apparent size of the kinases remains unchanged upon preincubation with 0.5 mM cAMP followed by sucrose density sedimentation in the presence of the nucleotide. The results indicate that (i) these diatom cAMP-dependent protein kinases are monomeric proteins, possessing both the cAMP-binding regulatory and catalytic domains on the same polypeptide chain, (ii) the enzymes do not dissociate into smaller species upon activation by binding cAMP, and (iii) self-phosphorylation of the enzymes by an intrapeptide reaction is cAMP dependent. The two diatom cAMP kinases are refractory to the heat-stable protein kinase modulator from rabbit muscle, but they respond differently to proteolytic degradation and to inhibition by arachidonic acid and several microbial alkaloids.

There is mounting evidence that the physiological action of adenosine 3′,5′-cyclic monophosphate (cAMP)[1] and cGMP in eukaryotic cells is mediated through the activation of cAMP-dependent and cGMP-dependent protein kinases (cAMP and cGMP kinases) (Walsh et al., 1968; Kuo & Greengard, 1969). These enzymes have similar substrate specificity, amino acid composition, self-phosphorylation, and mechanism of activation (Rosen et al., 1977; Edelman et al., 1987). They also share a conserved catalytic core with many other proteins such as tyrosine kinase, protein kinase C, myosin light chain kinase, the epidermal growth factor receptor, and the transforming protein $p60^{v\text{-}src}$ (Gill & McCune, 1979; Taylor, 1987). These characteristics have led many investi-

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[1] Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; cAMP kinase, cAMP-dependent protein kinase; cGMP kinase, cGMP-dependent protein kinase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; PMSF, phenylmethanesulfonyl fluoride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TCA, trichloroacetic acid; kDa, kilodalton(s); K-252a, (8*R**,9*S**,11*S**)-(−)-9-hydroxy-9-(methoxycarbonyl)-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7b,11a-triazadibenzo[*a*,*g*]cycloocta[*cde*]trinden-1-one.

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