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Rational Design and Expression of a Heparin-Targeted Human Superoxide Dismutase

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In order to improve the therapeutic effectiveness of human Cu,Zn superoxide dismutase (HSOD) by targeting it to cell surfaces and increasing its circulatory half-life, we have designed and expressed a heparin-binding derivative of HSOD. This design was based on the idea that structurally independent protein units, HSOD and the heparin-binding A+ helix from protein C inhibitor, could be combined with a carefully chosen linker, GlyProGly, to form a stable, bifunctional protein. The chimeric HSOD–GlyProGly–A+ protein was expressed and secreted to the periplasm of *E. coli* and had normal SOD activity. HSOD–GlyProGly–A+ had a significantly increased retention time relative to wild-type HSOD on a heparin affinity column, indicating that it was successfully targeted to heparin, and this binding was maintained at physiological ionic strength. When administered to mice, HSOD–GlyProGly–A+ had a half-life of ~15 minutes, twice that of wild-type HSOD. Our rational design approach should be generally applicable to the creation of bifunctional chimeric molecules.

Superoxide dismutases (SODs; E.C.1.15.1.1) are the enzymes responsible for scavenging superoxide radicals that are released during aerobic metabolism (1). Human Cu,Zn superoxide dismutase (HSOD) has been shown to be effective for treating inflammatory pathologies, reducing reperfusion injuries (2, 3), and decreasing blood pressure (4) in animal model systems. HSOD has recently received orphan drug status for the prevention of pulmonary dysplasia in premature infants (5). However, the short circulatory half-life of HSOD due to kidney clearance has limited its use in human medicine. A naturally occurring extracellular SOD (EC-SOD) that binds heparin on cell surfaces also has limited application due to heterogeneity and expression only in mammalian cell systems (6, 7). Interesting approaches to increase the half-life of HSOD include generating HSOD polymers (8) and coupling the protein to large molecular weight carriers (9, 10), but the aggregation state and homogeneity of these molecules are hard to control. Because endothelial tissue is a major site of superoxide-induced damage, our approach has been to target HSOD to these cell surfaces. This would result in increased half-life and, more importantly, allow

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us to control where the protein is delivered. Indeed, endothelium-bound SOD has been shown to prevent reperfusion damage (11) and to reduce blood pressure in spontaneously hypertensive rats (4). Targeting HSOD-GlyProGly-A+ to the extracellular matrix may also provide protection from oxidative damage within the sequestered microenvironments created by stimulated neutrophils in inflammatory disease states, as has been observed for secretory leukocyte protease inhibitor (12) and EC-SOD (11).

We used a rational approach to HSOD targeting by using molecular modeling to design an optimal linkage between the known structure of HSOD (13) and the modeled structure of the heparin-binding A+ helix from protein C inhibitor (PCI) (14) that would promote stable folding of the fusion protein. Based on this model, we have successfully created a new heparin-binding antioxidant protein with potential therapeutic value.

MATERIALS AND METHODS

Modeling and Rational Design. In designing a heparin-binding HSOD, we started with three-dimensional structures of HSOD (13) and the modeled A+ helix (14). Molecular graphics was used to identify an orientation for the A+ helix on the surface of HSOD that would stabilize the helix in order to minimize proteolysis, require only a short linker between HSOD and A+, and generate a two-helix heparin-binding motif in the HSOD dimer. Since the two termini of HSOD are spatially close, choice of terminus was not a design constraint. However, other experiments with HSOD have shown that fusions at the carboxyl terminus are well-tolerated (ref. 8 and unpublished results, R.A.H.). Using the molecular graphics package InsightII (Biosym Technologies, Inc., San Diego, CA), we placed the A+ helix in a surface groove of HSOD near the two-fold axis of the homodimer (Fig. 1A) such that the two A+ helices in the dimer formed

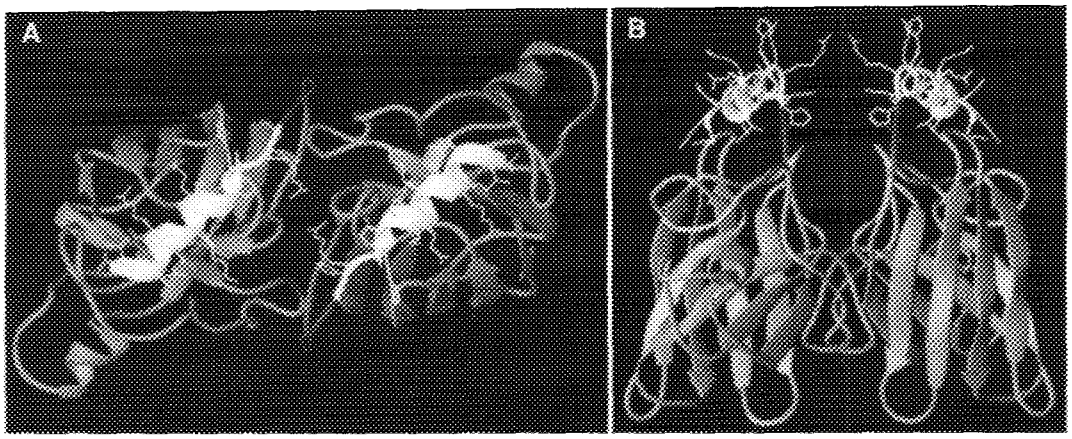

Fig. 1. Structural model of the HSOD-GlyProGly-A+ dimer. This design model was based on the X-ray structure of HSOD (13), homology modeling of GlyProGly and the A+ helix (14), and energy refinement of the complete structure (see Methods). The mainchain fold of the HSOD β-strands (ribbons with arrows), loops (tubes), and the short HSOD helix and longer A+ helix (coiled ribbons without arrows) were rendered using the program Ribbons, version 2.0 (30); the A+ helix and sidechains are shown in white, the GlyProGly linker and its Pro sidechain in dark grey, and HSOD in lighter grey. HSOD is active as a dimer, and this dimerization arranges the A+ helices in a two-helix motif similar to that found in other heparin-binding proteins (14). (A) View towards the A+ helix motif. These helices contain the Arg, Lys, and His sidechains responsible for heparin binding. The Pro sidechain of GlyProGly appears at top center in one monomer and at bottom center in the other. This linkage was designed to maintain access to the active site, which the short HSOD helix points towards (lower left and upper right). (B) Side view showing the nearly planar A+ helix pair packing against surface loops of the HSOD β-barrel. This view is roughly a 90° rotation about the horizontal axis in view (A).

a heparin-binding motif (14) similar to that found in platelet factor 4 (15) and such that the amino terminus of A+ was close to the carboxyl terminus of HSOD. This groove is formed by three surface loops of HSOD, which could move somewhat to accommodate the helix, and contains some nonpolar residues that would be buried by A+, stabilizing their association. We then determined by distance measurements in our model that a three-residue, turn-forming linker was required to connect the HSOD C-terminus to the N-terminus of A+. We chose GlyProGly because a Sequery (16) search of the Protein Data Bank (17) revealed that GlyProGly has a high preference to form turns in known protein structures, and because the large range of dihedral angles accessible to glycines (18) gives this peptide some flexibility to optimize interactions. Interestingly, GlyProGly occurs naturally in human EC-SOD (6) between the β-barrel domain homologous to HSOD and its heparin-binding peptide. We used the structure of GlyProGly in adenylate kinase (19) (Protein Data Bank code 3adk), which also links a β-strand and an α-helix, to model our GlyProGly linker. To complete the structural model of HSOD-GlyProGly-A+, bonds between the C-terminus of HSOD and the N-terminus of GlyProGly and between the C-terminus of GlyProPro and the N-terminus of the A+ helix were formed and stereochemically optimized by real-space refinement using the program Refi within Frodo (20). Any sidechain collisions were removed by manually changing torsional angles with InsightII, and unfavorable electrostatic and packing interactions were alleviated by steepest descents energy minimization with the program Discover (Biosym Technologies, Inc.). The resulting structural model of HSOD–GlyProGly–A+ is shown in Fig.1.

Construction of HSOD–GlyProGly–A+. HSOD–GlyProGly–A+ was constructed using the expression vector pPHSODIacI^q (21) containing the HSOD synthetic gene (8). The GlyProGly linker and A+ helix sequences were fused to the carboxyl terminus of HSOD by using cassette mutagenesis. The coding sequence was generated by reverse translation of the GlyProGly–A+ sequence and addition of a stop codon (TAA) followed by a *Sal* I cleavage site. An *Xma*I restriction site was also introduced into the linker sequence to allow future modification of the A+ sequence. A pair of synthetic oligonucleotides corresponding to bases 422-457 and to the complement of bases 426-462 was annealed and ligated to a second pair of annealed oligonucleotides corresponding to bases 458-517 and to the complement of bases 463-521. The resulting assembly was ligated into pPHSODIacI^q as a *Bam*HI-*Sal* I cassette, and the new vector was named pPHSODA+. Final confirmation of the clone's identity was done by dideoxynucleotide sequencing (22) of the *Nco*I-*Sal* I fragment containing the protein-coding portion of the fusion construct (Fig. 2).

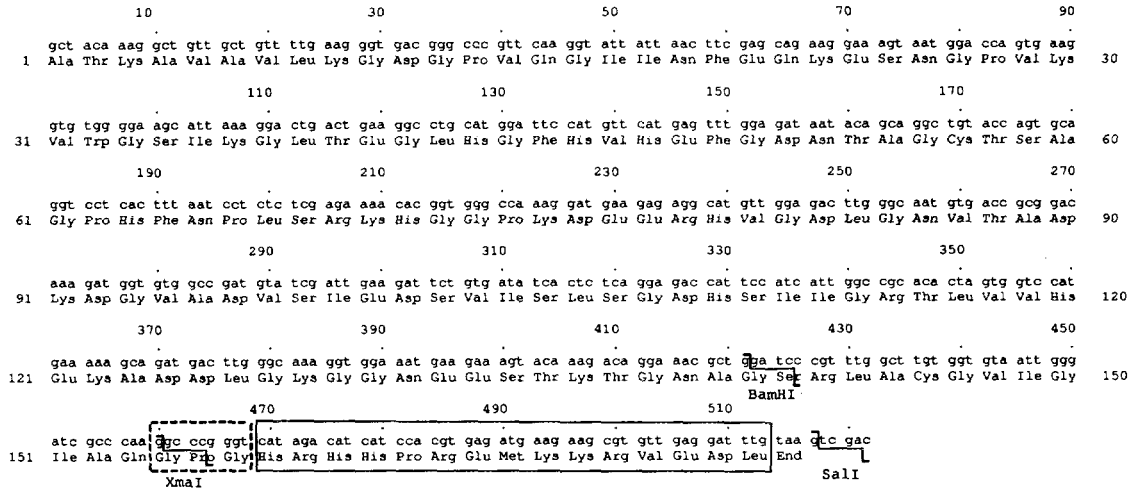

Fig. 2. HSOD–GlyProGly–A+ chimeric gene encoding the mature form of the protein. Numbers on the top correspond to nucleic acids, and numbers in the margins correspond to the amino acid sequence of the mature form of the fusion protein. The HSOD synthetic gene sequence starts at base 1 and ends at base 459. The GlyProGly linker is delimited by the dotted box (bases 460-468), while the A+ peptide is enclosed by the plain box (bases 469-513).

HSOD–GlyProGly–A+ Expression, Purification and Characterization. HSOD–GlyProGly–A+ protein was expressed in *E. coli* MC1061 (23) upon induction with 0.2 mM isopropylthio-β-D-galactoside (IPTG) in a Luria Bertani broth containing 200 μg per ml ampicillin and 250 μM $CuSO_4$. The periplasmic fraction of the bacterial cells was extracted using the osmotic shock procedure of Koshland and Botstein (24) modified by including 14mM NaEDTA in the hypertonic solution. Proper processing of the secretion signal was monitored by sequencing the amino terminus of the mature HSOD protein. HSOD–GlyProGly–A+ was further purified by heparin affinity chromatography on an Affi-Gel heparin column (BioRad, Richmond, CA). The protein was eluted with 200 ml of a 0.2 M Tris pH 7.0 buffer incorporating a linear gradient from 0.03 M to 0.4 M NaCl. Fractions of 5 ml were collected and tested by SDS-polyacrylamide gel electrophoresis (25), as shown in Fig. 3. The heparin-binding properties of HSOD–GlyProGly–A+ were also compared with those of HSOD by coeluting the two proteins on an Affi-Gel heparin column under the conditions described above (Fig. 3). The superoxide dismutase activity of HSOD–GlyProGly–A+ was assayed by the reduction of nitroblue tetrazolium on native 10% polyacrylamide gels (26) (Fig. 4A). The isoelectric point of HSOD–GlyProGly–A+ was determined with ultrathin acrylamide gels (Servalyte Precote pH 3-10) and the protein markers Mixture9 (Serva Biochemicals, Westbury, NY) (Fig. 4B).

Before animal testing, the pooled heparin column fractions containing HSOD–GlyProGly–A+ were dialyzed against 20 mM Tris-HCl pH 8.0 and loaded onto a 1.5 × 75 cm DEAE-Sepharose CL-6B chromatography column (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). The column was washed and eluted with a 0 to 0.15 M NaCl gradient in the same Tris-HCl buffer. Fractions containing HSOD–GlyProGly–A+ were pooled. To remove possible traces of endotoxin, the HSOD–GlyProGly–A+ was further purified by octyl-Sepharose chromatography. Groups of 2 BALB/c mice were injected intravenously with 2 mg of HSOD–GlyProGly–A+ or with recombinant HSOD (27) as a control. Blood samples were taken at 0, 2, 15, 60, and 240 minutes after injection. After 240 minutes a bolus of heparin was injected, and blood was taken 10 minutes thereafter, at 250 minutes. The HSOD-GlyProGly-A+ circulatory half-life was estimated based on residual SOD activity as assayed (26) in the serum samples

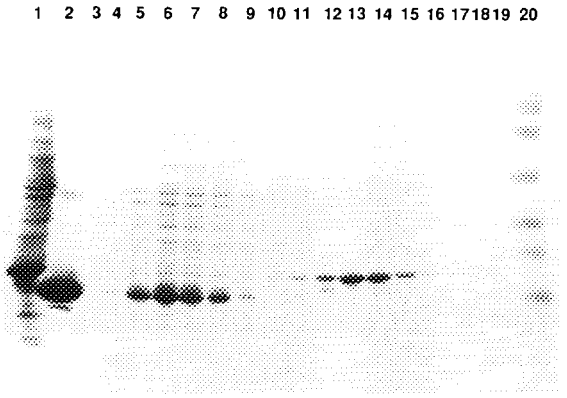

Fig. 3. SDS-polyacrylamide gel electrophoresis of fractions from the Affi-Gel heparin column. The *E. coli* periplasmic fraction containing HSOD–GlyProGly–A+ was mixed with an equivalent quantity of purified wild-type HSOD and loaded onto an Affi-Gel heparin column. The column was eluted with 200 ml of 0.2 M Tris,pH 7.0,buffer, incorporating a linear gradient from 0.03 M to 0.4 M NaCl. Fractions of 5 ml were collected and assayed on a 10%-20% polyacrylamide gradient gel run in the presence of β-mercaptoethanol and stained with Coomassie Brilliant Blue. Lane 1, *E. coli* periplasmic fraction containing HSOD–GlyProGly–A+; lane 2, 3 μg of purified wild-type HSOD; lane 3, 10 μl sample from column void volume. Lanes 4 to 19 correspond to 10 μl samples from heparin column fraction numbers 1, 3, 5, 6, 7, 9, 15, 18, 21, 23, 26, 28, 32, 35, and 40; lane 20, molecular mass standards. HSOD eluted at ~0.1 M NaCl, while HSOD–GlyProGly–A+ required nearly twice this salt concentration to be eluted from the heparin column.

RESULTS AND DISCUSSION

The pPHSODA+ vector permitted the expression of HSOD–GlyProGly–A+ at up to 15% of total *E. coli* protein. Estimation of the molecular weight by SDS-PAGE confirmed that HSOD molecular weight was increased by the amount (2180 D) expected for the addition of A+ peptide. Superoxide dismutation activity of the recombinant HSOD–GlyProGly–A+ protein was confirmed by gel assay (26) to be comparable to that of wild-type HSOD (Fig. 4A). The decreased motility of HSOD–GlyProGly–A+ in activity gels supported that positive charges (from the A+ helix) had been added to HSOD. After osmotic shock, the purification by heparin-agarose affinity column yielded >90% pure protein as estimated by SDS-PAGE. Further purification of HSOD–GlyProGly–A+ on DEAE and octyl-sepharose yielded >95% pure protein. (For HSOD, a single step of DEAE chromatography on the osmotic shock fraction produced 90% pure protein; data not shown.) Isoelectric focusing (Fig. 4B) showed the homogeneity of the preparation, as well as the expected difference in pI between wild-type HSOD (4.5) and HSOD–GlyProGly–A+ (6.5) due to the net positive charge on the HSOD–GlyProGly–A+ addition. On the heparin-agarose affinity column, the differential elution of HSOD and HSOD–GlyProGly–A+ showed that the fusion protein had a significantly higher affinity for heparin (Fig. 3). HSOD eluted at ~0.1 M NaCl, while HSOD–GlyProGly–A+ eluted at ~0.2 M NaCl. Our data showed the half-life of HSOD in mice to be ~7 minutes (consistent with previously published data, ref. 28), while the half-life of HSOD–GlyProGly–A+ was twice as long, at ~15 minutes.

A heparin-binding HSOD is expected to be useful therapeutically due to increased circulatory half-life and due to targeting the enzyme to cell surfaces, where its activity can have the most benefit. The heparin column results showed that HSOD–GlyProGly–A+ binds heparin at

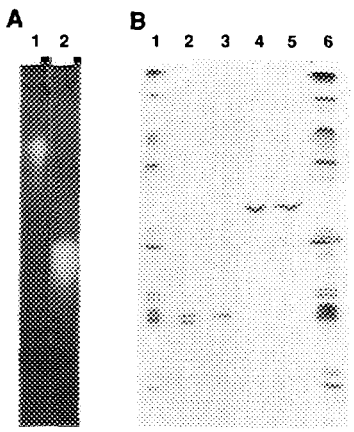

Fig. 4. Characterization of HSOD-GlyProGly-A+. (A) SOD activity gel. Equivalent amounts of HSOD and HSOD–GlyProGly–A+ were loaded onto a native 10% polyacrylamide gel, then separated and stained for SOD activity (26). HSOD–GlyProGly–A+, lane 2, has an SOD activity level comparable to that of wild-type HSOD, lane 1. (B) Isoelectric focusing of purified HSOD and HSOD–GlyProGly–A+. Lanes 1 and 6 contain a set of pI marker proteins; lanes 2 and 3, HSOD; lanes 4 and 5, HSOD–GlyProGly–A+. Even-numbered lanes were loaded with twice as much protein as odd-numbered lanes. HSOD has a pI of 4.5-4.6, while HSOD–GlyProGly–A+ has a much higher pI of 6.5.

physiological ionic strength (0.15 M) and thus has a similar ionic strength dependence to that observed for PCI, the donor of the A+ helix (14). This heparin affinity is relatively low when compared to that of EC-SOD, which requires 0.5 M salt to elute (6), or to that of antithrombin III, which requires >1 M NaCl to elute from a heparin column (28). However, with elution at an ionic strength slightly over 0.2 M NaCl, HSOD–GlyProGly–A+ should have a significant heparin binding affinity *in vivo*, resulting in a tendency to bind cell surfaces. In mice, we demonstrated a doubling in circulatory half-life. While the A+ helix is the major heparin-binding determinant of PCI, the H helix is also a likely contributor (14). Therefore, improvements to our design of HSOD–GlyProGly–A+ could include adding the H helix sequence and minimizing possible proteolysis of these charged helices by tethering them to the HSOD surface with disulfide bridges.

Recently, a hybrid protein involving fusion of the heparin-binding domain of EC-SOD to the C-terminus of HSOD has been shown to have affinity for the heparin-like proteoglycans on endothelial cells (29). Their approach takes advantage of the homology existing between the β-barrel domains of EC-SOD and HSOD to ensure that the two fusion moieties will be compatible. In contrast, our approach uses structural modeling and design to link functional units with self-determined structures taken from fusion partners lacking structural or evolutionary relationships. Thus, our design technique offers opportunities to create novel chimeric proteins by linking components that are not found together in nature.

By demonstrating that a human superoxide dismutase targeted to heparin could be engineered from unrelated protein units, we have begun to explore the possibilities of structural component-based design for developing proteins with new functionalities. Other proteins that might benefit from the addition of a targeting function include the serine protease inhibitors α-1-antitrypsin and secretory leukocyte protease inhibitor, and this targeting could be designed to achieve greater specificity as well as longer half-life. We are investigating other possibilities, including creating a synergy or cascade of more efficient reactions by coupling previously independent protein functional units, and stabilizing proteins by adding structural units that enable them to survive deleterious conditions such as proteolysis. Our technique for generating multifunctional proteins by fusing independent structural units with separate functions can be generalized to any protein folding unit for which a three-dimensional structure or structural model is available.

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