Genetically Engineered Polymers of Human CuZn Superoxide Dismutase

BIOCHEMISTRY AND SERUM HALF-LIVES*

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CuZn superoxide dismutase is a highly stable dimer of identical subunits with a combined molecular mass of 32,000 daltons. Two human superoxide dismutase genes have been joined in the same translational reading frame, using spacers of different lengths, to encode single chain proteins consisting of two identical human superoxide dismutase subunits. The first construct encodes two directly linked subunits; the terminal glutamine codon of the first gene was changed to a methionine codon and followed immediately by the second gene. The second construct encodes two subunits linked by a 19-amino-acid human immunoglobulin IgA1 hinge sequence. Both constructs produce high levels of catalytically active superoxide dismutase when expressed in *Escherichia coli*. The protein containing the IgA1 hinge sequence forms polymers up to 750,000 in molecular weight, which are linked together noncovalently by the hydrophobic bonding of the dimer interface. The polymers are soluble, thermostable, and of near normal specific activity. Site-directed in vitro mutagenesis was used to inactivate one of the two human superoxide dismutase subunits. The resulting human superoxide dismutase polymers have approximately 50% activity, thus confirming that the products of both genes are catalytically active. Large amounts of individual polymeric forms have been purified from recombinant yeast and tested for serum stability in rats. The serum half-life is approximately 7 min for both the two-chain wild type human superoxide dismutase dimer (Mr 32,000) and the single chain molecule consisting of a human superoxide dismutase dimer covalently linked by the immunoglobulin hinge region (Mr 34,000), whereas the higher molecular weight polymers (Mr ≥ 68,000) all have half-lives of approximately 145 min.

CuZn superoxide dismutases are thermostable cytoplasmic enzymes, found mainly in eukaryotes, which dismutate the superoxide radical to molecular oxygen and hydrogen peroxide, thus helping to protect cells against the toxic by-products of aerobic metabolism (Fridovich, 1979). Superoxide or more toxic molecules such as the hydroxyl radical and hypochlorous acid derived from superoxide are primarily responsible for the microbicidal activity of neutrophils and other short-lived phagocytic cells (Klebanoff and Clark, 1978; Fridovich, 1979; Weiss et al., 1985). The superoxide radical is also an important mediator of both the inflammatory response of neutrophils and of the damage that occurs during reperfusion of anoxic tissue after organ transplantation or when a blood clot is removed (Petrone et al., 1980; McCord, 1988). Thus, superoxide dismutase may be clinically useful as a nonsteroidal antiinflammatory agent and for reducing reperfusion damage to heart, kidney, and other organs (McCord, 1988). One potential limitation of the clinical usefulness of superoxide dismutase is that it is rapidly removed from blood by filtration in the kidney. The circulating half-life of bovine superoxide dismutase in rat blood is approximately 6 min (McCord et al., 1979).

Each CuZn superoxide dismutase molecule is a dimer of identical subunits and each subunit contains about 153 amino acids (depending on the species) and one copper ion and one zinc ion. The crystallographic structure of bovine CuZn superoxide dismutase, refined to a resolution of 2 Å, shows that the structural core of the subunit is a flattened Greek key β-barrel motif consisting of eight antiparallel β-strands joined by seven turns or loops (Tainer et al., 1982). The catalytic copper ion is liganded to the surface of the β-barrel by 4 histidine residues and occurs at the base of a channel formed by two loops extending from the β-barrel. In addition to forming the superoxide channel, the two loops encode specific functional subdomains involved in precollision electrostatic guidance of substrate (loop VII), zinc binding (loop IV), and dimer contact (loop IV) (Getzoff et al., 1983).

The dimer interaction is particularly strong in CuZn superoxide dismutases, and the subunits have not been separated without inactivating the enzyme. Thus, the bovine enzyme remains an active dimer in 8 M urea (Malinowski and Fridovich, 1979a) and 4% sodium dodecyl sulfate (Forman and Fridovich, 1973) and has a conformational melting temperature of 96 °C (Lepock et al., 1985; Roe et al., 1988). There is very tight packing of the complementary hydrophobic interfaces between the subunits (and between the two halves of the flattened β-barrel), and this may contribute to the high stability of the CuZn superoxide dismutases (Getzoff, 1982; Getzoff et al., 1986a).

The Greek key β-barrel found in CuZn superoxide dismutase is one of the most versatile structural motifs found in biological systems (Richardson, 1981; Blundell et al., 1981; Getzoff et al., 1986b). Protein domains with this fold are used for recognition and binding (immunoglobulins, pre-albumin), long-lived, and stable structural assemblies (lens crystallins,
icosahedral virus capsid proteins), electron transfer (plasto-
cyanin), and catalysis (serine proteases, CuZn superoxide
dismutases). The superoxide dismutase and immunoglobulin
Greek key β-barrels are structurally and organizationally sim-
ilar as well as sharing the same topological fold. Despite the
absence of any amino acid sequence homology in the DNA molecules containing the human superoxide dismutase and immunoglobulins, there is a close similarity in the relative position of the strand α carbon
atoms between superoxide dismutase and immunoglobulins.
In addition, two of the three hypervariable loops of immuno-
globulins, which are responsible for antigen binding, occur at
the same position as the two superoxide dismutase loops
encoding the functional subdomains described above (Rich-
ardson et al., 1976). It is possible that immunoglobulins and
CuZn superoxide dismutases share a common ancestral gene
(Richardson et al., 1976; McLachlan, 1980).

In an attempt to mimic the partially flexible domain linkage
that occurs between the Fab and Fe portions of the antibody
molecule, two human superoxide dismutase genes were joined
by a human immunoglobulin hinge region and expressed in
Escherichia coli and yeast. We have investigated the potential
that the products of such tandemly linked genes would
polymerize to produce high molecular weight forms, with
increased circulation times in blood.

**MATERIALS AND METHODS**

**General Procedures and Reagents—**DNA synthesis (Urdea et al., 1983) and DNA manipulations (Maniatis et al., 1982) were carried out as described previously. The DNA sequences of all cloned syn-
thetic DNAs, as well as all associated human superoxide dismutase sequences in the vector, were confirmed using the chain termination method (Sanger et al., 1977; Messing, 1983).

**Construction of Plasmids Encoding Two Directly Linked Human Superoxide Dismutase Subunits—**The 450-bp NcoI-SalI fragment from pSODNco5 (Hallewell et al., 1985), containing the human superoxide dismutase cDNA, was substituted between the unique NcoI and SalI sites of pSODCP2 (Steimer et al., 1986). The resulting plasmid (pSODCP2HSOD) was cut with SalI and partially digested with SstI. The 747-bp SstI-SalI fragment was isolated from an agarose gel and substituted between the unique SstI and SalI sites of pNco5A (Nativ et al., 1987) to produce pNco5AHSOD, the plasmid encoding expression of two directly linked human superoxide dismutase subunits under the control of the tac promoter (see Fig. 3).

**Plasmid Subunits Linked by an Immunoglobulin Hinge—**Complementary synthetic DNA molecules encoding the human IgA hinge se-
quence (Low et al., 1976) were made for the sequence between the Sau3AI (depicted as a bracketed BamHI site in Fig. 4) and NcoI sites (see Fig. 5). After annealing and phosphorylation, the synthetic linker was ligated with a 450-bp NcoI-EcoRI fragment containing the human superoxide dismutase cDNA (from plasmid pSODNco5; Hallewell et al., 1985) and vector plasmid pSODCP1 (previously cut with NcoI and EcoRI and phosphatased; Steimer et al., 1986). The resultant plasmid, pSODCP1SODHA1, contains tandem superoxide dismutase genes linked through the IgA1 sequence. This plasmid was cut with SalI and partially digested with SstI. The 804-bp SstI-SalI fragment was isolated from an agarose gel of the digest and substituted between the unique SstI and SalI sites of pNco5A (Nativ et al., 1987) to produce pNco5AHSODHA1, encoding E. coli expression of two directly linked human superoxide dismutase genes under the control of the tac and ampicillin promoters (see Fig. 3).

**Mutants—**The synthetic human superoxide dismutase gene (see Fig. 5) was made by synthesizing DNA molecules of between 40 and 75
nucleotides with overlapping complementary ends (Urdea et al., 1983). After annealing, the molecules were ligated together and cloned in plasmid pNco5A between its NcoI and SalI sites to make plasmid pNco5A1. The new hexanucleotide restriction sites were selected from all the possible sites, allowed by the degeneracy of the genetic code.

The plasmid pNco5A1 was inserted into plasmid pIE3 (a derivative of pUC18 containing the unique BamHI site) to make plasmid pIE3A (see Fig. 3). The plasmid pIE3A was cut with SstI and partially digested with SspI to produce plasmid pIE3A5, containing the unique SstI and SalI sites of pNco5A1, and the superoxide dismutase activity in sera determined using the pyrogallol method (Marklund and Marklund, 1974) and by 10 and 15% native polyacry-
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1 The abbreviation used is: bp, base pair(s).
buried. Holes and discontinuities in buried surfaces indicate gaps in the interface that are large enough to accommodate a water molecule. Computer graphics analysis was done using the graphics modeling language GRAMPS (O'Donnell and Olson, 1981) and the molecular modeling program GRANNY (Connolly and Olson, 1986).

RESULTS

Strategy for Covalently Joining Two Superoxide Dismutase Subunits—The genes used in these studies and their probable protein products are summarized schematically in Fig. 1. Three factors suggested that covalently joined human superoxide dismutase subunits should be constructed to investigate the nature of the dimer interaction and the potential of such molecules to polymerize by forming intermolecular dimer contacts. First, a large variety of multimeric structures are formed by the related immunoglobulin Greek key β-barrel (Richardson et al., 1976). Second, interaction between monomers is a prerequisite for protein polymerization; the well documented stability of the CuZn superoxide dismutase dimer interface suggested that, once formed, such polymers would be stable (Forman and Fridovich, 1973; Malinowski and Fridovich, 1979a). Last, the location of all the N and C termini in solvent exposed positions at the lower edge of the dimer interface suggested that a relatively short covalent interconnection that does not interfere with other parts of the molecule might be possible (see next section). Initially, two connections between subunits were investigated (see Fig. 1). Representing one extreme, the C terminus of one subunit was joined directly to the N terminus of the other. In the second construction, a 19-residue human immunoglobulin IγA1 hinge sequence was selected both for its presumed lack of immunogenicity and for the partial rigidity of its polyproline helix, which might favor polymer formation (Huber and Bennett, 1983). The hinge was shortened to remove free cysteine residues which could disrupt the molecule by forming aberrant disulfide bonds.

Structural Arrangement for Covalently Joining Two Superoxide Dismutase Subunits—In terms of the protein structure, the construction of directly linked and hinged subunits involves formation of direct and 19-residue hinge connections between the C terminus of one subunit and the N terminus of a second subunit. In the crystallographic structure of native bovine superoxide dismutase, the two flexible, solvent accessible termini are located 22 Å apart along a straight line (Fig. 2n). Direct connection across the 22-Å separation will distort the dimer by cutting through the bottom part of the dimer interface (Fig. 2b). This distortion may cause the exposure of hydrophobic areas normally covered by the residues near the N and C termini, and thus represents an extreme case for examining constraints in the design of covalently linked subunits. Yet, given the very strong dimer interaction, a construction with two human superoxide dismutase subunits directly joined end-to-end might fold to produce an active single chain enzyme.

In contrast, the 19-residue hinge connection should allow a minimum length of about 30 Å for helical conformation (Marquart et al., 1980) and a maximum length of over 50 Å for extended conformations. The hinged connection should therefore allow two topologically different possibilities: 1) the single chain monomer consisting of two covalently linked subunits also joined noncovalently at the dimer interface of each subunit and 2) a series of multimers consisting of the two-subunit monomers joined together by the noncovalent interactions of their dimer interfaces (see Fig. 1). The first case would produce a covalent connection around the lower part of the dimer interface, requiring a length of about 45 Å without disrupting the normal contact region. In the second polymeric conformation, the subunits of each monomer are in dimer contact with the subunits of other monomers, permitting wide variation in hinge conformation due to the surface exposure and mobility of the termini.

Expression of Two Directly Linked Human Superoxide Dismutase Subunits in E. coli—The plasmid pNco5AHSOD for E. coli expression of the directly linked subunits is shown in Fig. 3. To simplify the construction, the terminal glutamine codon of the first gene was replaced with a methionine codon (see Fig. 3 and "Materials and Methods"). This plasmid was transformed into the E. coli strain lacking the endogenous manganese and iron superoxide dismutases (sodA/sodB mutant, Carliz and Touati, 1986). The cell lysate shows a relatively complex pattern of active superoxide dismutase forms, but there is no evidence of polymerization (Fig. 4, lane 4, pNco5AHSOD). There is one distinct band, which comigrates with the wild type human superoxide dismutase control (Fig. 4, lane 3, pNco5A), and two or three less distinct bands against a diffuse background of superoxide dismutase activity. Analysis of plasmid DNA from the culture showed that about half the plasmid DNA was of the expected molecular weight, and half corresponded in size to the parental pNco5A plasmid containing a single copy of the human superoxide dismutase gene. Restriction enzyme analysis indicated that reciprocal recombination had occurred between the two homologous human superoxide dismutase genes to produce the smaller plasmid from which one of the tandem human superoxide dismutase genes had been lost. After such recombination the remaining wild type gene would produce native human superoxide dismutase, accounting for the wild type human superoxide dismutase that represents about half of the active superoxide dismutase observed on the gel. We speculate that the more diffuse superoxide dismutase activity bands, encoded by pNco5AHSOD, correspond to single chain monomers containing somewhat distorted and exposed hydrophobic dimer interfaces, as predicted from the structural analysis and shown schematically in Fig. 1.

The reciprocal recombination observed between the two directly joined identical genes was not seen when the same
Fig. 2. Stereo pairs showing computer graphics images of the dimer interface in bovine superoxide dismutase. a, this view down the 2-fold axis of the skeletal model represented as α carbon backbones shows that the dimer interface (horizontal, center) is formed between the two subunits (blue and red lines) of identical amino acid sequence and fold. The construction of directly linked and hinged subunits involves formation of direct and 19-residue hinge connections between the C terminus (orange circle and label C, left) of the lower subunit (red lines) and the N terminus (light blue circle and label N, right) of the upper subunit (blue lines) lying 22 Å apart along a straight line (label and dashed line). b, in this view roughly perpendicular to the dimer interface, the surface buried by the dimer contact (colored dots) is shown with the α carbon trace of one subunit (red lines). The surface is color-coded by electrostatic potential (red = most negative; yellow = negative; green = approximately neutral; cyan = positive; blue = most positive). Directly joined subunits connect the C terminus (orange circle and label C, left) of one subunit to the N terminus (light blue circle and label N, right) of the other subunit. The location of the active sites relative to this view are indicated by spheres showing the Cu (orange) and Zn (yellow) positions.
genes were joined by the IgAl hinge (see next section), indicating that such recombination is not a very common event. The postulated exposure of hydrophobic regions from the dimer interface in the directly linked human superoxide dismutase subunits may be toxic and slow cell growth. This could provide the selection for cells carrying the gene deletion, since this would decrease the expression level of the directly linked subunits.

Expression of Two Human Superoxide Dismutase Subunits Linked by the IgAl Hinge—To determine whether the IgAl hinge-linked human superoxide dismutase gene shown in Figs. 1 and 3 produces active enzyme, this gene was expressed in E. coli from plasmid pNco5AHSODHA1 (see "Materials and Methods" for construction). After cell lysis and centrifugation, the soluble proteins in the supernatant were separated on a polyacrylamide gel and stained for superoxide dismutase activity as shown in the last lane of Fig. 4. In contrast to the directly linked subunits, the IgAl hinge-linked construct shows a ladder of bands of superoxide dismutase activity of increasing intensity towards the bottom of the gel, suggesting that the molecule has polymerized. There is no evidence of reciprocal recombination to produce a band of activity corresponding to wild type human superoxide dismutase, and there was no detectable reciprocal recombination to produce deleted plasmid DNA (data not shown).

Design and Synthesis of Native and Mutant Human Superoxide Dismutase Genes—To facilitate construction and expression of mutant human superoxide dismutases by cassette mutagenesis, we first designed and cloned a human superoxide dismutase gene made from synthetic DNA, as shown in Fig. 5 (and see "Materials and Methods"). Replacement of the 2 free cysteine residues in human superoxide dismutase has been shown to increase the thermostability of the protein without decreasing the catalytic efficiency. 3 Therefore, in the synthetic gene, we changed the Cys-6 codon to an alanine codon and the Cys-111 codon to a serine codon. The alteration of the cysteine codons described above does not change the charge of the protein; the Cys-6, Cys-111 protein migrates at the same position as wild type on native polyacrylamide gels (data not shown). The synthetic gene was designed to include seven new hexanucleotide restriction sites, as well as the single StuI site also present in the human superoxide dismutase cDNA, inserted into the sequence by means of silent third position changes to amino acid codons. All of the new restriction sites are unique in the bacterial expression plasmid pNco5A, thus facilitating the rapid construction of mutants by cloning double-stranded synthetic DNA fragments and testing for altered activity on gels.

This method was used to create a plasmid (pNco5AD143) containing a mutant gene coding for a human superoxide dismutase in which the positively charged Arg-143 residue at the active center was replaced by a negatively charged aspartic acid residue (D143). The expression level of the mutant protein in E. coli is the same as the native protein (data not shown) and pulse radiolysis studies of the purified HSODD143 mutant protein show that its reaction rate at pH 8 is 2% of wild type, 4 in approximate agreement with our estimate from the activity gel (Fig. 6, lane 4, pNco5AD143). Mutations at Arg-143 had already produced proteins with reduced activity: the enzyme with Arg-143 replaced by the positively charged lysine had 50% of the wild type activity and replacement with the neutral amino acid isoleucine resulted in 11% of the wild type level (Beyer et al., 1987). These results confirm studies indicating that arginine 143 is important in local electrostatic attraction of the negatively charged superoxide radical (Getzoff et al., 1983). The mutant HSODD143 protein produced in the sodAsodB strain containing the plasmid pNco5AD143 has an increased net negative charge, and therefore migrates further down the activity gel toward the positive electrode (Fig. 6, lane 4).

Inactivation of the Second Superoxide Dismutase Subunit from the IgAl Hinge-linked Gene—To determine if both superoxide dismutase subunits in the IgAl-linked protein are

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3 R. A. Hallewell, K. C. Inlay, L. Laria, C. Gallegos, B. Irvine, N. M. Fong, E. D. Getzoff, J. A. Tainer, D. E. Cabelli, B. H. J. Biealski, P. Olson, G. T. Mullenbach, and L. S. Cousens, manuscript in preparation.

4 D. E. Cabelli and R. A. Hallewell, unpublished results.
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Fig. 4. Superoxide dismutase activity gel of E. coli sodAsodB cell extracts showing superoxide dismutases encoded by two human superoxide dismutase genes linked directly and by the immunoglobulin IgA1 hinge region. Gels were 10% polyacrylamide and each lane was loaded with 50 μg of cell lysate proteins or 50 ng of purified erythrocyte CuZn superoxide dismutase.

Fig. 5. Synthetic CuZn human superoxide dismutase gene used for construction of the Arg-143 to Asp-143 mutant. The hexanucleotide re-}

strictions sites shown are unique in pNco5AHSOD.

Functional, we inactivated the second, C terminal subunit. The C terminal wild type gene in plasmid pNco5AHSODHA1 was replaced by the HSODD143 gene, which codes for a human superoxide dismutase that is 2% active, to produce the E. coli expression plasmid pNco5AHSODD143HA1 (see “Materials and Methods”). The protein HSODD143HA1 produced in the sodAsodB strain from this plasmid is active and forms the characteristic ladder of polymeric bands (Fig. 6, lane 6). Thus, the first human superoxide dismutase domain presumably retains activity, and the second inactive mutant domain does not prevent polymerization. This suggests that the mutant subunit of HSODD143HA1 folds correctly to produce a near normal dimer interface. As expected for the increased net negative charge of the HSODD143HA1 mutant, each band migrates further down the gel toward the positive electrode than the equivalent wild type band. Each band of HSODD143HA1 activity is approximately 50% of the intensity shown when two wild type superoxide dismutase subunits are used (Fig. 6, lanes 5 and 6). This supports the conclusion that the first superoxide dismutase domain is fully active and the second domain largely inactive, and it indicates that both domains in wild type human superoxide dismutase contribute equally to superoxide dismutase activity. The superoxide dismutase activity band corresponding to the HSODD143HA1 monomer (see Fig. 1) contains one wild type subunit with normal activity and one inactive subunit in a single superoxide dismutase molecule (Fig. 6, lane 6). This confirms the conclusion that each subunit in the wild type dimer functions independently of catalytic activity in the other subunit (Malinowski and Fridovich, 1979b).

Purification and Characterization of the HSODHA1 Polymers—To increase the yield of the HSODHA1 polymers, a yeast system that had given high level expression of wild type human superoxide dismutase was used (Hallewell et al., 1987). The gene coding for the two IgA1 hinge-linked subunits was cloned into the pC1/1 vector (see “Materials and Methods”) giving the pC1/1SODHA1 plasmid shown in Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli, 1977) of the total soluble cell proteins in the yeast crude extract (Fig. 7A) showed protein of the predicted molecular mass (34,000 daltons) and no evidence of proteolytic breakdown or recombination to produce material of wild type molecular mass (16,000 daltons). The yield of HSODHA1 protein was approximately 40% of total soluble cell protein, which is comparable to that obtained previously for wild type human superoxide dismutase.

To purify the HSODHA1 protein, the clarified lysate was heated for 2 h at 65 °C and centrifuged to remove precipitated yeast proteins and other debris. After the heating step the HSODHA1 protein is at least 95% pure, and very little has been lost (Fig. 7A). Furthermore, heating does not alter the banding pattern on a native polyacrylamide gel stained for activity. Heating at 60 °C instead of 65 °C (Fig. 7A) gave decreased purity without increasing the yield significantly. After the heating step the HSODHA1 proteins were subjected to DEAE-Sepharose chromatography, which served mainly to remove nonproteinaceous contaminants.

The specific activity of the purified HSODHA1 protein was approximately the same as wild type human superoxide dismutase as measured using the pyrogallol assay (Merkland and Marklund, 1974). The size distribution of the HSODA1 species was analyzed by gel filtration chromatography on a calibrated AcA34 column (Fig. 7B), followed by native 15%
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human superoxide dismutase subunits linked by the IgA1 hinge or by a native N-terminal subunit linked to a 98% inactive C-terminal subunit. Samples are: purified human erythrocyte superoxide dismutase (HSOD, 50 ng, inactive C-terminal subunit. Samples are: purified human erythrocyte superoxide dismutase containing Asp-143 substituted for normal Arg-143 (pNco5A143), two wild type human superoxide dismutase subunits linked by the IgA1 hinge (pNco5AHSODHA1, lane 5), and an N-terminal wild type human superoxide dismutase subunit linked to the 98% inactive mutant human superoxide dismutase subunit (pNco5AHSODD143HA1, lane 6). Gel and lysis conditions are described in Fig. 4 legend.

polyacrylamide gel electrophoresis and staining both for proteins and for superoxide dismutase activity (Fig. 7C). Taken together, the sizing column and the activity gel show that superoxide dismutase molecules of molecular weights between approximately 33,000 and 750,000 are present and that gel mobility increases as the molecular weight decreases. A comparison of the gel stained for protein using Coomassie Blue with that stained for activity shows that all but the very high molecular weight forms are active, and a comparison of the intensities of staining indicates that the specific activities of the active species are approximately the same.

The three superoxide dismutase bands with molecular weights of approximately 33,000 presumably all correspond to the single chain HSODHA1 monomer (calculated molecular weight 34,000), comprising two superoxide dismutase subunits linked by the IgA1 hinge and noncovalently by the 19-residue hinge allows a structure of multiple ellipsoidal domains joined by hinges, rather than a single, larger, more compact ellipsoid. The nature of the multiple superoxide dismutase bands observed for the monomer and dimer fractions, which were also partially resolved on the 10% polyacrylamide gel (Fig. 6), has not been determined. One possibility is that they result from different conformations of the linked molecules. Alternatively, the multiple bands could reflect charge differences on the same conformation. Charge differences are probably responsible for the electrophoretic variants quite commonly observed in CuZn superoxide dismutases (Beyer et al., 1987).

Half-lives of the Superoxide Dismutase Polymers in Rat Blood—Rats were injected with 3–5 mg of wild type human superoxide dismutase (Mr 32,000), monomeric HSODHA1 (Mr 34,000), dimeric HSODHA1 (Mr 68,000), a high molecular weight fraction of HSODHA1 forms (Mr 82,000–340,000), and unfractionated HSODHA1. At various times after injection, blood was sampled, converted to serum, and tested for superoxide dismutase activity. Enzyme assays (Fig. 8) show that the HSODHA1 monomer (Mr 34,000), has a short half-life (approximately 7 min) similar to that of wild type human superoxide dismutase. The HSODHA1 dimer has a half-life...
Molecular weights are: superoxide dismutase, 34,000 for monomer SODHA1; 68,000 for dimer SODHA1, 82,000–340,000 for high molecular weight forms. 

of 135 min and the high molecular weight fraction a slightly greater half-life of 155 min. The monomeric HSODHA1 molecules in the unfractionated HSODHA1 preparation are presumably responsible for the intermediate half-life observed for this material. The results of the enzyme assays were qualitatively confirmed by superoxide dismutase activity gels as shown in Fig. 9.

**Fig. 8.** Loss of different molecular weight forms of CuZn superoxide dismutase from rat serum measured by enzymatic activity. Individual rats were injected with 3–5 mg of the purified superoxide dismutase, and immediately a blood sample was taken to determine the initial activity. Subsequent samples were taken at the times indicated. Molecular weights are: 32,000 for native superoxide dismutase, 34,000 for monomer SODHA1, 68,000 for dimer SODHA1, 82,000–340,000 for high molecular weight forms. 

of 135 min and the high molecular weight fraction a slightly greater half-life of 155 min. The monomeric HSODHA1 molecules in the unfractionated HSODHA1 preparation are presumably responsible for the intermediate half-life observed for this material. The results of the enzyme assays were qualitatively confirmed by superoxide dismutase activity gels as shown in Fig. 9.

**Fig. 9.** Loss of different molecular weight forms of CuZn superoxide dismutase from rat serum measured by superoxide dismutase activity gel. Left panel shows samples from a rat injected with native human superoxide dismutase (HSOD). Right panel shows samples from a rat injected with the unfractionated SODHA1 molecules. Injection and sampling conditions were as given in Fig. 8.

**DISCUSSION**

CuZn superoxide dismutases are highly stable dimers of identical subunits whose Greek key β-barrel motif is structurally similar to the immunoglobulin domain (Richardson et al., 1976). In this work, possibilities for the construction of covalently linked superoxide dismutase subunits were examined for direct and hinged connections between the C terminus of one subunit and the N terminus of a second subunit located 22 Å apart (Fig. 2). The direct connection of the solvent exposed termini represents an extreme condition for examining constraints for the design of covalently linked oligomeric enzymes. The 19-residue hinge connection allowing long connections of variable conformation between the solvent exposed termini should represent a relatively unconstrained condition.

Two topologically different possibilities can result from the two subunits linked by the IgA1 hinge: 1) a monomer forming an intramolecular system similar to the native dimer and 2) an intermolecular system consisting of a series of multimers with covalent links between the dimeric forms (Fig. 1). The results show the existence of large amounts of both the new monomeric 34,000 molecular weight form and polymeric forms measuring up to 750,000 in molecular weight. Thus, both topological possibilities are experimentally observed. The soluble polymers are unlikely to contain any free dimer interfaces because the dimer interface is hydrophobic and stabilized by strong interactions (Getzoff et al., 1986a). An exposed dimer interface would probably cause the polymer to become membrane-bound or insoluble. The smallest polymer is a dimer of two 34,000-dalton polypeptide chains. Larger polymers are probably integral multiples of these chains (Fig. 7), linked together by dimer interfaces, and circularized to prevent exposure of free dimer interfaces. The superoxide dismutase crystal structure suggests that such circularized polymers are topologically possible, without structural distortion (Fig. 2).

Approximately half of the IgA1 hinge-linked subunits underwent self-dimerization to form the 34,000-dalton monomer, and half participated in intermolecular reactions to form polymers (Fig. 7B). This indicates that the dimer interface of any given subunit has about an equal probability of participating in the intra- or intermolecular reaction. The ratio of these two interactions depends on both the protein concentration and the length and flexibility of the intramolecular hinge.

The polymerization reaction will also be affected by the complementarity of the dimer interfaces of the two linked superoxide dismutase domains. To investigate this we substituted bovine CuZn superoxide dismutase for the second human superoxide dismutase subunit using the recently cloned cDNA. If homologous dimer interfaces are preferred, the hybrid molecule would favor the formation of polymers by promoting bovine-bovine and human-human dimer interfaces between different molecules. The proportion of molecules in the monomer form did not change significantly in this construction indicating that there was no preferential interaction of homologous dimer interfaces. However, there is a very high sequence conservation of the dimer contact residues between bovine superoxide dismutase and human superoxide dismutase: the 14 residues with greater than 20 Å of buried surface in the dimer contact are all invariant, and sequence differences for residues with greater than 10 Å of buried surface occur only at positions 17 (bovine superoxide dismutase Thr versus human superoxide dismutase Ile) and 153 (bovine superoxide dismutase Lys versus human superoxide dismutase Glu), based upon analysis of the bovine superoxide dismutase crystallographic structure (Getzoff, 1982).

In antibody structures, the immunoglobulin hinge provides a partially flexible linkage between the Fab and Fc domains. Immunoglobulin hinges have a high proline content and often form polyproline helices in antibody structures. However, hinge regions can appear disordered in crystal structures of antibodies lacking the usual disulfide bridges between the two heavy chain hinge regions (Silverton et al., 1977; Marquart et al., 1980). The immunoglobulin hinge is known to provide segmental flexibility in antibodies (Huber and Bennett, 1983). It may thus be ideal for covalently joining independently folding domains without interfering with normal activity and folding. The human immunoglobulin hinge region was chosen in part for its presumed lack of immunogenicity, but it is interesting to speculate whether a much simpler sequence such as polyglycine would also have produced the polymers.

Half-life studies on the hinged HSODHA1 molecules reveal that the very rapid loss of superoxide dismutase from the circulation, presumably by filtration in the kidney, can be prevented by an increase in molecular weight from 34,000 to 68,000, which increases the half-life from 7 to about 135 min. This result is in agreement with previous studies indicating 5. I. Laria and R. A. Hallewell, unpublished results.
that the molecular sieve in the kidney glomerulus prevents the rapid removal of molecules greater than 40,000 in molecular weight (Heinemann et al., 1974). There is also evidence of a slow component in serum disappearance of the multimeric forms of superoxide dismutase, similar to that observed for the Fab fragment of immunoglobulin (Arend and Silverblatt, 1975), so that large amounts of superoxide dismutase remain in circulation 4 h after injection (Figs. 8 and 9). The failure of the immunoglobulin hinge sequence to increase the serum half-life of the 34,000-dalton monomer shows that the IgA1 hinge itself contributes very little to serum stability. Above 68,000 daltons, molecular mass does not appear to be an important limiting factor in the serum stability of superoxide dismutase, and molecules of at least 340,000 do not have an appreciably longer half-life. This leads to the conclusion that other modifications to the superoxide dismutase structure are required to increase serum half-life further. Such alterations might include the addition of specific binding sites to the molecule to increase adherence to the walls of the blood vessels or targeting of the molecule to specific cell types.

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REFERENCES

Arend, W. P., and Silverblatt, F. J. (1975) Clin. Exp. Immunol. 22, 502–513
Beauchamp, C., and Fridovich, I. (1971) Anal. Biochem. 44, 276–287
Beggs, J. D. (1978) Nature 275, 104–109
Beyer, W. F., Jr., Fridovich, I., Mullenbach, G. T., and Hallewell, R. (1987) J. Biol. Chem. 262, 11182–11187
Blundell, T., Lindley, P., Miller, L., Moss, D., Slingsby, C., Tickle, L., T urnell, B., and Wistow, G. (1981) Nature 289, 771–777
Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
Carlioz, A., and Touati, D. (1986) EMBO J. 5, 623–630
Connolly, M. L. (1983) Science 221, 709–713
Connolly, M. L., and Olson, A. J. (1985) Comput. & Chem. 9, 1–6
Connolly, M. L., and Fridovich, I. (1973) J. Biol. Chem. 248, 2645–2649
Fridovich, I. (1979) Adv. Inorg. Biochem. 1, 67–90
Getzoff, E. D. (1982) The Refined 2Å Structure of Copper, Zinc Superoxide Dismutase: Implications for Stability and Catalysis. Ph.D. dissertation, Duke University
Getzoff, E. D., Tainer, J. A., Weiner, P. K., Kollman, P. A., Richardson, J. S., and Richardson, D. C. (1983) Nature 306, 287–290
Getzoff, E. D., Tainer, J. A., and Olson, A. J. (1986a) Biophys. J. 49, 191–206
Getzoff, E. D., Hallewell, R. A., and Tainer, J. A. (1986b) in Protein Engineering: Applications in Science, Industry and Medicine (Inouye, M., ed.) pp. 41–69, Academic Press, New York
Hallewell, R. A., Masiarz, F. R., Najarian, R. C., Puma, J. P., Quiroga, M. R., Randolph, A., Sanchez-Pescador, R., Scandella, C. J., Smith, B., Steiner, K. S., and Mullenbach, G. T. (1985) Nucleic Acids Res. 13, 2017–2034
Hallewell, R. A., Mills, R., Tekamp-Olson, P., Blacher, R., Rosenberg, S., Otting, F., Masiarz, F. R., and Scandella, C. J. (1987) Biotechnology 5, 363–366
Heinemann, H. O., Maack, T. M., and Sherman, R. L. (1974) Am. J. Med. 56, 71–82
Huber, R., and Bennett, W. S. (1983) Biopolymers 22, 261–279
Klebanoff, S. J., and Clark, R. A. (1978) The Neutrophil: Function and Clinical Disorders, pp. 642–896, North-Holland Publishing Co., Amsterdam
Laemmli, U. K. (1977) Nature 227, 680–685
Lepeck, J. R., Arnold, L. D., Torrie, B. H., Andrews, B., and Kruuv, J. (1985) Arch. Biochem. Biophys. 241, 243–251
Low, T. L. K., Liu, Y. S. V., and Putnam, F. W. (1976) Science 191, 390–392
Malinowski, D. P., and Fridovich, I. (1979a) Biochemistry 18, 5055–5060
Malinowski, D. P., and Fridovich, I. (1979b) Biochemistry 18, 237–244
Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Marklund, S., and Marklund, G. (1974) Eur. J. Biochem. 47, 469–474
Marquart, M., Deisenhofer, J., and Huber, R. (1980) J. Mol. Biol. 141, 369–391
McCord, J. M. (1986) N. Engl. J. Med. 312, 159–163
McCord, J. M. (1988) J. Free Radicals Biol. Med. 4, 9–14
McCord, J. M., Stokes, S. H., and Wong, K. (1979) in Advances in Inflammation Research (Weissman, G., Samuelson, B., and Paolelli, R., eds) Vol. I, pp. 273–280, Raven Press, New York
McLachlan, A. D. (1980) Nature 285, 267–268
Messer, J. (1983) Methods Enzymol. 101, 20–78
Nativig, D. O., Imlay, K., Touati, D., and Hallewell, R. A. (1987) J. Biol. Chem. 262, 1497–14701
O’Donnell, T. J., and Olson, A. J. (1981) Comput. Graphics 15, 133–142
Petrone, W. P., English, D. K., Wong, L., and McCord, J. M. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 1159–1163
Richardson, J. S. (1981) Adv. Protein Chem. 34, 168–339
Richardson, J. S., Richardson, D. C., Thomas, K. A., Silvertone, E. W., and Davies, D. R. (1976) J. Mol. Biol. 102, 221–235
Roe, J. A., Butler, A., Scholler, D. M., Valentine, J. S., Marky, L., and Breuer, K. J. (1988) Biochemistry 27, 950–968
Sanger, F., Nicken, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
Silvertone, E. W., Navia, M. A., and Davies, D. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5140–5144
Steiner, K. S., Higgins, K. W., Powers, M. A., Stephana, J. C., Gynes, A., George-Nascimento, C., Luciw, P. A., Barr, P. J., Hallewell, R. A., and Sanchez-Pescador, R. (1986) J. Virol. 54, 9–16
Tainer, J. A., Getzoff, E. D., Beem, K. M., Richardson, J. S., and Richardson, D. C. (1982) J. Mol. Biol. 160, 181–217
Tainer, J. A., Getzoff, E. D., Richardson, J. S., and Richardson, D. C. (1983) Nature 306, 284–287
Urdea, M. S., Merryweather, J. P., Mullenbach, G. T., Coit, D., Heberlein, U., Valenzuela, P., and Barr, P. J. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 7461–7465
Weiss, P. K., Langridge, R., Blaney, J. M., Schaefer, R., and Kollman, P. A. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 3754–3758
Weiss, J. J., Lampert, M. B., and Test, S. T. (1985) Science 222, 625–629

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