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THERMOSTABILIZATION OF RECOMBINANT HUMAN AND BOVINE CuZn SUPEROXIDE DISMUTASES BY REPLACEMENT OF FREE CYSTEINES

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SUMMARY Human CuZn superoxide dismutase (HSOD) has two free cysteines: a buried cysteine (Cys6) located in a β-strand, and a solvent accessible cysteine (Cys111) located in a loop region. The highly homologous bovine enzyme (BSOD) has a single buried Cys6 residue. Cys6 residues in HSOD and BSOD were replaced by alanine and Cys111 residues in HSOD by serine. The mutant enzymes were expressed and purified from yeast and had normal specific activities. The relative resistance of the purified proteins to irreversible inactivation of enzymatic activity by heating at 70°C was HSOD Ala6 Ser111 > BSOD Ala6 Ser109 > BSOD Cys6 Ser109 (wild type) > HSOD Ala6 Cys111 > HSOD Cys6 Ser111 > HSOD Cys6 Cys111 (wild type). In all cases, removal of a free cysteine residue increased thermostability.

CuZn superoxide dismutases (SODs) are highly stable enzymes, predominantly found in the peroxisomes of eukaryotes (1), which protect cells against the toxic effects of the superoxide radical produced as a by-product of aerobic metabolism (2). Each molecule is a dimer of identical subunits and each subunit contains about 153 amino acids and one Cu and one Zn ion. The enzyme has a flattened Greek key β-barrel structural motif consisting of eight antiparallel β-strands joined by seven turns or loops (3). The CuZn SODs are unusually stable proteins: bovine CuZn superoxide dismutase (BSOD) remains active in 8M urea (4) and 4% sodium dodecyl sulphate (5) and has a conformational melting temperature of 89-104°C, depending on the heating rate, the pH, and the oxidation state of the Cu ion (6,7). Human CuZn superoxide dismutase (HSOD) has two free cysteine residues (Table I). The bovine crystal structure indicates that Cys6 is located adjacent to the dimer interface with the side chain pointed toward the interior of the β-barrel (3). Thus, the sulfhydryl is probably inaccessible to solutes at physiological temperatures. The second cysteine (Cys111) is, at present, unique to humans (8). It is solute accessible and reactive, and it often becomes blocked during purification (9). The single disulfide bond between cysteine residues Cys57 and Cys146 (HSOD numbering) is conserved in all fifteen species whose residues have

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been aligned (8). Reduction of the disulfide bond causes a large loss of protein stability (10). Here we report the expression and purification of BSOD and HSOD from yeast, the effects on enzyme stability of replacing buried or solvent accessible cysteines in HSOD, and the comparative effect of replacing the homologous buried Cys6 in HSOD and BSOD.

MATERIALS AND METHODS

Expression of Bovine SOD in Yeast and Construction of Mutants

A full length BSOD cDNA was cloned, sequenced, and used for oligonucleotide directed *in vitro* mutagenesis, as described previously (11,12), to insert all the restriction sites shown in Fig. 1. The gene encoding the BSOD Cys6 to Ala6 mutant was made by substituting double-stranded synthetic DNA between the NcoI and BstEII sites of the BSOD cDNA shown in Fig. 2. The NcoI-SalI fragments containing BSOD or HSOD coding sequences were expressed in yeast strain 2150-2-3 *leu*$^-$ using the pC1/1PGAP plasmid, as described previously (13). The mutant HSOD cDNAs encoding Cys6 to Ala6 and Cys111 to Ser111 were made as described previously (11,12) and the double mutant made by utilising the unique StuI site that lies between the codons for amino acids 6 and 111. To ensure that no errors had occurred in the mutagenesis or cloning, the mutant HSODs were re-sequenced from plasmid DNA prepared from the yeast strains.

Purification of Wild Type and Mutant SODs

A 10 litre culture of each yeast strain was grown, harvested, and lysed in 20 mM Tris-HCl pH 8, as described previously (12,13). The lysate was centrifuged, and the pellet washed twice with buffer. The washes were pooled with the original supernatant. After centrifugation, 1 liter of extract was obtained (approximately pH 6), and stored at -20°C. The extract was thawed and heated for 2 hr at 65°C. After centrifugation to remove the precipitated protein and debris, 850 ml of clarified extract was obtained and adjusted to pH 8 with NaOH. Buffer was added to a final volume of 8.5 liters, resulting in a conductivity of 1.2 mmho. This was loaded onto a 400 ml column of DEAE-sepharose CL-6B (Pharmacia). The column was washed with the same buffer, and eluted with a gradient up to 0.15 M NaCl in the Tris-HCl buffer. The peak was collected in 3 pools corresponding to 10-35 mM, 35-50 mM, and 50-65 mM NaCl. The pools were dialyzed against distilled water, sterile filtered, and stored in aliquots at -20°C. Protein concentrations were measured by the Coomassie method (14) and by dry weight determination, and the lower of the two values taken. Samples loaded on SDS-polyacrylamide gels were reduced by heating for 3 min. at 100°C in gel loading buffer (15) containing 20 mM dithiothreitol prior to loading on the gel.

Determination of Free Cysteine

DTNB (5,5'-dithiobis(2-nitrobenzioc acid)) was reacted with SODs at room temperature at pH 7.3 in 3mls containing: 150 μg/ml SOD, 100 mM sodium phosphate, 2 mM EDTA, 0.67 mM DTNB, and 6M guanidine chloride (for determination of free cysteines under denaturing conditions). Reactions were initiated by addition of DTNB, and the A_{412} measured at 10 minute intervals until no increase in absorbance was observed. Extinction coefficients under the same native and denaturing conditions were determined using L-cysteine (16,17).

Thermostability Assays

Heating reactions (1 ml) contained 200 μg/ml (6.25μM) of a freshly thawed aliquot of SOD in 100 mM sodium phosphate pH 7.8. Tubes were pre-incubated for a few minutes at 70°C prior to addition of SOD. Samples were removed to ice at the times indicated in Fig. 5 and assayed for residual activity at 20°C using the pyrogallol method (18).

RESULTS

Construction of Mutants and Expression in Yeast

Based on the conservative, naturally occurring replacements for free cysteine residues in the bovine and yeast sequences (8), the human and bovine mutants shown in Table I were made by *in vitro* mutagenesis of the human and bovine cDNAs (see Methods). To make wild type BSOD

Table I. Cysteine residues in native and mutant CuZn SODs. Residues are numbered by alignment with the human sequence (8); there is a 2 amino acid deletion in BSOD after C6 so that S111 is S109 in BSOD numbering. There is a disulfide bond between the conserved cysteine residues C57 and C146.

Species	Free Cysteines	Total Cysteines			
		6	57	111	146
Human	HSOD C6 C111 (wild type)	Cys	Cys	Cys	Cys
	HSOD C6 S111	Cys	Cys	Ser	Cys
	HSOD A6 C111	Ala	Cys	Cys	Cys
	HSOD A6 S111	Ala	Cys	Ser	Cys
Bovine	BSOD C6 S109 (wild type)	Cys	Cys	Ser	Cys
	BSOD A6 S109	Ala	Cys	Ser	Cys

(C6S109) and the BSOD A6S109 mutant, we first cloned the BSOD cDNA using the HSOD cDNA (20) as a hybridization probe. The sequence of the cDNA is shown in Fig. 1 together with new restriction sites placed in the sequence using M13 site-directed mutagenesis. The NcoI and SalI sites facilitated the yeast expression and the BstEII site facilitated construction of the BSOD A6S111 mutant by cassette mutagenesis as described in Methods. Native BSOD and all the mutant SODs were expressed in yeast from the glyceraldehyde 3-phosphate dehydrogenase promoter carried on the pC1/1 yeast shuttle plasmid (see methods). All the yeast strains accumulated about 30% of total cellular protein as recombinant SOD.

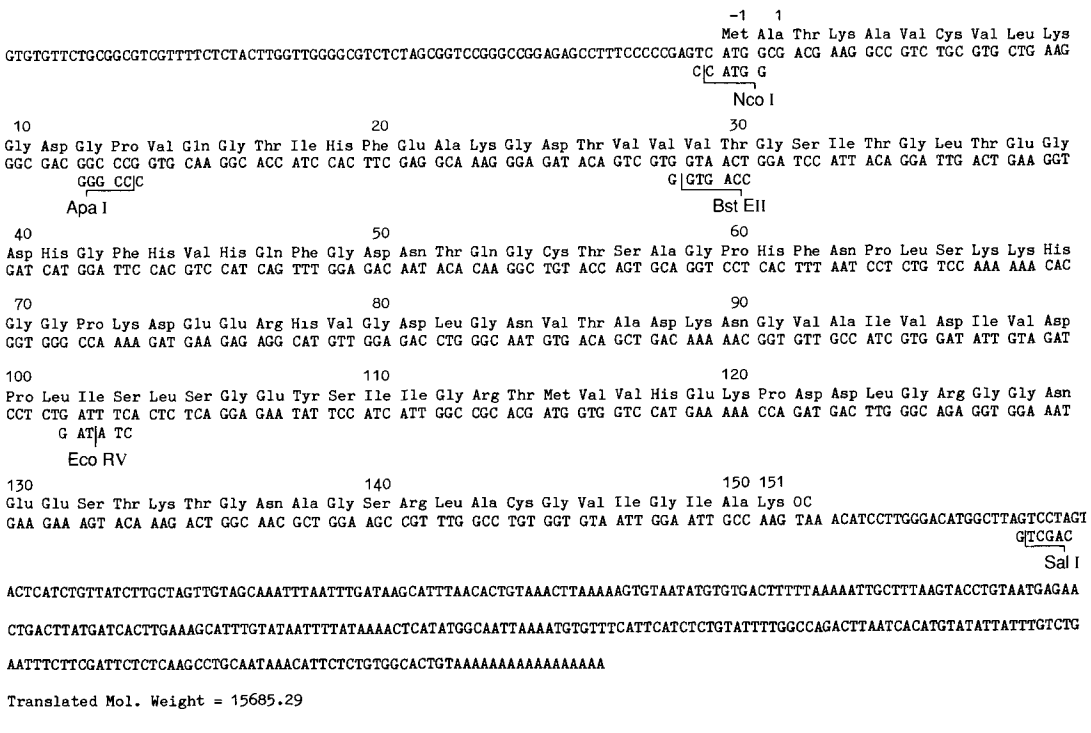

Fig. 1. cDNA sequence of bovine CuZn SOD and predicted amino acid sequence. The restriction sites shown underneath the sequence were inserted using M13 site-directed *in vitro* mutagenesis.

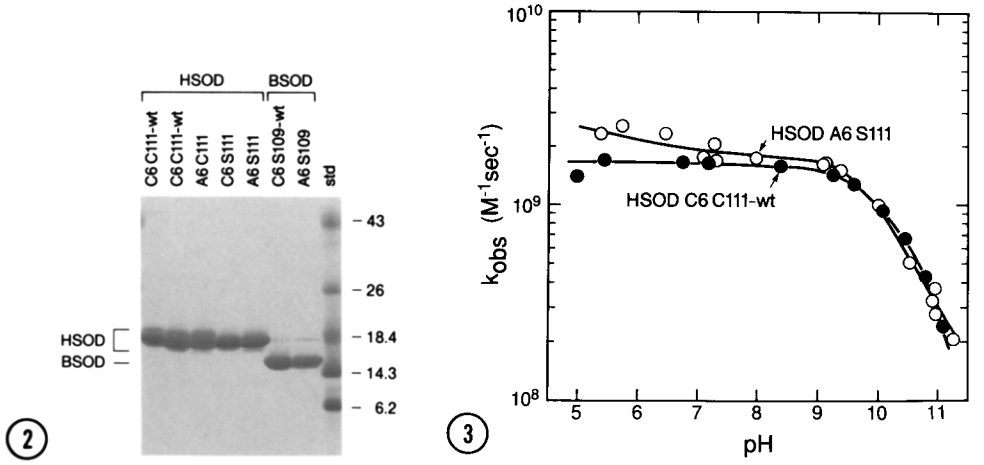

Fig. 2. SDS-10-20% polyacryamide gel stained with Coomassie blue showing purification of recombinant wild type and mutant SODs. Lanes 1 and 2 contain wild type HSOD from erythrocytes and yeast, respectively. All other lanes contain mutant SODs made in yeast. 10 µg of protein was loaded in each lane.

Fig.3. Reaction rates vs. pH of native HSOD C6C111-wt and HSOD A6S111 measured using pulse radiolysis (19).

Purification and Characterization of Mutants

An SDS-polyacrylamide gradient gel of purified wild type and mutant proteins is shown in Fig. 2. Wild type HSOD from human erythrocytes (lane 1) and from yeast (lane 2) were purified by conventional methods (12). The mutant SODs were purified by the rapid, heat-step procedure described in Methods. All HSODs containing the C111 residue show multiple bands in Fig. 2 whereas all SODs lacking this residue show predominantly a single band. This suggests that the C111 residue is partially blocked after purification by the conventional or rapid methods, and implies that multiple blocking groups are present. Note that gradient rather than linear SDS-polyacrylamide gels are required to detect the multiple bands in C111 containing HSODs. The specific activity of all of the mutant SODs appeared to be approximately the same as their wild type parental enzymes, as measured by the pyrogallol method at pH 7.8 (18). The reaction rates of HSOD A6S111 and wild type HSOD C6C111 were the same over a broad pH range (Fig. 3).

To confirm the free cysteine content of the mutants and to quantitate the fraction of blocked C111 residues, the free cysteine content of both native and denatured SODs was measured using the DTNB (5,5'-dithiobis (2-nitrobenzoic acid)) method (21) as shown in Table II. As expected, HSOD A6S111 and BSOD A6S109 had no DTNB reactive residues in the native or denatured protein. HSOD C6S111 and wild type BSOD C6S109, which contain only buried cysteine, each had one fully reactive cysteine residue, but only when denatured with guanidine hydrochloride. The solvent accessible C111 residue of both wild type HSOD C6C111 and HSOD A6C111 was 75-90% blocked, as measured by DTNB reaction.

Thermostability of Native and Mutant SODs

The thermostability of the SODs was determined by activity assays following heat treatment at 70°C in 100 mM sodium phosphate pH 7.8 (Fig. 4 and METHODS). Heating did not cause

Table II. Free cysteine residues per subunit of wild type or mutant human and bovine CuZn SOD. Free cysteine content was determined using the DTNB method (21). Proteins were denatured in 6M guanidine hydrochloride.

Free Cysteines	Measured Free Cysteine Residues per SOD Subunit	
	Native	Denatured
HSOD C6C111-wt (erythrocyte)	0.25	1.13
HSOC C6C111-wt (yeast)	0.12	1.17
HSOD C6S111	0.00	1.00
HSOD A6C111	0.08	0.08
HSOD A6S111	0.09	0.11
BSOD C6S109-wt	0.08	0.81
BSOD A6S109	0.00	0.00

significant loss of protein, as measured using the Coomassie method (14), indicating that loss of activity was not due to SOD precipitating or adhering to the tube. Heat inactivation was not easily reversible: enzyme activity lost by heating was not regained by storage at 5°C for 24 hrs. Half-times of inactivation are: 21 min. for HSOD C6C111-wt; 34 min. for HSOD C6S111; 51 min. for HSOD A6C111; 89 min. for BSOD C6S109-wt; 125 min. for BSOD A6S109; >180 min. for HSOD A6S111. Thus, in all cases, removal of a free cysteine residue increased thermostability.

DISCUSSION

In this work we have shown that replacement of the buried or surface exposed free Cys residues in HSOD or BSOD increases the stability of the enzyme activity to irreversible thermal inactivation. Similarly, mutations that remove free thiols from T4 lysozyme lead to increased resistance to thermal inactivation (22). The results suggest that strategies for stabilizing proteins should include removal of any free thiols by conservative replacement of the Cys residues. In both

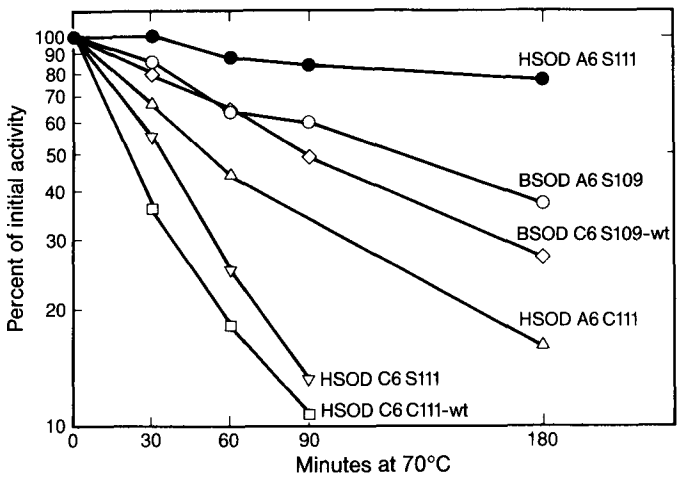

Fig. 4. Effect of heating on enzyme activity of wild type and mutant HSODs and BSODs. The experiment was repeated three times with similar results.

HSOD and BSOD there is no loss of catalytic activity upon conservative replacement of free Cys with homologous residues chosen from a sequence alignment of SODs from 15 species (8).

We have recently shown, using differential scanning calorimetry techniques, that the order of resistance to thermal inactivation of the SODs is the same as the order of their ability to refold correctly after unfolding (23,24). In contrast, the conformational stability of the proteins is only slightly increased for HSOD mutants and significantly decreased for the BSOD mutant where the transition temperature for unfolding of wild type BSOD is 88.3°C versus 84.1°C for the mutant with Cys6 replaced by Ala (23,24). Thus, the greater resistance to thermal inactivation of the SODs with free Cys replaced by Ala or Ser is largely due to their increased ability to refold correctly after unfolding, and not to an increase in conformational stability. Since our activity assay to determine thermostability was done at room temperature, allowing refolding to occur after a timed exposure to 70°C, it essentially reflects irreversible denaturation. We are currently investigating the mechanism for such denaturation to determine if it is due to the formation of incorrect intermolecular or intramolecular disulfide bonds that block correct refolding or to another process.

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