Chimeras of human extracellular and intracellular superoxide dismutases. Analysis of structure and function of the individual domains

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Heterologous expression/protein fusion/subunit interaction

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Introduction

Aerobic organisms have developed an array of defense mechanisms against oxygen free radicals, important components of which are superoxide dismutases (SODs). These metalloenzymes are present in most aerobic organisms and serve to catalyze the dismutation of superoxide radicals. In all mammalian cells, two different types of CuZn-containing SOD enzymes are produced, one intracellular (CuZnSOD) and one extracellular form (EC-SOD). In humans most EC-SOD is found in extracellular matrices, associated with heparan sulfate proteoglycans, and much smaller amounts are found in plasma, lymph, synovial fluid and cerebrospinal fluid. Every biological macromolecule can serve as a target for the damaging action of the abundant oxygen radical. A wide range of degenerative processes and diseases have also been coupled with the action of oxygen radicals such as aging (Orr and Sohal, 1994), reperfusion damage of ischemic tissue (Hatori et al., 1992), oncogenesis, inflammatory actions (Fridovich, 1986) and motor neurone degeneration (McCord, 1994). Interest has therefore evolved in the therapeutic potential of SOD enzymes and a wide range of clinical applications have been suggested.

Extra- and intra-cellular Cu- and Zn-containing SOD enzymes catalyze the dismutation reaction with about the same efficiency and have very similar kinetic parameters. The central part of the human (h)EC-SOD sequence is clearly homologous with the sequence of the intracellular hCuZnSODs. However, hEC-SOD, in contrast to intracellular hCuZnSOD, is a glycoprotein and it contains one N-terminal and one C-terminal domain with no counterparts in intracellular hCuZnSOD.

The three-dimensional structure of CuZnSOD is well known (Tainer et al., 1982) but determination of the three-dimensional structure of EC-SOD by X-ray crystallography has failed owing to difficulties in crystallizing the protein. The cause of this failure is probably the heterogeneous glycosylation of hEC-SOD. A variant lacking the glycosylation site has been produced, but its solubility was found to be too low for the protein to be useful for crystallization (Edlund et al., 1992). The protein is unfortunately too large for structure determination by NMR. With the exception of the first 10 amino acids, the sequence of the central domain of hEC-SOD (amino acids 60–194) can be modeled on the structure of hCuZnSOD (Figure 1B).

One of the characteristic properties of hEC-SOD is its binding to sulfated glycosaminoglycans such as heparin and to heparan sulfate (Marklund, 1982). The C-terminal domain of EC-SOD has been shown to be essential for this binding (Inoue et al., 1990; Sandstrom et al., 1992).

Further, we showed recently that a fusion protein composed of the C-terminal domain from hEC-SOD fused to human carbonic anhydrase II (HCAII), forms a monomeric protein that binds to heparin-Sepharose with approximately the same affinity as hEC-SOD. Previous NMR results demonstrate that the C-terminal domain of both the fusion protein and hEC-SOD moves independently from the rest of the protein and that the central part of the domain is involved in conformational exchange (Tibell et al., 1997).

hEC-SOD is a tetrameric protein whereas intracellular hCuZnSOD is a dimer. It has been proposed by Carlsson et al. (1996) that the tetrameric hEC-SOD is held together by the N-terminal domain as a dimer of two dimers, which in turn is held together by a dimer contact area analogous to that found in the dimeric intracellular hCuZnSOD. In CuZnSOD, the dimer interface is located close to where the N- and C-termini protrude from the globular protein. If we assume that the central part of hEC-SOD (residues 49–196) is structurally homologous to hCuZnSODs, the N- and C-terminal domains of hEC-SOD must be folded close to each other (Figure 1A) and, at least partly, occupy the area corresponding to the subunit interaction surface in hCuZnSOD. Despite strong similarities between the sequences of hEC-SOD and hCuZnSOD, many of the residues that are involved in the dimer contacts and which are evolutionarily conserved in the...
CuZnSODs are not conserved in hEC-SOD. This is true especially for the regions that correspond to the N- and C-termini of CuZnSODs (Tibell et al., 1996). Earlier results from our laboratory (Tibell et al., 1996; Stenlund et al., 1997) have shown that the principal component in the formation of tetramers in hEC-SOD is the N-terminal domain.

Studies of structural and functional relationships of the different domains of EC-SOD require genetic engineering and mutagenesis, which are far more easy to achieve in prokaryotic than in eukaryotic systems. However, the exploration of hEC-SOD has been partly hindered by its limited availability. Since the production of hEC-SOD in CHO cells gives relatively small amounts of the protein (Tibell et al., 1987), considerable effort has been devoted, without success, to the expression of hEC-SOD in prokaryotes such as Escherichia coli. Attempts to produce the enzyme in yeast have also been discouraging. Interestingly, expression of the intracellular hCuZnSOD in E.coli (Hartman et al., 1986) and yeast (Hallewell et al., 1987) gives high yields of protein. We report here the production and characterization of two fusion proteins, which is part of a project designed to overcome some of these problems. One of these proteins comprises the N-terminus of hEC-SOD fused to the N-terminus of hCuZnSOD (FusNCZ) and the other comprises the N-terminal and C-terminal domains of hEC-SOD fused to the N- and C-termini of hCuZnSOD, respectively, resulting in a mimic of hEC-SOD (PseudoEC-SOD). In this study we also compared the characteristics of the fusion proteins and hEC-SOD. In addition, a number of mutated forms of PseudoEC-SOD were used to elucidate the nature of different domains of EC-SOD require genetic engineering and mutagenesis, which are far more easy to achieve in prokaryotic than in eukaryotic systems. However, the exploration of hEC-SOD has been partly hindered by its limited availability. Since the production of hEC-SOD in CHO cells gives relatively small amounts of the protein (Tibell et al., 1987), considerable effort has been devoted, without success, to the expression of hEC-SOD in prokaryotes such as Escherichia coli. Attempts to produce the enzyme in yeast have also been discouraging. Interestingly, expression of the intracellular hCuZnSOD in E.coli (Hartman et al., 1986) and yeast (Hallewell et al., 1987) gives high yields of protein. We report here the production and characterization of two fusion proteins, which is part of a project designed to overcome some of these problems. One of these proteins comprises the N-terminus of hEC-SOD fused to the N-terminus of hCuZnSOD (FusNCZ) and the other comprises the N-terminal and C-terminal domains of hEC-SOD fused to the N- and C-termini of hCuZnSOD, respectively, resulting in a mimic of hEC-SOD (PseudoEC-SOD). In this study we also compared the characteristics of the fusion proteins and hEC-SOD. In addition, a number of mutated forms of PseudoEC-SOD were used to elucidate the nature of the subunit interaction of hEC-SOD and the role of the dimer contact surface present in intracellular hCuZnSOD.

Results and discussion

Expression and isolation of FusNCZ and PseudoEC-SOD

The fusion proteins FusNCZ and PseudoEC-SOD (Figure 2) were produced in E.coli resulting in high yields (Figure 3, lanes 1 and 7). After cell disruption, essentially all of the enzyme remained in the supernatant (Figure 3, lanes 2 and 8), indicating that the fusion proteins are highly soluble in bacteria. Moreover, almost all of the detected SOD activity was sensitive to 1 mM KCN, indicating that the activity corresponded to CuZnSOD and not to bacterial SOD enzymes.

Isolation procedures for the fusion proteins were developed, an example of which is given for PseudoEC-SOD in Table I. The isolation of FusNCZ followed the same scheme with the exception that the heparin-Sepharose step was replaced with a phenyl-Sepharose chromatographic step.

PseudoEC-SOD was purified by a four-step purification method with high yield (Table I). An initial heat incubation efficiently removed contaminating proteins with almost no loss of fusion protein (Figure 3, lane 3). The subsequent chromatographic steps mainly removed specific protein contaminants without any dramatic increase in specific activity. However, size-exclusion chromatography on Superose 12 removed the other major contaminant, nucleic acids (Figure 3, lane 4). The increase in yield to over 100% after the ion-exchange chromatographic step might reflect stabilization of the protein or removal of inhibitory agents.

Both intracellular and extracellular CuZnSOD enzymes are heat stable to 60–70°C (Tibell et al., 1993) and the heat treatment provided a very efficient purification step with high recovery (Table I). To assess whether the heat treatment changed any of the characteristics of the fusion proteins, all proteins and mutated variants were isolated in two parallel alternative purification sequences, one with and one excluding the heat-incubation step. When the properties of the resulting proteins were compared, no difference in physico-chemical properties [as measured by specific activity, size-exclusion chromatography, circular dichroism (CD) and heparin affinity] could be detected for any of the proteins (data not shown). However, when the heat incubation step was excluded, small amounts of contaminant remained in the sample.

During the production and isolation of the fusion proteins, three different problems were identified. The copper content in the resulting fusion proteins was often insufficient and the copper was often apparently replaced by zinc. Partial proteolysis of PseudoEC-SOD (especially of the C-terminal domain) often occurred and became more pronounced on prolonged...
A chimeric extracellular superoxide dismutase mimic

Table I. Purification protocol for PseudoECSOD

<table>
<thead>
<tr>
<th>Isolation step</th>
<th>Total activity (U)</th>
<th>Total protein (total A280)</th>
<th>Specific activitya</th>
<th>A280/A260 (%)</th>
<th>Total recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant after cell disruption</td>
<td>3800</td>
<td>2200</td>
<td>1.7</td>
<td>1.5</td>
<td>100</td>
</tr>
<tr>
<td>Incubation at 60°C</td>
<td>3796</td>
<td>223</td>
<td>17</td>
<td>0.4</td>
<td>99</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>5820</td>
<td>201</td>
<td>29</td>
<td>0.5</td>
<td>136</td>
</tr>
<tr>
<td>Heparin-Sepharose</td>
<td>4927</td>
<td>38</td>
<td>77</td>
<td>0.6</td>
<td>116</td>
</tr>
<tr>
<td>Superose 12</td>
<td>2814</td>
<td>34</td>
<td>83</td>
<td>1.4</td>
<td>89</td>
</tr>
</tbody>
</table>

a Estimated from pyrogallol assay measurements. 1 U = amount SOD required for 50% inhibition.
b Total activity (U)/total A280.

Table II. A comparison of physico-chemical properties of fusion and native proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular mass (kDa)</th>
<th>pI</th>
<th>Extinction coefficientc</th>
<th>Heparin NaCl (M)d</th>
<th>Antibody reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native Subunit</td>
<td>(SDS–PAGE)</td>
<td>Predicteda</td>
<td></td>
<td>Anti-hCuZnSOD</td>
</tr>
<tr>
<td>FusNCZ</td>
<td>124</td>
<td>26</td>
<td>21.37</td>
<td>21.30</td>
<td>4.6</td>
</tr>
<tr>
<td>Pseudo-ECSOD</td>
<td>132</td>
<td>30</td>
<td>24.55</td>
<td>24.56</td>
<td>5.6</td>
</tr>
<tr>
<td>V24D/F50E/G51E</td>
<td>132</td>
<td>30</td>
<td>24.6</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>hCuZnSOD</td>
<td>151</td>
<td>32</td>
<td>26.66</td>
<td>N/A</td>
<td>4.6</td>
</tr>
<tr>
<td>hrEC–SOD</td>
<td>32</td>
<td>19</td>
<td>15.80</td>
<td>15.83</td>
<td>5.7</td>
</tr>
</tbody>
</table>

a Calculated from amino acid sequences (and oligosaccharide sequence in the case of hrEC–SOD).
b As measured by mass spectrometry.
c Measured as described by Gill and von Hippel (1989).
d Elution concentration of NaCl from heparin-Sepharose.
e Purified by heparin-Sepharose chromatography as pseudoEC-SOD.

Table III. Activity and metal content in SOD enzymes

<table>
<thead>
<tr>
<th>Protein</th>
<th>% of ECSOD activitya</th>
<th>% Cu2+</th>
<th>% Zn2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>hEC-SOD</td>
<td>100</td>
<td>95</td>
<td>169</td>
</tr>
<tr>
<td>Pseudo-ECSOD</td>
<td>49</td>
<td>56</td>
<td>71</td>
</tr>
<tr>
<td>FusNCZ</td>
<td>48</td>
<td>41</td>
<td>131</td>
</tr>
<tr>
<td>V24D/F50E/G51E</td>
<td>1.5</td>
<td>9</td>
<td>120</td>
</tr>
</tbody>
</table>

Metal contents were determined by electrothermal atomic absorption spectrometry in a Perkin-Elmer Zeeman 330 + HGA instrument.
a Pyrogallol activity assay.

The first problem was alleviated by altering the copper and zinc concentrations in the growth medium (Table III) to 3 mM copper and 30 µM zinc. Coexpression with the human copper chaperone for SOD (CCS) (Culotta et al., 1997) might be an alternative to improve further the in vivo incorporation of copper. Addition of protease inhibitors and heat treatment during the isolation process reduced the proteolysis but did not prevent it completely. We conclude that the C-terminal domain is most sensitive to proteolysis since prolonged storage of PseudoEC-SOD often resulted in loss of heparin affinity. The observation that no proteolysis problems were observed in the FusNCZ protein, which does not contain the C-terminal domain, supports this conclusion. Contaminating nucleic acid was degraded by addition of DNase and RNase to the homogenization buffer (a second addition was made after the initial heat incubation if this step was applied). Neither the ion-exchange nor the heparin-Sepharose chromatographic step separated the nucleic acid fragments from the fusion proteins. Hydrophobic chromatography on phenyl-Sepharose partly removed the nucleic acid fragments but size-exclusion chromatography was the most efficient method for removal of nucleic acids (Table I).

The heparin-Sepharose chromatographic step cannot be used for the purification of FusNCZ, since this fusion protein lacks the heparin-binding C-terminal domain (Figure 1). An alternative purification step with phenyl-Sepharose was therefore developed.

Characterization of the proteins

A number of analyses were carried out to verify the identity of the fusion proteins. Amino acid sequencing of the N-terminus of FusNCZ and PseudoEC-SOD was confirmed by N-terminal sequencing (not shown). The fusion proteins reacted with antibodies directed against hCuZnSOD and with antibodies directed against hEC-SOD (Table I).

One of the characteristic properties of hEC-SOD is its binding to sulfated glycosaminoglycans such as heparan sulfate and heparin and to heparin-Sepharose (Marklund, 1982; Karlsson et al., 1988). Several reports have shown that the C-terminal domain of EC-SOD is essential for binding to heparin (Inoue et al., 1990; Sandstrom et al., 1992; Tibell et al., 1997). The affinity of the proteins for heparin-Sepharose, as determined by the NaCl concentration needed to elute them from a heparin-Sepharose column, is compared in Table II. PseudoEC-SOD was shown to elute at an NaCl concentration of 0.45 M, which is lower than that needed for the elution of hEC-SOD, 0.55 M (Table II).

The specific activities of the fusion proteins were approximately the same for PseudoEC-SOD as for hCuZnSOD and hEC-SOD if the occupancy of Cu and Zn in the metal sites is taken into account (Table III) since the measured activity is...
results imply that the N-terminal domain contributes fusion proteins is absent in the spectrum of hCuZnSOD. These the shoulder at ~222 nm in the spectra of hEC-SOD and the wavelengths in the fusion proteins and hEC-SOD. Furthermore, hCuZnSOD spectrum is more intense and shifted to lower hCuZnSOD spectrum at ~190 nm, to a band at ~194 nm as in slight shift of the positive band, which has a maximum in the corresponding spectrum of hEC-SOD. When compared with of FusNCZ and Pseudo-ECSOD are very similar to the of hCuZnSOD in the tetramerization of the fusion proteins. However, the role of the dimer interaction of hCuZnSOD to the dimeric hCuZnSOD protein induces tetramerization. This clearly shows that fusion of the N-terminal domain of hEC-SOD to the dimeric hCuZnSOD protein induces tetramerization of the fusion proteins. However, the role of the dimer interaction of hCuZnSOD in the tetramerization of the fusion proteins cannot be determined from these results.

The CD spectra of PseudoEC-SOD and Fus NCZ were recorded in the far-UV (Figure 4) and near-UV (results not shown) regions and compared with the corresponding spectra for hCuZnSOD and hEC-SOD. The spectra in the far-UV region (Figure 4) indicate a well defined secondary structure for all four proteins. As can be seen in Figure 4, the spectra of FusNCZ, Pseudo-ECSOD and EC-SOD are tetramers, whereas hCuZnSOD is a dimer. The CD spectra of hCuZnSOD (F50E/G51E ) was reported to have ~10% of the specific activity of the native form (Bertini et al., 1994). This seems to be the case also for the PseudoEC-SOD mutant V24D/ F50E/G51E (Table III) if the copper content is considered.

The isoelectric point for PseudoEC-SOD was about one pH unit higher than for hEC-SOD, which probably reflects the higher pI for the fused hCuZnSOD.

Molecular masses of the purified fusion proteins were determined by mass spectrometry (Table II). These values matched the predicted molecular masses well, while the apparent subunit molecular masses of the fusion proteins, as measured by SDS–PAGE, were 28 and 30 kDa for FusNCZ and PseudoEC-SOD, respectively, and deviated about 22% from the predicted molecular masses (Table II). This deviation is in the same range as that reported earlier for hCuZnSOD (Table II and as reported by Hartman et al., 1986). The results from analysis of the native molecular mass by size-exclusion chromatography show that FusNCZ, PseudoEC-SOD and EC-SOD are tetramers, whereas hCuZnSOD is a dimer. The explanation of this most probably is that the tryptophan residues are mobile and therefore do not contribute to the spectra (results not shown).

In summary, we have reported here, for the first time, the successful production and purification of an hEC-SOD analogous protein (PseudoEC-SOD) in E.coli, with characteristics closely resembling those of hEC-SOD. This protein can be produced in large quantities and can easily be genetically manipulated. Hence PseudoEC-SOD might be a very helpful tool for further investigation of the action of EC-SOD and might have interesting medical applications.

Introduction of mutations in subunit interaction interfaces

Our previous studies have shown that fusion of the N-terminal domain of hEC-SOD with the monomeric protein human carbonic anhydrase resulted in a tetrameric fusion protein (Tibell et al., 1996). The hydrophobic side of a predicted amphipathic α-helix (formed by residues 14–32) in this N-terminal domain was shown to be essential for the subunit interaction in this hybrid protein (Stenlund et al., 1997). To investigate the relevance of these results in a protein with very close structural and functional resemblance to hEC-SOD, five mutant forms of PseudoEC-SOD were constructed. In three of the mutant forms, amino acid residues on the hydrophobic side of the predicted α-helix at positions 17, 24 and 31 (I17D, V24D and V31D) were altered. In a fourth mutant variant, amino acid residues 50 and 51 in the hCuZnSOD sequence were altered (F50E/G51E). These mutations are at the dimer interface of hCuZnSOD and have been shown earlier to produce a monomeric species of hCuZnSOD (Bertini et al., 1994). In the fifth mutant variant, amino acid residue 24 in the N-terminal domain and amino acid residues 50 and 51 in the hCuZnSOD part of the fusion protein (V24D/ F50E/G51E) were altered. This variant combines mutations that have been shown to disrupt subunit interactions in N-terminal fusion proteins and hCuZnSOD. The five mutant variants of PseudoEC-SOD were produced, isolated and characterized by size-exclusion chromatography (Table IV) and CD spectroscopy (Figure 5).

The mutations in positions 24 and 31 in the α-helix of the N-terminal domain resulted in the formation of dimeric proteins. The corresponding mutations in the fusion protein with HCAII have been shown earlier to form a mixture of monomers

<table>
<thead>
<tr>
<th>Fusion protein</th>
<th>Mutation</th>
<th>Monomer</th>
<th>Dimer</th>
<th>Tetramer</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCuZnSOD</td>
<td></td>
<td>×</td>
<td>×</td>
<td></td>
</tr>
<tr>
<td>hEC-SOD</td>
<td></td>
<td>×</td>
<td>×</td>
<td></td>
</tr>
<tr>
<td>FusNCZ</td>
<td></td>
<td>×</td>
<td>×</td>
<td></td>
</tr>
<tr>
<td>PseudoEC-SOD</td>
<td>I17D</td>
<td>×</td>
<td>×</td>
<td></td>
</tr>
<tr>
<td></td>
<td>V24D</td>
<td>×</td>
<td>×</td>
<td></td>
</tr>
<tr>
<td></td>
<td>V31D</td>
<td>×</td>
<td>×</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F50E/G51E</td>
<td></td>
<td></td>
<td>×</td>
</tr>
<tr>
<td></td>
<td>V24D/F50E/G51E</td>
<td>×</td>
<td>×</td>
<td></td>
</tr>
</tbody>
</table>
and dimers (Stenlund et al., 1997). The mutation in position 17 of the N-terminal domain resulted in a pure tetramer in PseudoEC-SOD but, as described earlier, in 70% tetramer and 30% monomer in the HCAII fusion (Stenlund et al., 1997). Hence the subunit interaction seems to be somewhat more stable in the PseudoEC-SOD than in the fusion proteins with carbonic anhydrase. This conclusion is supported by the CD results since, according to the CD spectra in the far-UV region (Figure 5), the secondary structure seems to be less disturbed in the PseudoEC-SOD mutants than in the corresponding mutants of the HCAII fusion with the N-terminal domain (Stenlund et al., 1997). An explanation of these observations could be that the central hCuZnSOD-domain in PseudoEC-SOD has a stabilizing effect on the N-terminal domain. Alternatively, the dimer interface of the hCuZnSOD part of PseudoEC-SOD can become functional when the N-terminal interactions are perturbed. However, on comparing the subunit interaction region in hCuZnSOD with the corresponding residues in the modeled central domain of hEC-SOD (Figure 6), several of the residues shown to be involved in the dimer interaction, which are conserved among CuZnSOD enzymes, are not conserved in hEC-SOD (Tibell et al., 1996). Inspection of the models shows that these residues are predominantly found close to the area where the N- and C- termini protrude in CuZnSOD and where the N- and C-terminal domains in hEC-SOD most likely fold (Figures 1 and 6). Therefore, the possible interactions that involve direct subunit contacts between the central domains of hEC-SOD might be less extensive than observed for the well characterized subunit contacts in hCuZnSOD. To test further whether the interactions between the N-terminal domains are sufficient for the formation of stable tetramers, we decided to destroy the hCuZnSOD-dimer contact surface in PseudoEC-SOD. We chose the F50E/G51E double mutation because it has been shown to give monomeric hCuZnSOD (Bertini et al., 1994). The resulting PseudoEC-SOD variant was purely tetrameric (Table IV), strongly indicating that the interactions between the N-terminal domains are sufficient for the formation of stable tetramers. The mutation (V24D) in the N-terminal domain disturbs the subunit contact in PseudoEC-SOD and gives dimers (Table IV). If this mutation is added to the F50E/G51E variant (V24D/F50E/G51E), the resulting protein is monomeric (Table IV).

These results together with results from the earlier studies of N-terminal fusions with HCAII (Tibell et al., 1996; Stenlund et al., 1997) clearly show that the N-terminal domain is responsible for the tetramer formation in PseudoEC-SOD and that the hydrophobic side of the predicted amphipathic α-helix is essential for the subunit interaction. The dimer interaction of intracellular hCuZnSOD is not essential for subunit interactions (tetramer formation) in PseudoEC-SOD and, since the corresponding putative subunit contacts in the central domain of hEC-SOD are less extensive (Figure 6), we propose this dimer contact to be of less importance.

Materials and methods

Construction and production of the fusion proteins

The cDNA coding the human intracellular CuZnSOD was isolated from a human lymphocyte cDNA library (CD4 Positive Jurkat T. Clontech) made in the cloning vector λgt 11, by polymerase chain reaction (PCR) using the oligonucleotides 5’CACGCCCGCTGCGCCAGTGCACGGCCGCAGGCAGGCCGGTGTGGCAGTGTCTG3’ and 5’GTGAAAGTGAAGACTGTAATTGCGACGACCCAGTTGCAATGACCC3’. The gene fusions were made using a plasmid, pACNN, constructed for expression of another fusion protein, FusNN, described earlier (Tibell et al., 1996). To construct the gene fusion corresponding to the protein FusNCZ (where the N-terminal 49 amino acids of hEC-SOD are fused to the N-terminal of hCuZnSOD) we used sticky-feet mutagenesis (McPherson et al., 1991) using the isolated PCR-fragment.

The 25 amino acid C-terminal of hEC-SOD was isolated from a human placenta cDNA library (kindly provided by J.L.Millan) by PCR using the oligonucleotides 5’GTGTTAGTGAACTGTAATTGGCGACGACCCAGTTGCAATGCC3’ and 5’CAGTGAACCAAGTGAAGCTGACGACCCAGTTGCAATGCC3’. The resulting DNA-fragment was subsequently fused to the 3’-end of the FusNCZ gene by sticky-feet mutagenesis, resulting in a fusion called PseudoEC-SOD.

For construction of the five mutants we used site-directed mutagenesis essentially according to the method of Kunkel (1985) with the slight modification described by Mårtensson et al., (1995). DNA sequencing of the entire coding region,
using the chain-termination method of Sanger et al. (1977), confirmed the fusion proteins and the mutations.

The fusion proteins were produced essentially as described earlier (Mårtensson et al., 1995) with the modification of adding 3 mM CuSO₄ and 30 µM ZnSO₄ at the time of induction. Cultures of 2–2.5 l with an OD₆₆₀ of ~1.2 were harvested by centrifugation. The bacteria were resuspended in a homogenization buffer containing 50 mM potassium phosphate, pH 7.8, 0.01 mg/ml DNase, 0.01 mg/ml RNase, 0.3 mM PMSF and 1 mM EDTA. In addition, Complete protease inhibitor cocktail tablets (Boehringer Mannheim) were dissolved in the homogenization cocktail according to the manufacturer’s instructions. The resuspended bacteria were homogenized using a bead-beater and after centrifugation for 45 min at 20 000 r.p.m., the supernatant was collected for protein purification.

**Purification**

**Heat denaturation.** Contaminating proteins were denatured by incubating 10 ml samples of the supernatant from the homogenized cells at 60°C for 20 min followed by centrifugation.

**Anion chromatography.** The sample containing fusion protein was applied to an anion-exchange chromatographic column (40x2.6 cm), Q-sepharose FF, pre-equilibrated with 20 mM potassium phosphate buffer, pH 7.0. After applying the sample, the column was washed with this buffer plus 0.2 M NaCl and the fusion protein was eluted in a pulse of 0.5 M NaCl in the buffer. The flow-rate was 7 ml/min during the entire chromographic process.

**Hydrophobic chromatography.** To a sample containing the fusion protein, sodium phosphate was added to a final concentration of 1 M and the pH was adjusted to 9.0. A phenyl-Sepharose FF column (10x1 cm) was equilibrated with 1 M sodium phosphate, pH 9.0, the sample was applied and the column was washed with this buffer. The fusion protein was eluted with 250 mM sodium phosphate, pH 9.0, and strongly binding contaminating proteins were washed out with 10 mM sodium phosphate, pH 9.0. The flow rate was 2 ml/min throughout the chromatographic process.

**Heparin-Sepharose.** The sample containing fusion protein was applied to a heparin-Sepharose column (14.5x1.6 cm) pre-equilibrated with 10 mM sodium phosphate buffer, pH 7.5, and washed after application of the sample with this buffer. The fusion protein was eluted with 500 mM sodium phosphate, pH 9.0, and strongly binding contaminating proteins were extracted with 10 mM sodium phosphate, pH 9.0. The flow rate was 6.6 ml/min during the entire process.

**Size-exclusion chromatography.** Size-exclusion chromatography was performed on a Superose 12 HR 10/30 column (Pharmacia Biotech), using 10mM sodium phosphate buffer, pH 7.6, and 0.15 M NaCl as eluent, an elution rate of 0.5 ml/min, an injection volume of 200 µl and protein concentrations of ~1.5 mg/ml. If necessary, the samples were concentrated before application.

**Purification procedures.** FusNCZ, PseudoEC-SOD and the mutated PseudoEC-SOD protein variants were purified either with or without initial heat incubation of the supernatant after cell disruption. The next purification step was anion chromatography on Q-Sepharose FF, followed by either phenyl-Sepharose chromatography for FusNCZ or dialysis and heparin-Sepharose chromatography for the PseudoEC-SOD proteins. Size-exclusion chromatography and a final concentration step removed nucleic acids.

**Analysis of fusion proteins**

The SOD activity was measured according to Marklund and Marklund (1974). The N-terminal amino acid sequence was determined in an Applied Biosystems Model 477A sequencing system. Protein extinction coefficients were determined according to the method of Gill and von Hippel (1989). SDS–PAGE was performed on a discontinuous polyacrylamide gel system (Jergil and Olsson, 1974) containing 14.9% acrylamide and 0.1% SDS. For Western blotting, the proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, MA) by standard methods. Blots were developed using highly purified rabbit anti-EC-SOD, rabbit anti-hCuZnSOD or rabbit anti-HCAII and alkaline phosphatase-labeled anti-rabbit-IgG antibodies (Dakopats, Copenhagen, Denmark). Isoelectric focusing was carried out using a Phastgel IEF 3–9 gradient medium and a Pharmacia PhastSystem instrument. Metal contents were determined by electrothermal atomic absorption spectrometry in a Perkin-Elmer Zeeman 303 + HGA apparatus. Analytical heparin-Sepharose chromatography was carried out at room temperature as described previously (Karlsson et al., 1988). The apparent molecular weight of each of the native fusion proteins was estimated by chromatography on a Superose 12 HR 10/30 column (Pharmacia Biotech) (Stenlund et al., 1997). Circular dichroism (CD) spectra were recorded at 23°C on a CD6 spectrodichrograph (Jobin-Yvon Instruments, Longjumeau, France) essentially as described by Freskgård et al. (1994). Far-UV spectra of the proteins were recorded in a 0.5 mm quartz cell at a protein concentration of 0.25–0.4 mg/ml in 5 mM sodium phosphate, pH 7.6, and presented as mean residue weight ellipticities (MRW) (Freskgård et al., 1994) on the basis of a molecular weight of 24.55 kDa and 228 amino acids.

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**References**

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