Evolutive and structural characterization of *Nostoc commune* iron-superoxide dismutase that is fit for modification

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ABSTRACT. Superoxide dismutase (SOD) has extensive clinical applications for protecting organisms from toxic oxidation. In this study, the integrated iron-superoxide dismutase gene (*fe-sod*) coding sequence of *Nostoc commune* stain CHEN was cloned from genomic DNA and compared to *sods* from other reported algae. These analyses of immunology and phylogenetics indicated that this Fe-SOD is considerably homologous with SODs from lower prokaryotes (Fe-SOD or Mn-SOD) but not those from higher animals (Cu/Zn-SOD). In addition, the *N. commune* Fe-SOD shows 67 to 93% protein sequence identity to 10 other algal Fe-SODs (or Mn-SODs) and 69 to 93% gene sequence identity. Rare nonsynonymous substitutions imply that algal SODs are being subjected to strong natural selection. Interestingly, the *N. commune* Fe-SOD enzyme molecule has a compact active center that is highly conserved (38.1% of residues are absolutely conserved), and 2 loose ends localized outside the molecule and inclined to mutate (only 11.5% of residues are absolutely conserved). Based on associative analyses of evolution, structure, and function, this special phenomenon is attributed to function-
dependent evolution through negative natural selection. Under strong natural selection, although the mutation is random on the gene level, the exterior region is inclined to mutate on the protein level owing to more nonsynonymous substitutions in the exterior region, which demonstrates the theoretical feasibility of modifying Fe-SOD on its ends to overcome its disadvantages in clinical applications.

Key words: Nostoc commune; Iron-superoxide dismutase; Phylogenetics; Structure and function; Natural selection

INTRODUCTION

The superoxide dismutase (SOD) family is a group of functionally anti-oxidative stress proteins found in all 3 kingdoms of life. Three classes of SODs occur (Fe-SOD, Mn-SOD, and Cu/Zn-SOD), each differing in the metals at their catalytic active sites. Fe-SOD and Mn-SOD are found in Eubacteria, Eubacteria-derived eukaryotic mitochondria, and Archaeabacteria (Regelsberger et al., 2004; Gottlieb et al., 2005; Brioukhanov et al., 2006; Chen et al., 2007; Seo et al., 2007). The Cu/Zn enzyme is confined almost exclusively to the cytoplasm of eukaryotic organisms.

The theory of free radicals and tumors holds that reactive oxygen species originating in redundant radicals attack organisms, leading to genetic mutations and tumors (Behrend et al., 2003; Li et al., 2008; Takenaka et al., 2011). SOD is supposed to be the main cellular superoxide anion-degrading enzyme that inhibits tumor growth (Zhang et al., 2002; Hu et al., 2005). However, SOD has many critical disadvantages in clinical applications: it cannot target tumor cells (Oyanagui et al., 1991), resulting in a low curative efficacy, and it can barely permeate cell membranes because of its large molecular weight (Delanian et al., 2001; Jubeh et al., 2005; Rengel et al., 2005), which leads to a low cellular effect. In previous SOD modifications, we fused fe-sod with the LC-1 single-chain variable fragment antibody (ScFv) gene and achieved an SOD-ScFv fusion protein that could actively recognize SPC-A-1 (a human lung adenocarcinoma cell line) lung tumor cells and permeate cell membranes (Lu et al., 2006). However, the modification of SOD for better effectiveness is still worthy of further research and development.

As promising green foods in the 21st century, Nostoc commune and Spirulina platensis have abundant Fe-SOD enzymes (Shirkey et al., 2000; Choudhary et al., 2007) that we have purified and characterized in previous studies. SOD activity assays have indicated that the specific activities of these enzymes are 2700 and 4200 U/mg, respectively. They have been applied to treat inflammation and cancer (Lu et al., 2006). Detailed investigation of the enzyme sequences and structures, which are related with catalytic function, is necessary to prepare them for further modification and application. In this study, we cloned fe-sod from N. commune stain CHEN and raised an anti-SOD antibody to conduct immunological and phylogenetic analyses of Fe-SOD. To extend this analysis, we characterized the phylogensis of additional genes of the SOD family from 14 lower prokaryotes and 4 representative eukaryotes. The evolution/structure/function-based predictive analysis in the current study presented an interesting and special evolution characterization, which we considered to be the result of a “special law of nature” under strong selection stress. This insight theoretically prepares us for modifications of SOD that overcome its current disadvantages.
MATERIAL AND METHODS

The pUCm-T vector *Escherichia coli* BL21 (DE3): *hsdS gal* (*λcIt857 ind1 Sam7 nin5 lacUV5-T7*) was available at our institution. *N. commune* strain CHEN was cultivated in BG11 culture medium. *E. coli* BL21 colonies were maintained in Luria-Bertani culture medium. Cu/Zn-SOD (8000 U/mg) was obtained from Baoan Corporation in Shanghai, China.

Cloning of *fe-sod*

*N. commune* stain CHEN were grown in BG11 culture medium (150 mg NaNO₃, 4 mg K₂HPO₄, 7.5 mg MgSO₄ · 7H₂O, 3.6 mg CaCl₂ · 2H₂O, 5.8 mg Na₂SiO₃ · 9H₂O, 0.6 mg citric acid, 0.6 mg ferric ammonium citrate, 0.1 mg ethylenediaminetetraacetic acid, 2 mg Na₂CO₃, 0.1 mL A₅ solution, and 100 mL H₂O) at 25°C and exposed to light (intensity of 2000 Lux) each day for 10 h. After 45 days, a 0.1 g *N. commune* sample was taken, and its genomic DNA was isolated using the method of Kim et al. (1997). The SOD fragment was amplified from the genomic DNA and primed with SOD-forward (5ꞌ-GAGGATTTGACACGATGGCATTTG-3ꞌ) and SOD-backward (5ꞌ-CTGACACCTAATTAAGCTTTGGCC-3ꞌ) according to a method published previously (Lu et al., 2006). The achieved *sod* was sequenced. Homology searches were performed with the Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/BLAST), and 13 Fe-SODs (or Mn-SODs) showing the best homology (including 10 reported SODs from algae) were recorded (see Table 1). These sequences were aligned using Clustal X, and neighbor-joining (NJ) was performed using the Molecular Evolutionary Genetics Analysis 4 software to construct phylogenetic trees. Branch supports were estimated with a bootstrap analysis of 3000 replicates for NJ. Sequence alignments and analysis were carried out with the GeneDoc software. The tertiary structure was predicted by submitting the protein sequence to the SWISS-MODEL server (http://www.expasy.org/swissmod/SWISS-MODEL.html) through ExPASy, and the resultant tertiary model was visualized using RasMol 2.6.

Immunoblot analyses of Fe-SOD from *N. commune*

The native Fe-SOD protein was purified from *N. commune* stain CHEN (or *S. platensis*) using DEAE-Sepharose Fast Flow and Sephadex G-100 in an orderly manner (Lu et al., 2006). The SOD activity was assayed using the pyrogallol autoxidation method (Marklund and Marklund, 1974). The purified *N. commune* Fe-SOD was used to raise anti-SOD antiserum. The antiserum was then applied to react with SODs from *N. commune*, *S. platensis*, and cattle erythrocyte cells via Western blotting.

RESULTS

Cloning and immunologic characterization of Fe-SOD from *N. commune* stain CHEN

Primers were designed against the sequence ends of *fe-sods* from other algae (*Nostoc linckia* AAL25194 and *Anabaena variabilis* ATCC 29413, ABA20587). The achieved *fe-sod* sequence (accession No. AAV84021) is 603 bp and contains initiation (ATG) and stop (TAA)
codons. Purified native *N. commune* stain CHEN Fe-SOD was used to raise the anti-SOD antiserum. To ensure the activity of this antiserum, we reacted it with *N. commune* stain CHEN Fe-SOD. The result showed that this antiserum could effectively recognize *N. commune* stain CHEN Fe-SOD (Figure 1, lane 1). Moreover, the antiserum could recognize native Fe-SOD from *S. platensis*. However, it barely reacted with Cu/Zn-SOD from cattle erythrocyte cells. We, therefore, concluded that the Fe-SOD from *N. commune* stain CHEN was more antigenically related to that of *S. platensis* than to that of Cu/Zn-SOD from higher animals.

**Phylogenetic analysis**

We analyzed the phylogenetic relationships among 14 Fe-SODs (or Mn-SODs) from lower prokaryotes (including 10 reported SODs from algae) and 4 representative Cu/Zn-SODs (fruit fly, horse, house mouse, and human; see Table 1) using the NJ method of Molecular Evolutionary Genetics Analysis 4 (Figure 2; Tamura et al., 2007). The branch points represent speciation events designated S1-S3. Figure 2 shows that the 14 Fe-SODs (or Mn-SODs) from lower prokaryotes form a coherent grouping, whereas the 4 Cu/Zn-SODs from eukaryotes form another grouping (S1 separates eukaryotes from prokaryotes, implying the commencement of divergence between Fe-SOD/Mn-SOD and Cu/Zn-SOD within the common ancestry). In the prokaryote grouping, the SODs from *Myxococcus xanthus* DK 1622, *Magnetospirillum magnetotacticum* MS-1, and *Acinetobacter* sp ADP1 branch relatively early (S2 splits algae from other prokaryotes), but the analysis failed to distinguish an outgroup from Fe-SOD and Mn-SOD. In the eukaryote grouping, the SOD from the lower animal (fruit fly) branches relatively early (S3), whereas those from mammals (horse, house mouse, and human) branch later. Together with the result from the immunological and phylogenetic analyses, these results suggest that the Fe-SODs (or Mn-SODs) and Cu/Zn-SODs are remote from each other evolutionarily and evolved in different directions.
Table 1. Taxonomic placement and classes of superoxide dismutases (SODs) of each species and GenBank accession numbers included in the phylogenetic analyses.

<table>
<thead>
<tr>
<th>Codes</th>
<th>Group</th>
<th>Species</th>
<th>Classes of SODs</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Algae</td>
<td><em>Nostoc commune</em> stain CHEN</td>
<td>Fe-SOD</td>
<td>AA94021</td>
</tr>
<tr>
<td>2</td>
<td>Algae</td>
<td><em>Nostoc punctiforme</em> PCC 73102</td>
<td>Fe-SOD</td>
<td>ZP_00108516</td>
</tr>
<tr>
<td>3</td>
<td>Algae</td>
<td><em>Nostoc commune</em> stain DRH1</td>
<td>Fe-SOD</td>
<td>AAF25009</td>
</tr>
<tr>
<td>4</td>
<td>Algae</td>
<td><em>Nostoc linckia</em></td>
<td>Fe-SOD</td>
<td>AAL25194</td>
</tr>
<tr>
<td>5</td>
<td>Algae</td>
<td><em>Nostoc sp</em> PCC 7120</td>
<td>Fe-SOD</td>
<td>NP_486978</td>
</tr>
<tr>
<td>6</td>
<td>Algae</td>
<td><em>Anabaena variabilis</em> ATCC 29413</td>
<td>Mn-SOD</td>
<td>ABA20587</td>
</tr>
<tr>
<td>7</td>
<td>Algae</td>
<td><em>Plectonema boryanum</em> UTEX 'B 485'</td>
<td>Mn-SOD</td>
<td>ABA69954</td>
</tr>
<tr>
<td>8</td>
<td>Algae</td>
<td><em>Thermosynechococcus elongatus</em> BP-1</td>
<td>Mn-SOD</td>
<td>BAC09071</td>
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<td>9</td>
<td>Algae</td>
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<td>Mn-SOD</td>
<td>ABDD0958</td>
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<td>10</td>
<td>Algae</td>
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<td>Mn-SOD</td>
<td>ABDD3447</td>
</tr>
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<td>11</td>
<td>Prokaryote</td>
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<td>Mn-SOD</td>
<td>ABF87575</td>
</tr>
<tr>
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<td><em>Magnetospirillum magnetotacticum</em> MS-1</td>
<td>Mn-SOD</td>
<td>ZP_00054068</td>
</tr>
<tr>
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<td>Prokaryote</td>
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<td>Mn-SOD</td>
<td>YP_046036</td>
</tr>
<tr>
<td>14</td>
<td>Algae</td>
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<td>Mn-SOD</td>
<td>AAQ22734</td>
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<td>15</td>
<td>Eukaryote</td>
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<td>16</td>
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<tr>
<td>17</td>
<td>Eukaryote</td>
<td><em>Equus caballus</em> (horse)</td>
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<td>BAA76921</td>
</tr>
<tr>
<td>18</td>
<td>Eukaryote</td>
<td><em>Mus musculus</em> (housemouse)</td>
<td>Cu/Zn-SOD</td>
<td>NP_035564</td>
</tr>
</tbody>
</table>

Figure 2. Phylogenetic relationships among members of the superoxide dismutase (SOD) family. Phylogenetic trees were constructed using the neighbor-joining method. The lengths of the branches reflect evolutionary distance. Number at the branch nodes indicates percentage bootstrap support for 3000 replicates. Speciation events are designated *S*₁, *S*₂, and *S*₃. The groups ‘Algae’, ‘Prokaryote (without algae)’, and ‘Eukaryote’ are shown by I, II and III, respectively. [F], [M] and [C] represent Fe-SOD, Mn-SOD and Cu/Zn-SOD, respectively.

Evolutive characterization of *N. commune* stain CHEN Fe-SOD

Fourteen prokaryote SODs were aligned and compared with one another using the GeneDoc software (significant similarity was found with the 4 Cu/Zn-SODs describe above; data not shown). As shown in Figure 3, of 200 common residues in the 14 SODs, 31 are variable, 121 are conserved, and 60 are absolutely conserved. In addition, 37 of the 60 absolutely conserved residues are from 3 conserved regions: G1FNNAQQVWNHTFFFW (69-84), AAATQFGSGWAWL (120-132), and DVVEHAYY (162-169). According to the DVVEHAYY-conserved region, a downstream primer...
(5'-AATGTAGGGCGTCTTC-3') was designed, and we successfully amplified partial *S. platensis* *fe-sod* (accession No. AAQ22734) using genomic DNA (the upstream primer was above SOD-forward). In addition, we found 3 peculiar mutant residues (Ser81, Met162, and Asp166; see Figure 3) in the SOD from *S. platensis*, and these residues are absolutely conserved in the other 10 algal SODs. This heterogeneity introduces difficulty to the forecasting and designing of a downstream primer to clone integrated *S. platensis* *fe-sod* CDS (studies are proceeding now).

Figure 3. Alignment of superoxide dismutases (SODs) from 14 reported low prokaryote in Genbank. Variable residues are displayed as black letters on a white background; conserved residues are displayed as white letters on a gray background (subscript lowercase); absolutely conserved residues are displayed as white letters on a black background (subscript uppercase); three conserved regions are displayed as subscript arrowhead. The Fe³⁺ bound sites are in subscript box; characterized residues distinguishing Fe-SODs from Mn-SODs are in subscript circle. The followed entry in the matrix was concluded form alignment, indicating the total number of variable residues, conserved residues, and absolutely conserved residues from total 200 residues. The number in bracket indicates the number of corresponding residues in interior region (31-169 amino acids)/exterior region.
Many important signature amino acid residues were also revealed: 2 unique and conserved amino acid residues (Thr138 and Asn149) distinguish algae from other lower prokaryotes (see Figure 2, S.); in addition, _N. commune_ stain CHEN Fe-SOD exhibits 4 residues (Leu6, Gln90, Thr94, and Lys173) that are absent in other SODs. Further, the residues implicated in the formation of the active site and metal ion binding are absolutely conserved in these SODs - such as 4 Fe³⁺-bound sites in the SOD active center (His28, His80, Asp162, and His166; a water molecule or a hydroxyl ion is the fifth site) as well as the residues Ala75, Gln76, Trp78, and Ala147, which are proposed as primary candidates to distinguish Fe-SODs from Mn-SODs (Parker and Blake, 1988a,b; Van Camp et al., 1990). Interestingly, of 139 interior residues (we define 31-169 amino acids as the interior region and the others as the exterior region), 19 are variable, 95 are conserved, and 53 are absolutely conserved; each of the 3 conserved regions described above are included. However, of 61 exterior residues, 12 are variable, 26 are conserved, and only 7 are absolutely conserved. Conclusively, it is significant that the interior residues show a lower mutation rate than that of the exterior residues.

**Structure and function analysis**

The _N. commune_ stain CHEN amino acid sequence was submitted to the SWISS-MODEL server using ExPASy (Schwede et al., 2003; Kopp and Schwede, 2006), and the structure was predicted. Figure 4 shows that the structure can be divided into 2 domains, the N-terminal domain involving an extended region followed by 2 alpha-helices (Ala22-Tyr29, His32-Tyr44) and the C-terminal domain containing 3 alpha-helices (Ser66-Asn79, Gly97-Gly108, and Arg176-Asn185), and 3 beta-sheets (Ser127-Gly136, Gly137-Pro145, and Lys155-Asp162) inserted sequentially between the fourth and fifth helices (see Figure 3). This structure is comparable to that in a report of an X-ray analysis of Fe-SOD from _Mycobacterium tuberculosis_, implying that the prediction is considerably reliable (Cooper et al., 1995).

**Figure 4.** The tertiary structure of Fe-SOD from _Nostoc commune_ stain CHEN by RasMol 2.6. The amino acid sequence of superoxide dismutase (SOD) was submitted to SWISS-MODEL server, so that the tertiary structure was predicted (BLAST search P < 0.00001, SIM > 25%). Alpha-helices, beta-sheet, and two ends of protein sequence are in pink, yellow, and blue, respectively.
In addition, the 4 Fe\(^3\)-bound sites are close to one another even though they are apart in the primary structure. Further, the active center and Fe\(^3+\)-bound sites are inside the SOD molecule, whereas the ends of the sequences (beside 31-169 amino acids) are outside and relatively loose. Accordingly, these ends also have a mutation rate higher than that of the interior (see Figure 3, matrix). To characterize this coincidence in detail, we compared \textit{N. commune} stain CHEN Fe-SOD with 10 SODs from other algae and determined the mutation rates of various regions. Table 2 shows that the mutation rates of the exterior region in the 10 controls are significantly higher than those of the interior region on the protein level but relatively equal on the gene level.

<table>
<thead>
<tr>
<th>9 algae species(^a)</th>
<th>Protein mutation rate (%)(^b)</th>
<th>Gene mutation rate (%)(^b)</th>
<th>Sequence identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exterior/interior</td>
<td>Exterior/interior</td>
<td>Gene/protein</td>
</tr>
<tr>
<td>2</td>
<td>11.5/4.3</td>
<td>8.6/6.2</td>
<td>93/93</td>
</tr>
<tr>
<td>3</td>
<td>13.1/5.7</td>
<td>7.7/9.8</td>
<td>90/92</td>
</tr>
<tr>
<td>4</td>
<td>13.2/12.9</td>
<td>13.1/13.7</td>
<td>87/87</td>
</tr>
<tr>
<td>5</td>
<td>18.0/11.5</td>
<td>19.3/17.1</td>
<td>82/86</td>
</tr>
<tr>
<td>6</td>
<td>21.3/10.7</td>
<td>21.1/15.8</td>
<td>82/86</td>
</tr>
<tr>
<td>7</td>
<td>18.0/17.9</td>
<td>22.4/25.7</td>
<td>75/82</td>
</tr>
<tr>
<td>8</td>
<td>29.5/23.7</td>
<td>29.5/30.7</td>
<td>69/73</td>
</tr>
<tr>
<td>9</td>
<td>41.0/23.7</td>
<td>34.9/28.4</td>
<td>68/70</td>
</tr>
<tr>
<td>10</td>
<td>41.0/24.5</td>
<td>34.0/30.5</td>
<td>67/69</td>
</tr>
</tbody>
</table>

\(^a\)The codes of the 9 algae species are the same with Table 1. \(^b\)Mutation rate = number of variable residues (or nucleotides) / number of total residues (or nucleotides) \times 100%. \(^c\)Exterior: region beside of 31-169 amino acids (or 93-507 nucleotides). Interior: region of 31-169 amino acids (or 93-507 nucleotides).

**DISCUSSION**

When gene sequences initially begin to diverge during evolution, mutations usually accumulate most rapidly in the third codon, and most substitutions are synonymous. As a result, nucleotide identity between 2 gene sequences is usually lower than amino acid identity between the 2 corresponding proteins. Typical of this situation are the \textit{p-vac} and \textit{c-vac} genes of \textit{Halobacterium halobium} and the \textit{c-vac} gene of \textit{Haloferax mediterranei} (Horne et al., 1988; Englert et al., 1990). For these genes, the gene identity is much lower than the corresponding amino acid identity (84.7 vs 97.4\%, 86.4 vs 96.1\%, and 85.5 vs 98.7\%, respectively). However, the algal \textit{sods} do not conform to the behavior displayed in these genes. The Basic Local Alignment Search Tool 2 sequence results of the comparison of \textit{N. commune} CHEN Fe-SOD with 10 other algal SODs, \textit{sods} showed relatively equivalent gene and amino acid identities (67-93\% identity between gene sequences and 69-93\% identity between amino acid sequences; see Table 2). These results imply that most of the encoding triplets are connected by nonsynonymous nucleotide substitutions, which indicates that these \textit{sods} from algae continue to be subjected to strong and changing selection. This selection may be related to intracellular or extracellular stress from oxygen radicals.

Interestingly, this strong selection seems to lead to a special evolutive result: the conserved residues are mostly localized on the interior of protein sequences, whereas the exterior parts exhibit a higher degree of variability. A similar phenomenon was reported in 8 sets of homol-
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ogous protein families as early as 1980 (Go and Miyazawa, 1980), but the mechanism remains unclear. The statistics in Table 2 present this evolutionary characterization in detail. We designed an Re/Ri (mutation rate of exterior/mutation rate of interior) for the data in Table 2 to visualize the diversity of mutation rates in various regions. An Re/Ri of >1 represents an exterior mutation rate that is higher than that of the interior, whereas an Re/Ri of 1 implies random mutation. The Re/Ri fluctuated near 1 on the gene level, implying that nucleotide mutations occur randomly on the entire sequence; however, the Re/Ri of the 10 proteins studied were all above 1 to varying degrees. Remarkably, the Re/Ri is high in 2, 3, and 6 controls, suggesting a significant imbalance of mutation distribution in these species. Given the distinct Re/Ri values on the gene and protein levels, we can easily conclude that of the total equally distributed nucleotide substitutions on the gene level, most of the nonsynonymous substitutions accumulate at the ends of gene sequences, leading to higher mutation rate on the exterior of the protein, whereas synonymous substitutions are mostly localized at the interior of the gene, inducing a conserved protein interior.

We attribute this imbalanced distribution of nonsynonymous mutation to a special law of nature. According to this law, exterior-mutating proteins tend to survive, whereas interior-mutating proteins are likely to be eliminated. We tried to uncover the special law of nature using associative analysis of its structure and function: nonsynonymous substitutions localized at the gene interior usually destroy the enzyme active center. Figures 3 and 4 show the sites of catalysis, and Fe$^{3+}$-bound sites are all between the 31-169-amino acid region, constituting the enzyme activity center; they are absolutely conserved. Subsequently its antioxidant function is impaired. These substitutions go against the special law of nature and are therefore likely to be eliminated. On the contrary, although exterior nonsynonymous substitutions on sod also lead to exterior-mutating proteins, they do not impair enzymatic activity because of their special structure. Figure 4 shows that the exterior residues are on the surface of the protein and well removed from the site of catalysis; they are not presumed to participate in the formation of the enzyme activity center. Hence, these substitutions survive under the special law of nature. In a word, this law was function-based during algal SOD evolution. We attribute the action of the special law of nature to an attempt by the organism to maintain enzymatic catalytic function and survive oxidative stress. Accordingly, we fused an ScFv gene fragment on one end of N. commune fe-sod, which was presumed to be enzyme function-independent, and the resulting fusion protein retained considerable enzyme activity but had higher anti-tumor efficacy (Lu et al., 2006).

In sum, the fe-sod CDS from N. commune stain CHEN was cloned, and its gene and protein sequences exhibited many unusual and remarkable features: 1) the rare nonsynonymous substitutions within algal sods implied that they are being subjected to strong natural selection; 2) under this strong selection, although the mutation was random on the gene level, on the protein level, the exterior region is inclined to mutate (Re/Ri > 1) owing to the higher frequency of nonsynonymous substitutions in the exterior region; and 3) the interesting evolution characterization was function-based to maintain SOD activity under strong natural selection. These features show the theoretical feasibility of modifying the ends of Fe-SOD to overcome its disadvantages in clinical applications.

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