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Amino acid sequence of iron-superoxide dismutase from *Pseudomonas ovalis*

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The amino acid sequence of iron-superoxide dismutase from *Pseudomonas ovalis* was deduced by the analyses of peptides derived from limited hydrolysis of the aminoethylated or pyridylethylated apoprotein with trypsin, *Staphylococcus aureus* V8 protease, and dilute acid hydrolysis. The polypeptide chain contains 195 amino acid residues and has a calculated M_r of 21 421. The sequence is highly homologous (65% identity) to the recently published sequence of the iron-superoxide dismutase from *Photobacterium leiognathi*. It is also homologous to the known sequences of the manganese-superoxide dismutase by sharing 33–53% identical residues. Alignment of the superoxide dismutase sequences and the available structural information from X-ray crystallography suggest that the ligands to the iron in the *P. ovalis* superoxide dismutase are His-26, His-74, Asp-156 and His-160, which align with the ligands to the manganese in the *Thermus thermophilus* manganese-superoxide dismutase. The sequence information of the *P. ovalis* dismutase will facilitate refinement of the X-ray crystallographic data that are now available at 2.9 Å resolution.

Metalloprotein; Primary structure; Iron coordination; Iron-superoxide dismutase; Sequence homology

1. INTRODUCTION

Superoxide dismutases (EC 1.15.1.1) are metalloproteins that catalyze the reaction, $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$, and scavenge the superoxide radicals toxic to cells. Three classes of superoxide dismutases have been distinguished according to the bound metal ions; the copper/zinc-, manganese-, or iron-containing enzymes, which exhibit different spectra of phylogenetical distributions [1].

Among the isozymes, the copper/zinc-superoxide dismutase is clearly distinct from the other classes of dismutase with respect to its amino acid sequence and three dimensional structure. This isozyme has been the most extensively studied, and its structure-function relationships are reviewed in recent publications [2,3].

NH_2-terminal sequence analyses of the manganese- and iron-superoxide dismutases from more than 17 species suggested that these isozymes are structural homologs [4,5]. This has been supported by the complete sequence of *Escherichia coli* [6]. *Bacillus stearothermophilus* [7], yeast [8] and human liver [9] manganese-dismutase and the first complete sequence of *Photobacterium leiognathi* iron-superoxide dismutase [10]. X-ray diffraction studies of the manganese-dismutase from *Thermus thermophilus* [11] and of the iron-dismutases from *Pseudomonas ovalis* [12] and *E. coli* [13] have further demonstrated that these isozymes have very similar three dimensional structures. However, with a few exceptions [14–16], the metal requirement of the manganese- and iron-dismutases is specific, indicating that the metal-

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ligand environments, or the active site geometries of these isozymes are different. Thus, more detailed studies are required to establish the structure-function relationships of the manganese- and iron-superoxide dismutases.

This report describes the amino acid sequence of the iron-superoxide dismutase from *P. ovalis*. This is the first complete sequence of this class of dismutase for which the crystal structure has also been resolved by an X-ray diffraction study.

2. MATERIALS AND METHODS

P. ovalis iron-superoxide dismutase was purified according to Yamakura [17]. The purified protein was reduced and aminoethylated or pyridylethylated in 8 M urea before sequence analysis. The alkylated protein (2–10 mg) was subjected to separate proteolysis with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Millipore), *Staphylococcus aureus* V8 protease (Miles), and dilute acid hydrolysis with 0.01 M HCl at 110°C for 5 h. The resulting fragments were separated mainly by means of high-performance reversed-phase chromatography on a macroreticular polystyrene resin [18] or on silica-based packing materials with a programmed gradient of acetonitrile in 0.1% trifluoroacetic acid or in 0.1% heptafluorobutyric acid. Methods used for the sequence determination of proteins and peptides including amino acid analysis, sequential Edman degradation, carboxypeptidase digestions and hydrazinolysis have been described [19–21]. A portion of the sequence was established or confirmed on a model 470A gas-phase sequenator (Applied Biosystems) according to the protocol provided by the supplier.

3. RESULTS AND DISCUSSION

The amino acid sequence of iron-superoxide dismutase from *P. ovalis* is shown in fig. 1, together with the peptides used for the sequence determination. The sequence was deduced primarily based on the analyses of the complete set of tryptic peptides of the aminoethylated apoprotein, which were isolated by high-performance reversed-phase chromatography on a macroreticular polystyrene resin eluted with a trifluoracetic acid-acetonitrile solvent system [18]. Overlaps among the tryptic peptides were obtained by isolating peptides of two additional cleavages. *S. aureus* V8 protease digestion and dilute acid hydrolysis, of the pyridylethylated polypeptide chain. One of the large dilute acid peptides, D3, was sub-digested with V8 protease and a fragment, D3S3, was isolated and used for proving the alignment between T4 and T5. Most of the peptide sequence was determined by our standard sequential degradation procedure and carboxypeptidase digestions. However, five peptides (T5a, T5b, T12, D3 and D3S3) were subjected to analyses on a model 470A gas-phase sequenator to establish or to confirm the sequence. Thus, most of the residues in fig. 1 were identified more than twice in the sequence analysis.

A 35-residue NH_2-terminal sequence of this protein was reported by Harris et al. [4]. The reported sequence and an equivalent sequence derived from our own NH_2-terminal analysis (fig. 1) perfectly fit the proposed sequence shown in fig. 1. The analysis of the intact protein by hydrazinolysis [19] showed Ala to be the COOH-terminus, also compatible with the results in fig. 1.

The final sequence thus obtained comprises 195 amino acids, and the composition calculated from the sequence agrees with the previous compositional analysis of the protein after acid hydrolysis [22]. A characteristic feature of the composition is the relatively high content of tryptophan and histidine residues, and the lack of methionines. The three cysteine residues were identified as the alkylated derivatives in the sequence analysis. The previous titration experiment using Ellman's reagent [17] and the X-ray analysis of this protein [12] suggest that all the cysteine residues have free SH-groups in the intact protein and that there are no disulfide bridges. An earlier compositional analysis [17] and the X-ray analysis [12] assumed the presence of an unknown 'electron dense' cofactor in this enzyme, but we have had no evidence for such a substance during the sequence study. The M_r of the polypeptide chain computed from the sequence is 21 421, comparable to the value (M_r 19 500) estimated by dodecyl sulfate gel electrophoresis [17].

Reinspection of fig. 1 shows that the *S. aureus* protease has cleaved the polypeptide chain at a COOH-terminus of glycine at least three times, twice at a dipeptide sequence Gly-Ser resulting in

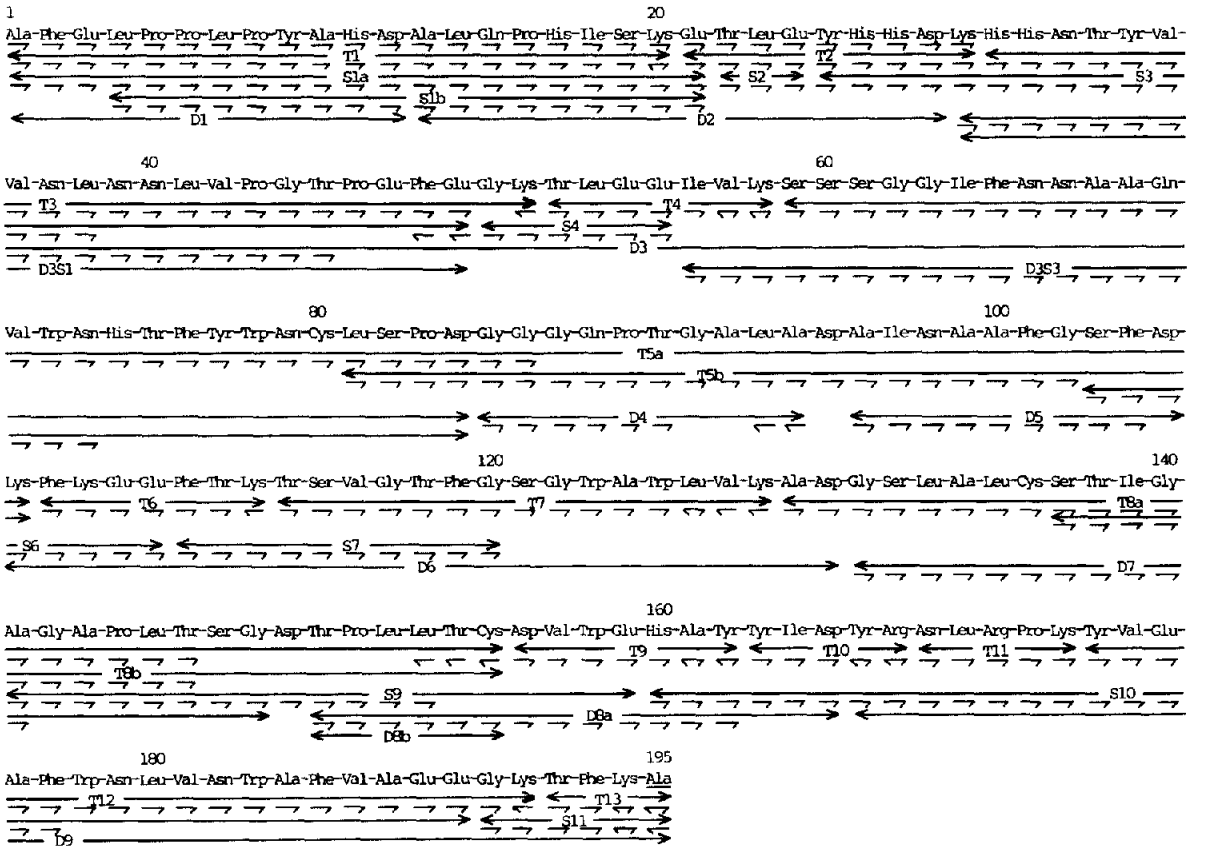

Fig. 1. Amino acid sequence of iron-superoxide dismutase from *P. ovalis* and peptides used for the sequence determination. T refers to tryptic peptides, S to *S. aureus* V8 protease peptides, and D to dilute acid peptides. D3S refers to *S. aureus* protease peptides of the peptide D3. (→) Sequence determined by Edman degradation and (←) that determined by carboxypeptidase digestion. (—) The COOH-terminal Ala identified by hydrazinolysis of the intact protein.

fragments S6 and S7 and once at a sequence Gly-Ala producing fragment S9. This is somehow unexpected from the known specificity and our previous experience in the use of this proteolytic enzyme, although we have noticed that a Gly-Ser bond in the equivalent sequence of *Ph. leiognathi* superoxide dismutase was also cleaved by this protease in the published sequence analysis [10]. In the present study we have also noticed that dilute acid hydrolysis was extremely useful in proving the necessary overlaps, where the complete set of peptides was isolated by following reversed-phase chromatography except for free aspartic acids at positions 95 and 149. This is attributable to the recent advances in the liquid chromatography technique which allows more efficient peptide purification than conventional techniques.

In fig. 2, the amino acid sequence of the iron-superoxide dismutase from *Ph. ovalis* is aligned with the recently determined sequence of *Ph. leiognathi* iron-superoxide dismutase and the sequences of manganese-containing dismutases from four different sources. Gaps have been inserted to maximize the homologies among the sequences, and the resulting scores are listed in table 1. The *P. ovalis* iron-dismutase shows the highest homology (65.1%) to the *Ph. leiognathi* counterpart, but is also homologous to the manganese enzymes. Thus, the homology scores between the two iron- and four manganese-superoxide dismutases are 33.3–52.7%, which is the same order of homology as the manganese enzymes show among themselves (38.8–59.9%). In the sequence, a total of 40 residues is found at identical positions of all the superoxide dismutases listed in fig. 2. Thus, the iron- and manganese-superoxide dismutases show

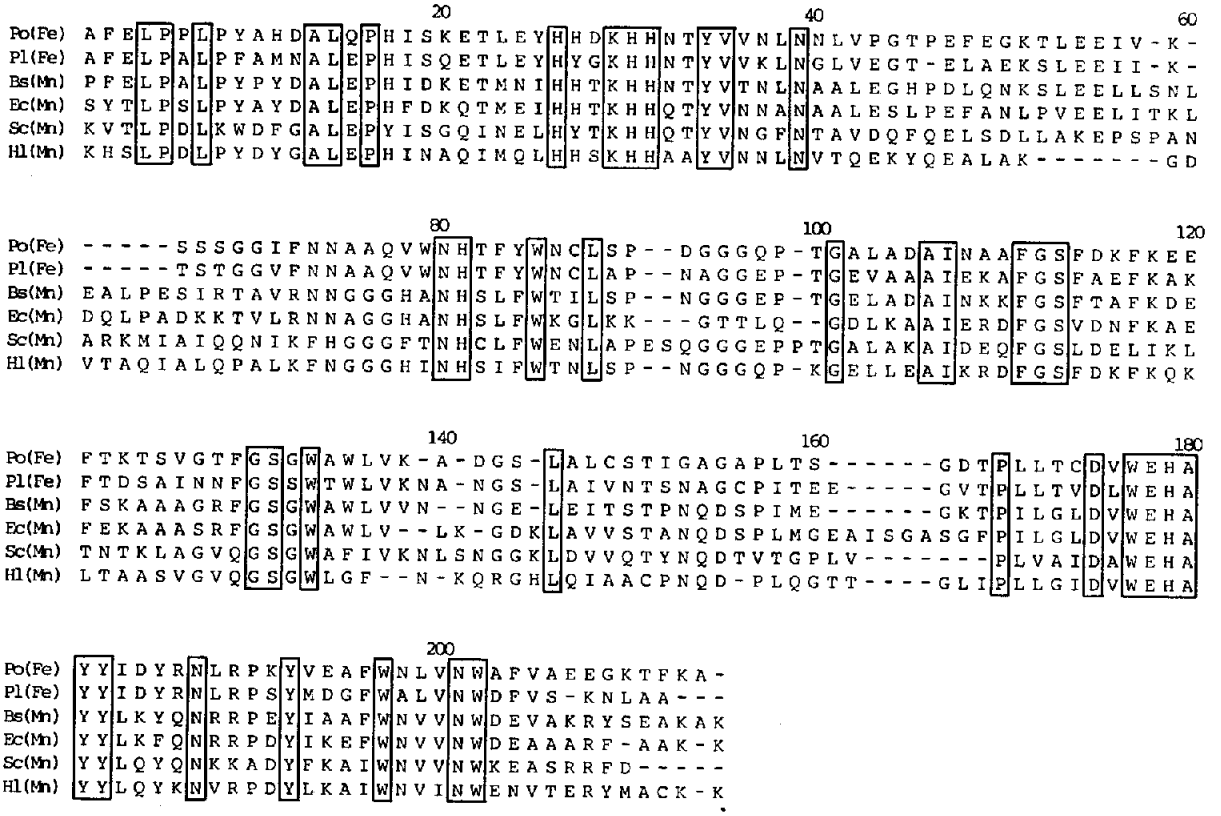

Fig. 2. Comparison of the amino acid sequence of *P. ovalis* (Po), *Ph. leiognathi* (Pl), *B. stearothermophilus* (Bs), *E. coli* (Ec), Yeast (Sc), and human liver (Hl) superoxide dismutases. Gaps have been introduced to obtain maximal homologies amongst the sequences. Boxes indicate positions where residues are identical.

extensive sequence homology over the entire polypeptide chain. This should account for the similar tertiary structural folds of both types of superoxide dismutase observed by X-ray crystallography [11–13].

The X-ray structure of the *T. thermophilus* manganese-superoxide dismutase determined at a 2.4 Å resolution [11] has assigned residues His-28, His-83, Asp-165 and His-169 as ligands to the manganese. In the aligned sequences shown in fig. 2 these residues align with His-26, His-81, Asp-175 and His-179. For the *P. ovalis* iron-superoxide dismutase the ligands to the iron have been tentatively assigned as residues 26, 69, 151, and 155 in the X-ray analysis at a 2.9 Å resolution [13]. If we assume that the X-ray analysis could not identify

Table 1

Sequence homology between iron- and manganese-superoxide dismutase[a]

	P. leiognathi	*B. stearo-thermophilus*	*E. coli*	Yeast	Human liver (Mn)
P. ovalis (Fe)	65.1	52.7	42.2	33.3	39.4
Ph. leiognathi (Fe)		49.8	39.3	34.1	35.0
B. stearothermophilus (Mn)			59.9	39.3	47.8
E. coli (Mn)				38.8	42.4
Yeast (Mn)					42.4

[a] Values are given as percentage of identical residues from the total numbers of residues in fig. 2

five additional residues between residues 26 and 69 at this level of resolution, then the metal ligands correspond to His-26, His-74, Asp-156, and His-160 (fig. 1). These residues perfectly align with the ligands to manganese in the aligned sequences (fig. 2). It is also likely that the *E. coli* iron-superoxide dismutase shares the same ligands to the iron, which have tentatively been assigned to residues 26, 69, 148 and 152 by X-ray analysis [13]. Therefore it appears that the two types of dismutase isozymes have common ligands for the metal coordination, the residues positioned at 26, 81, 175 and 179 in fig. 2.

There are superoxide dismutases from three bacteria, *Bacteriodes fragilis, Propionibacterium shermanii* and *Streptococcus mutans*, that bind either iron or manganese without affecting their activities [14–16]. But, in general, the metal requirements of the manganese- and the iron-superoxide dismutases are specific as demonstrated by reconstitution experiments, where the manganese-substituted *P. ovalis* iron-dismutase [23] and the iron-substituted *E. coli* and *B. stearothermophilus* manganese-dismutases [24,25] retained almost no enzymatic activites. This suggests that both types of isozymes have different geometries in the active site residues. It is expected that the sequence information of the *P. ovalis*-dismutase will facilitate further refinement of the X-ray data and give more insight into a molecular mechanism of the catalytic reaction [26] as well as of the H_2O_2-dependent inactivation reaction [27] of the superoxide dismutase.

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