

Comparison of the Crystal Structures of the Human Manganese Superoxide Dismutase and the Homologous *Aspergillus fumigatus* Allergen at 2-Å Resolution¹

Sabine Flückiger,* Peer R. E. Mittl,[†] Leonardo Scapozza,[‡] Helmi Fijten,* Gerd Folkers,[‡] Markus G. Grütter,[†] Kurt Blaser,* and Reto Crameri^{2*}

Manganese superoxide dismutase (MnSOD) of *Aspergillus fumigatus*, a fungus involved in many pulmonary complications, has been identified as IgE-binding protein. It has been shown also that MnSODs from other organisms, including human, are recognized by IgE Abs from individuals sensitized to *A. fumigatus* MnSOD. Comparison of the fungal and the human crystal structure should allow the identification of structural similarities responsible for IgE-mediated cross-reactivity. The three-dimensional structure of *A. fumigatus* MnSOD has been determined at 2-Å resolution by x-ray diffraction analysis. Crystals belonged to space group $P2_12_12_1$ with unit cell dimensions of $a = 65.88$ Å, $b = 98.7$ Å, and $c = 139.28$ Å. The structure was solved by molecular replacement using the structure of the human MnSOD as a search model. The final refined model included four chains of 199–200 amino acids, four manganese ions, and 745 water molecules, with a crystallographic R -factor of 19.4% and a free R -factor of 23.3%. Like MnSODs of other eukaryotic organisms, *A. fumigatus* MnSOD forms a homotetramer with the manganese ions coordinated by three histidines, one aspartic acid, and one water molecule. The fungal and the human MnSOD share high similarity on the level of both primary and tertiary structure. We identified conserved amino acids that are solvent exposed in the fungal and the human crystal structure and are therefore potentially involved in IgE-mediated cross-reactivity. *The Journal of Immunology*, 2002, 168: 1267–1272.

Superoxide dismutases (SOD³; EC 1.15.1.1) are metalloenzymes that catalyze the dismutation of toxic superoxide radicals to oxygen and hydrogen peroxide and are considered as the first line of defense against the toxicity of oxygen-related radicals (1). The SODs can be subdivided into three classes, depending on the metal cofactor used: iron SOD and manganese SOD (MnSOD), which are homologous in sequence and share a similar $\alpha\beta$ fold (2), and the structurally unrelated Cu, ZnSOD with a Greek key β -barrel fold (3). While Cu, ZnSOD occurs primarily in eukaryotes and iron SOD in prokaryotes, MnSOD is found in both prokaryotes and mitochondria (1). Prokaryotic and eukaryotic MnSODs differ in their usually dimeric and tetrameric structure, respectively (4). Exceptions are the tetrameric MnSODs from the extreme thermophiles *Thermus thermophilus* and *Thermus aquaticus* (5, 6). The crystal structures have been solved for the MnSODs of *Bacillus stearothermophilus* (7), *Thermus thermophilus* (8), *Homo sapiens* (4, 9), *Propionibacterium*

shermanii (10), and *Escherichia coli* (11). In all MnSOD structures known, the active center is exceptionally highly conserved and three histidines and one aspartic acid residue have been identified as metal ligands (10). The MnSOD of *Aspergillus fumigatus*, a fungus involved in many pulmonary complications ranging from benign colonization of the lung to life-threatening diseases (12), has been isolated as an IgE-binding protein from a cDNA library displayed on the surface of filamentous phage (13). Furthermore, it has been shown that the homologous proteins from *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and *E. coli* are able to bind IgE from individuals sensitized to *A. fumigatus* MnSOD (14). These data suggest that MnSODs from phylogenetically distant species represent a family of cross-reactive structures involved in allergic reactions. The most surprising observation was that both *A. fumigatus* and human MnSOD were recognized by IgE Abs from subjects allergic to *A. fumigatus* MnSOD and elicited specific immediate type skin reactions in these individuals (15). Moreover, both human and *A. fumigatus* MnSOD induced proliferation in PBMC of *A. fumigatus*-allergic subjects who showed specific IgE responses and reacted in skin tests to MnSOD (15). These data provide strong evidence for in vitro and in vivo humoral and cell-mediated autoreactivity to human MnSOD in patients allergic to *A. fumigatus*. IgE-mediated autoreactivity to self Ags is an emerging phenomenon that may contribute to exacerbation and/or perpetuation of severe atopic diseases in the absence of exogenous allergen exposure (16, 17). To elucidate whether the mechanisms of these IgE-mediated autoimmune reactions are based on molecular mimicry between conserved B cell epitopes present on allergens and on the structurally related human counterparts, exact comparisons of the three-dimensional structure of the proteins are required. Furthermore, to identify the solvent-exposed amino acids accessible for Ag/Ab interactions on the surface of an allergen, its crystal structure is needed. In this study we

*Swiss Institute of Allergy and Asthma Research, Davos, Switzerland; [†]Institute of Biochemistry, University of Zürich, Zürich, Switzerland; and [‡]Department of Applied Biosciences, Swiss Federal Institute of Technology, Zürich, Switzerland

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² Address correspondence and reprint requests to Dr. Reto Crameri, Swiss Institute of Allergy and Asthma Research, Obere Strasse 22, CH-7270 Davos, Switzerland. E-mail address: crameri@siaf.unizh.ch

³ Abbreviations used in this paper: SOD, superoxide dismutase; rms, root-mean-square; MnSOD, manganese SOD; PDB, Brookhaven Protein Data Bank.

present the crystal structure of *A. fumigatus* MnSOD at 2-Å resolution. The structure of the human MnSOD has been determined at 2.2-Å resolution and deposited in the Brookhaven Protein Data Bank (PDB) with the ID 1ABM (4). We combined data on conserved amino acid residues in the two homologous sequences with data on the solvent accessibility of the residues in the two crystal structures and identified the amino acids potentially involved in IgE-mediated cross-reactivity as a first step to engineer MnSOD molecules devoid of cross-reactive B cell epitopes.

Materials and Methods

Cloning, expression, and purification of *A. fumigatus* MnSOD

A. fumigatus MnSOD was originally isolated from a cDNA library displayed on the surface of filamentous phage using IgE of individuals sensitized to the fungus as ligand (15). The sequence encoding the mature fungal enzyme was cloned as *Bgl*II/*Hind*III fragment into *Bam*HI/*Hind*III-restricted high level expression vector pQE16 (Qiagen, Hilden, Germany), thereby eliminating the [His]₆ fusion tag, and transformed into competent *E. coli* strain M15 by electroporation (18). Single transformants were picked and grown at 30°C in 2YT medium to an OD₆₀₀ of 0.7, induced by 2 mM isopropylthiogalactoside and harvested after another 4 h of incubation by centrifugation at 6,000 × *g* for 10 min at 4°C. Pellets were stored at −80°C until use. Precipitated cell pellets from 1 liter of culture were thawed by transfer to an ice/water bath for 20 min and subsequently frozen for 20 min at −80°C. This cycle was repeated three times to release recombinant protein (19). Thereafter, cell pellets were resuspended in 10 ml of H₂O and placed in an ice/water bath for 60 min. Samples were then centrifuged at 10,000 × *g* (15 min at 4°C) and the supernatants containing the recombinant protein were separated from the pellets. Recombinant MnSOD was stepwise precipitated using ammonium sulfate and precipitates were resuspended in TBS buffer (pH 7.4) and analyzed by SDS-PAGE (4–20%; NOVEX, San Diego, CA) under denaturing, reducing conditions. For further purification the fractions containing recombinant MnSOD were loaded onto a Fractogel EMD trimethyl-aminoethyl Sepharose (M) column (Merck, Darmstadt, Germany) and eluted with a linear NaCl gradient at pH 8. Thirty to 40 mg of pure MnSOD were typically isolated from 1 liter of culture. The protein was reconstituted by dialysis against water containing 0.1 mM MnCl₂. Molecular size and purity were analyzed by denaturing, reducing SDS-PAGE and native-like folding was confirmed by determination of the enzymatic activity (14). For crystallization experiments the recombinant protein was concentrated to 15 mg/ml by ultrafiltration (Centrex UF-2; Schleicher & Schuell, Dassel, Germany).

Crystallization and data collection

A. fumigatus MnSOD was crystallized by the hanging-drop vapor-diffusion method. Among the crystallization conditions screened, the best results were achieved with polyethylene glycol 400 as precipitant. The solution of a crystallization drop was prepared by mixing 2 μl of protein solution (15 mg/ml protein in water, 0.1 mM MgCl₂) with 2 μl of reservoir solution (27% polyethylene glycol 400, 100 mM Tris-HCl, pH 8). The droplets were equilibrated against 500 μl of reservoir solution at 23°C. Rhombohedral crystals grew within a few days.

X-ray diffraction analysis was performed using CuK_α radiation generated by a Nonius FR 591 rotating anode generator (Nonius, Delft, The Netherlands) equipped with a double-focusing mirror system (XRM-216; Prophys, Zürich, Switzerland). Data were recorded on an imaging plate detector (300 mm; Mar Research, Norderstedt, Germany) with a detector to crystal distance of 120 cm. Crystals were flash-frozen in a stream of cold nitrogen gas after soaking in cryoprotectant solution (22% (v/v) glycerol in reservoir buffer) and measurements were performed at 100 K. Under these conditions crystals were stable and diffracted x-rays to 2-Å resolution. A data set was collected for one crystal (85 × 85 × 850 μm) and processed with the DENZO and SCALEPACK crystallographic data reduction package (20). The crystal belonged to space group *P*2₁2₁2₁ with the unit cell parameters *a* = 65.88 Å, *b* = 98.7 Å, and *c* = 139.28 Å. Assuming four molecules of 22 kDa in the asymmetric unit, the calculated Matthews coefficient *V*_M was 2.57 Å³/Da, corresponding to a solvent content of 52.5% (21). Statistics on data collection are given in Table I.

Molecular replacement

The structure of *A. fumigatus* MnSOD was solved by molecular replacement using the program AMORE (22). The structure of the human MnSOD dimer (PDB ID 1ABM) (4), which shares 45% sequence identity with *A. fumigatus* MnSOD, was used as a search model. The cross-rotation func-

Table I. Data collection and refinement statistics

Data collection	
Resolution range (Å)	50–2.0 (2.07–2.0) ^a
Completeness (%)	91.0 (34.8)
<i>R</i> _{sym} ^b	8.3 (33.3)
Number of measured reflections	257,369
Number of unique reflections	56,585
Refinement	
Resolution range (Å)	500–2.0
<i>R</i> -factor (%) ^c	19.4
Free <i>R</i> -factor (%) ^d	23.3
Number of protein atoms	6314
Number of water molecules	745
Number of manganese ions	4
rmsd bond length (Å) ^e	0.006
rmsd bond angles (degrees)	1.1

^a In parentheses, data for the outermost resolution shell are given.

$$^b R_{\text{sym}} = \frac{\sum_{hkl} \sum_i |I_{i(hkl)} - \bar{I}_{(hkl)}|}{\sum_{hkl} \sum_i I_{i(hkl)}}$$

$$^c R\text{-factor} = \frac{\sum_{hkl} ||F_{\text{obs}}| - k|F_{\text{calc}}||}{\sum_{hkl} |F_{\text{obs}}|}$$

^d Free *R*-factor calculated with 10% of the total reflections held aside through refinement for cross-validation.

^e rmsd, rms deviation.

tion calculated with data between 8- and 3.4-Å resolution yielded two peaks of similar heights (8.7 and 8.55 σ, respectively) that were substantially higher than the third-highest peak (3.5 σ). The first translation function was calculated for the search model that was rotated according to the Eulerian angles of the highest peak of the cross-rotation function using data between 8- and 3.3-Å resolution. A significant 9-σ peak was observed for the correctly positioned search model. To find the shift for the second dimer, the search model was rotated according to the Euler angles of the second-highest peak in the cross-rotation function while the parameters for the first dimer were held constant. A 22-σ peak was observed for the correct translation of the second search model. Two copies of the dimeric search model were transformed by α = 90.58°, β = 37.25°, γ = 21.56°, α = 0.1953, β = 0.4101, γ = 0.0802 and α = 86.93°, β = 36.75°, γ = 18.34°, α = 0.6880, β = 0.9146, γ = 0.7541, respectively, to yield the correctly positioned tetramer. After 10 cycles of rigid-body refinement (8- to 3-Å resolution) the *R*-factor was 45.3%.

Refinement

The refinement of the *A. fumigatus* MnSOD model was performed with the crystallography and NMR system program (23). In the initial stages the simulated annealing protocol was used followed by several cycles with the refine protocol that combined simulated annealing with energy minimizing and B factor refinement. Manual corrections of the model were performed by examination of the (2*F*_o − *F*_c) and (*F*_o − *F*_c) maps with program O (24). The manganese atoms were placed in the active sites and a total of 745 solvent molecules were added using the water pick protocol implemented in the crystallography and NMR system. Noncrystallographic symmetry restraints were gradually omitted and data were extended to 2-Å resolution. After several cycles of manual fitting, refinement and addition of water molecules the final *R*-factor was 19.4% and the free *R*-factor was 23.3%. The statistics of the final model are summarized in Table I. The stereochemistry of the *A. fumigatus* MnSOD model was checked with PROCHECK (25). The Swiss-Pdb Viewer (26) was used for figure production.

Calculation of the solvent-accessible area

The solvent-accessible surface area of the final model was calculated with the program Naccess (S. J. Hubbard and J. M. Thornton, Department of Biochemistry and Molecular Biology, University College, London, U.K.) using a probe radius of 1.4 Å (27). The relative residue-accessible area is the ratio of the accessible area of an amino acid in the model to the accessible area of the amino acid in a tripeptide.

Accession number

Structural data is accessible from the PDB (ID code 1KKC).

Results

The overall structure of *A. fumigatus* MnSOD

The crystal structure of *A. fumigatus* MnSOD was solved at 2-Å resolution by molecular replacement and refined to a crystallographic *R*-factor of 19.4% (free *R*-factor 23.3%). The final parameters of refinement are given in Table I. The final model of *A. fumigatus* MnSOD consists of four chains with 199 or 200 amino acid residues (chain A: Gln¹⁴ to Lys²¹³; chain B: Gln¹⁵ to Lys²¹³; chain X: Gln¹⁵ to Gly²¹⁴; chain Y: Gln¹⁴ to Lys²¹³), four manganese atoms, and 745 water molecules. A total of 99.6% of the non-glycine and non-proline residues have main chain dihedral angles within the allowed regions of the Ramachandran plot (28) as determined by PROCHECK. For the three residues (Asp¹⁶⁰ of chains A, X, and Y) that fell in the disallowed region clear electron density was visible. One *cis* proline per monomer (Pro³⁰) in analogy to human MnSOD has been detected.

A. fumigatus MnSOD forms a homotetramer in the crystal (Fig.

1, A and B) which is in agreement with structures of MnSODs from other eukaryotic organisms. The structures of each of the four chains in the crystallographic asymmetric unit are very similar. Superposition of the backbones of chains A and B and X and Y, respectively, results in root-mean-square (rms) deviations of 0.23 and 0.29 Å, respectively (0.51 Å for all atoms in both cases). The dimer formed by chains A and B and the dimer formed by chains X and Y can be superimposed with a rms deviation of 0.27 Å for the main chain atoms and 0.61 Å for all atoms. The two dimers pack tightly together to form the tetramer that has a 222 non-crystallographic symmetry. The overall fold of one monomer and the active site geometry are shown in Fig. 1, C and D. Each chain contains seven α -helices and five strands of β -sheet (Fig. 1C). In the active site of each subunit manganese is bound by three histidines, His⁴⁰, His⁸⁸, and His¹⁷⁸, one aspartic acid, Asp¹⁷⁴, and one water molecule (Fig. 1D). This is in accordance with the metal coordination residues found in all known MnSOD structures (10).

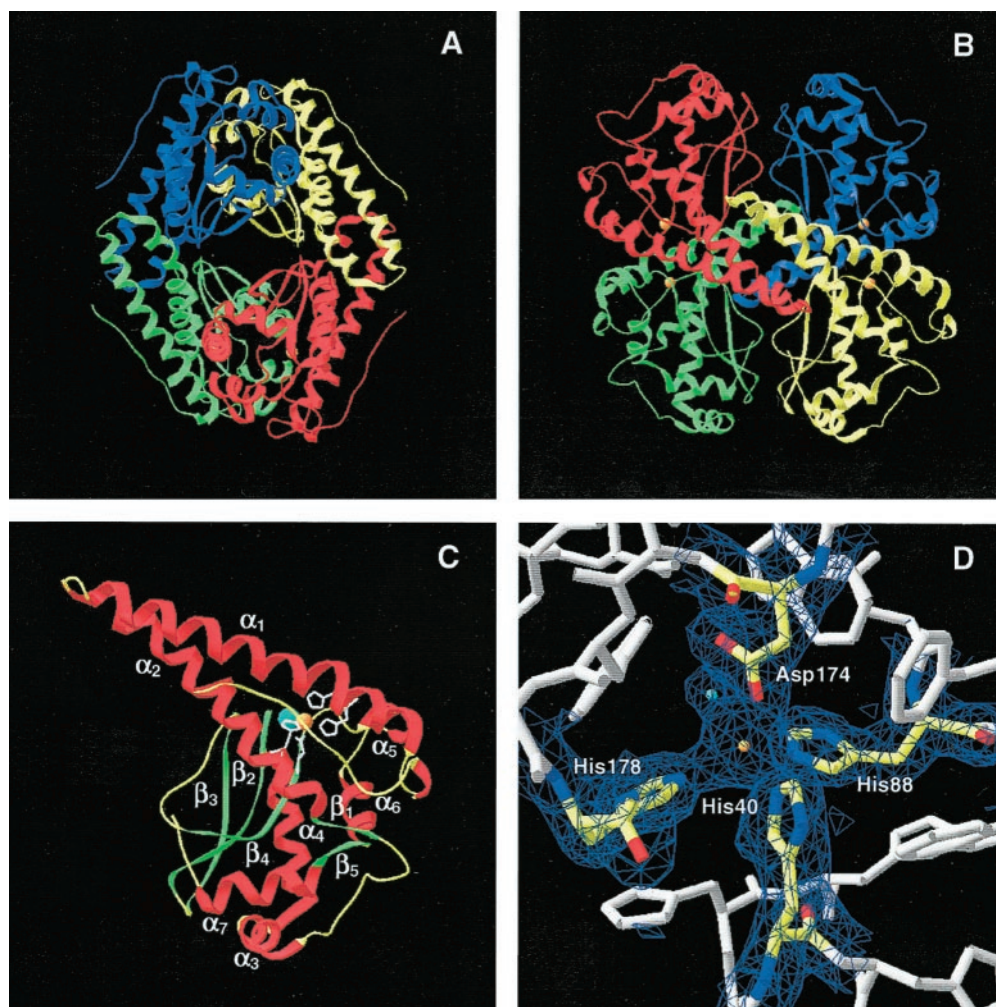


FIGURE 1. A and B, Ribbon diagram showing the assembly of the *A. fumigatus* MnSOD tetramer. The two views are related by a 90° rotation about a vertical axis. The four subunits are indicated by different colors: A, yellow; B, blue; X, red; Y, green. Manganese ions are shown as orange spheres. C and D, Subunit fold and active site geometry of *A. fumigatus* MnSOD. C, The overall structure of one monomer is shown in ribbon representation: α -helices, red; β -sheets, green; turns, yellow. Each subunit contains seven α -helices (α_1 – α_7) and five β -sheets (β_1 – β_5). The manganese ion is shown as orange sphere. The protein ligands coordinating the manganese ion are drawn in a white stick representation. The water molecule occupying the fifth coordination site is shown as light blue sphere (enlarged for better clarity). D, Close-up view of the active site of one subunit of *A. fumigatus* MnSOD. The amino acid residues surrounding the active site are drawn in a stick representation. The manganese ion (orange sphere) is bound by His⁴⁰, His⁸⁸, Asp¹⁷⁴, and His¹⁷⁸. The fifth coordination site is occupied by a water molecule (light blue sphere). The $(2F_o - F_c)$ electron density map contoured at 1 σ is of good quality.

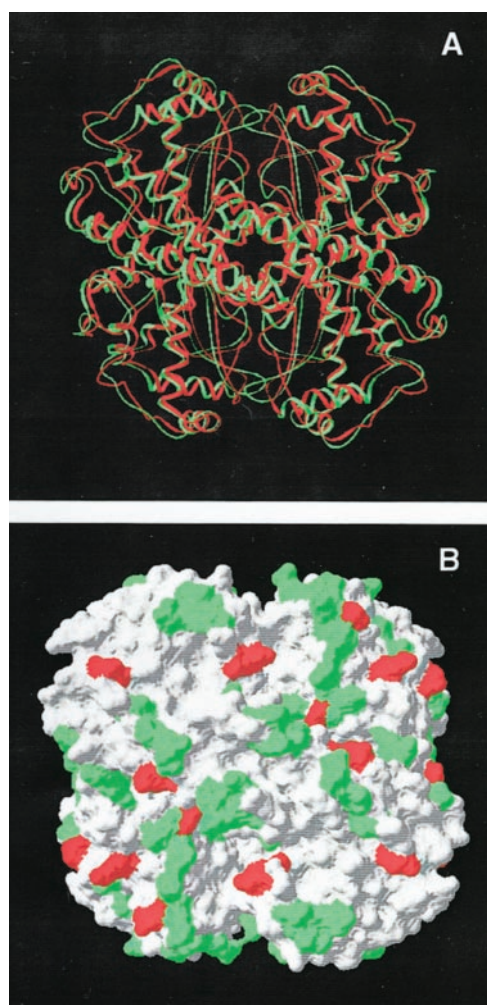


FIGURE 2. A, Superposition of the *A. fumigatus* and the human (PDB ID 1ABM) MnSOD tetramer. The main chain atoms of the human MnSOD (green) were superimposed onto the main chain atoms of the fungal MnSOD (red) with a rms deviation of 1.48 Å. The similarity of the overall structures is apparent. B, The molecular surface of the *A. fumigatus* MnSOD tetramer, determined with a probe radius of 1.4 Å, showing putative IgE-binding amino acid residues. Amino acids that are identical in the fungal and the human sequence and at least 50% solvent exposed in both crystal structures are shown in red. Additional amino acids that are identical or similar and at least 30% solvent exposed are shown in green.

Structural alignment of the fungal and human MnSOD and putative IgE-binding residues

MnSODs belong to a family of highly conserved proteins. On the sequence level *A. fumigatus* MnSOD shares 45% identity and 65% similarity with the human MnSOD. Both enzymes are tetrameric with high similarity in the overall structure. Superposition of the backbones of the two tetramers gives a rms deviation of 1.48 Å (Fig. 2A) while the monomers can be superimposed with a rms deviation in the range of 0.9 Å. Obvious differences in the two structures are detected only in some loop regions. These include the two regions from residues 99 to 101 and 104 to 108 with insertions, the turn between residues 148 and 151, and the region within residue 160 and 170 with deletions in the fungal sequence compared with the human MnSOD sequence (Fig. 3). The program Naccess was used to calculate the solvent-accessible surface area of the two structures and the relative residue-accessible area, using a probe radius of 1.4 Å. Only those residues that are at least partly exposed to solvent can contribute to IgE binding. Therefore, the amino acids that are identical or similar in the fungal and human sequence and solvent exposed in both structures are potentially involved in the IgE-mediated cross-reactivity between the two proteins. Fig. 3 shows a sequence alignment of chain A of *A. fumigatus* and human MnSOD. A total of 101 of the 224 residues in the alignment are identical, and 145 are identical or similar. Of the 101 identical amino acids, 17 are at least 30% and 10 thereof are at least 50% solvent exposed in both structures. These amino acids are considered to be the most likely candidates involved in Ab-mediated (IgE) cross-reactivity of the two proteins. If the similar residues are taken into account, a total of 34 and 20 are at least 30 and 50% solvent exposed, respectively. These putative IgE-binding residues that could be involved in several B cell epitopes elicited by a polyclonal immune response are scattered over the whole sequence (Fig. 3), in agreement with the findings that B cell epitopes elicited by natural exposure are discontinuous (29).

The contribution of amino acid residues to the binding of specific serum IgE to an allergen can be determined by site-directed mutagenesis and testing of the IgE-binding capacity of the mutants. To narrow down the initial number of residues to be subjected to further investigations, the sequences of other MnSODs have been included to identify conserved residues. It has been shown also that the MnSODs from *S. cerevisiae*, *D. melanogaster*,



FIGURE 3. Sequence alignment of *A. fumigatus* and human MnSOD (33). The first line shows the amino acid sequence numbers; the second line shows the secondary structure assignment of *A. fumigatus* MnSOD (h, α -helices; s, β -sheets). Asterisks indicate identical (101) and colons indicate similar (44) amino acids in the two sequences. Of the 101 identical amino acids, 10 are at least 50% solvent exposed in the two structures (shown in red boxes). An additional 24 amino acids are identical or similar in the two sequences and at least 30% solvent exposed in both structures (shown in green boxes).

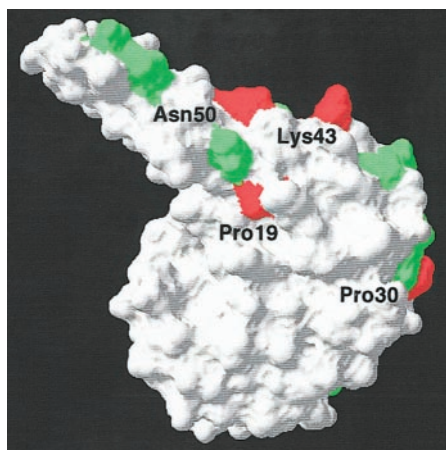


FIGURE 4. The molecular surface of subunit A of *A. fumigatus* MnSOD, determined with a probe radius of 1.4 Å, showing putative IgE-binding amino acid residues. Amino acids that are identical in the sequences of *A. fumigatus*, human, *S. cerevisiae*, *D. melanogaster*, and *E. coli* MnSOD and at least 30% solvent exposed in the crystal structure of the *A. fumigatus* and the human MnSOD tetramer are shown in red and labeled. Pro³⁰ and Asn⁵⁰ are >50% solvent exposed. Of the amino acids that are similar in the five sequences, 11 are at least 30% solvent exposed in the fungal and human structure (shown in green).

and *E. coli* are able to bind IgE from *A. fumigatus*-sensitized individuals (14), suggesting IgE cross-reactivity between these organisms. Sequence alignment of the MnSODs from these organisms together with the MnSODs from *A. fumigatus* and human results in an identity of 19% and a similarity of 34% of the alignment length (48 identical plus 38 similar amino acids). Of the identical residues only four (Pro¹⁹, Pro³⁰, Lys⁴³, and Asn⁵⁰) are at least 30% and only two (Pro³⁰ and Asn⁵⁰) are at least 50% solvent exposed in the fungal and the human crystal structure (Fig. 4). Therefore, Asn⁵⁰ and Lys⁴³ could be the first suitable targets for substitution by site-directed mutagenesis. If the similar amino acids shared between the MnSODs studied are included, five additional residues (Gln²⁹, Glu³⁸, Thr⁴⁷, Glu⁵⁷, and Asn¹⁹⁷) are 30–50% and six (Thr¹⁷, Gln³⁵, Lys⁶¹, Asp¹²³, Lys¹²⁴, and Lys¹⁹³) are at least 50% solvent exposed. The latter could form the second group of candidates for mutational studies.

Discussion

The number of known allergen structures, elucidated either by x-ray crystallography or homology modeling, is increasing. However, so far no characteristic structural or functional features of allergens have been detected that would allow the prediction of the allergenicity of a protein. It seems that in principle any protein has the potential to act as an allergen (30). In contrast to allergenicity, which is an intrinsic property of many different molecular structures, Ab-mediated cross-reactivity among homologous proteins is largely determined by common structural features shared between protein families (29). The only reliable method to determine the complete structure of a B cell epitope is to cocrystallize the Ag with a mAb Fab and determine the structure of the complex by x-ray crystallography. The atomic details of interaction are known for more than 30 Ag-Ab combinations (30). Recently, the first structure of an allergen-Fab complex, the major birch pollen allergen Bet v 1 complexed with the Fab of a murine monoclonal IgG1 Ab, has been elucidated (31). Because crystallization and x-ray crystallography require homogeneous reagents, polyclonal human serum IgE cannot be used. Furthermore, monoclonal human IgE is difficult to obtain. Therefore, mAbs from other sources

must be generated for crystallization of Ag/Ab complexes. An alternative approach to identify IgE-binding epitopes is to determine shared features of cross-reactive allergens on the level of primary and tertiary structure. We used this approach to identify amino acid residues potentially involved in IgE-mediated cross-reactivity between MnSODs of different origin. Notably, humoral and cell-mediated autoreactivity to human MnSOD in patients allergic to *A. fumigatus* has been clearly shown (15). The crystal structure of *A. fumigatus* MnSOD was determined at 2-Å resolution and comparison with the structure of the human MnSOD, which had been determined at 2.2-Å resolution (4), revealed a high similarity between the two structures. Although the fungal and the human sequence share 101 identical amino acids, only 17 thereof are >30% and only 10 are >50% solvent exposed in both structures and therefore likely to be accessible for Ag-Ab interactions (Fig. 2B). Thus a large portion of the conserved amino acid residues is located in the core of the protein and inaccessible for Ag/Ab interactions, including the highly conserved residues defining the active center of the enzyme (Fig. 1D). Because Ab-mediated cross-reactivity between two proteins is dependent on shared structural features on the surface, at least some of the conserved, solvent-exposed amino acids identified in this study must be involved in the binding of polyclonal IgE. These residues are distributed over the whole sequence (Fig. 3), in agreement with the discontinuous structure found in B cell epitopes (29). In contrast, they are clustered over the whole surface (Fig. 2B), indicating that the entire surface of the MnSOD is potentially antigenic, as postulated by Laver et al. (29). The known B cell epitopes, which occupy a buried surface in the range of 540–890 Å² (32), are formed by 15–22 amino acid residues on different surface loops, and their allergenicity is absolutely dependent upon conformation of the native proteins (29). Therefore, correct protein folding is essential for B cell epitope mapping. In the case of recombinant MnSOD, a native-like conformation could be confirmed by measurement of the enzymatic activity of the protein. Moreover, inhibition experiments using different MnSODs and serum of individuals sensitized to *A. fumigatus* MnSOD showed that the enzymes share common IgE-binding epitopes (14). Sequential site-directed mutagenesis of the conserved, surface-displayed amino acid residues described above will allow experimental determination of the contribution of each single residue to the clinically observed cross-reactivity of *A. fumigatus*-allergic individuals toward MnSODs from different species.

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