INFECTION AND IMMUNITY, Aug. 1996, p. 3326–3332 Vol. 64, No. 8
0019-9567/96/$04.00+0
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The Cu,Zn Superoxide Dismutases of *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nidulans*, and *Aspergillus terreus*: Purification and Biochemical Comparison with the *Aspergillus fumigatus* Cu,Zn Superoxide Dismutase

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Received 11 March 1996/Returned for modification 19 April 1996/Accepted 13 May 1996

Cu,Zn superoxide dismutases (SODs) have been purified to homogeneity from *Aspergillus flavus* and *A. niger*, which are significant causative agents of aspergillosis, and from *A. nidulans* and *A. terreus*, which are much rarer causative agents of disease, using a combination of isoelectric focusing and gel filtration fast protein liquid chromatography. The purified enzymes have been compared with the previously described SOD from the most important pathogen in the genus, *A. fumigatus* (M. D. Holdom, R. J. Hay, and A. J. Hamilton, Free Radical Res. 22:519–531, 1995). The N-terminal amino acid sequences of the four newly purified enzymes were almost identical and demonstrated homology to known Cu,Zn SODs from a range of organisms including that from the previously described SOD from *A. fumigatus*. SOD activity was detectable in the culture filtrates of all species, and intracellular Cu,Zn SOD activity as a proportion of total protein was highest in early-log-phase cultures. The specific activities of the purified enzymes were similar, and all four of the newly described enzymes were inhibited by potassium cyanide and diethyldithiocarbamate, known Cu,Zn SOD inhibitors. Sodium azide and *o*-phenanthroline demonstrated inhibition at concentrations from 5 to 30 mM, and EDTA also exhibited a varying degree of inhibition of SOD activity. However, there were differences in the nonreduced molecular masses, the reduced molecular masses, and the isoelectric points of the four newly described SODs and the *A. fumigatus* enzyme; these varied from 55 to 123 kDa, 17.5 to 19.5 kDa, and 5.0 to 5.9, respectively. Of particular note was the observation that the *A. fumigatus* enzyme was thermostable compared with the SODs from the other species; in addition, the *A. fumigatus* enzyme retained all of its activity at 37°C relative to 20°C, whereas the SODs of *A. nidulans* and *A. terreus* lost significant activity at the higher temperature. *Aspergillus* Cu,Zn SOD plays a hypothetical role in the avoidance of oxidative killing mechanisms, and our data suggest that the thermotolerant *A. fumigatus* Cu,Zn SOD would be more effective in such a protective system than, for example, the equivalent enzyme from the more rarely pathogenic *A. nidulans*.

Members of the genus *Aspergillus* are causative agents of a range of disease states in humans which include allergic bronchopulmonary aspergillosis, aspergilloma, and invasive aspergillosis. The incidence of disease has increased significantly, and invasive aspergillosis is a frequent cause of morbidity and mortality in immunocompromised patients (6, 25), especially those in whom there is persistent neutropenia. Of more than 130 members of the genus, 16 species have been documented as causative agents of human disease (19), although only 3 species cause the vast majority of infections. Thus, *Aspergillus fumigatus* is the most common cause of aspergillosis, with *A. flavus* (which may cause invasive aspergillosis, particularly in the paranasal sinuses) and *A. niger* (which may cause invasive pulmonary aspergillosis and aspergilloma) being the second and third most common causative species, respectively (19). Other species, including *A. nidulans* (3) and *A. terreus* (20), are very much rarer causes of disease in humans. Most members of the genus are ubiquitous in nature and are found typically in soil and associated with decaying organic debris. Interestingly, *A. niger* is probably the most common member of the species in the natural environment (19). This would suggest that although individuals may be exposed to a large number of different *Aspergillus* species, only a limited number of species are capable of initiating disease with any regularity, presumably as a result of their possession of various virulence determinants.

A number of these potential virulence determinants have now been investigated, primarily in *A. fumigatus*, and they include a range of proteinases (5, 18, 28), gliotoxins (8, 21), and various phospholipids which may inhibit complement activity (31, 32). The latter may play a role in inhibiting the recognition of fungal elements by immune effector cells; however, as yet, there have been no attempts to identify mechanisms which may be important in protecting the fungal cell from the direct killing mechanisms used by cells such as the neutrophils, which play a major role in the destruction of hyphae (30). Several studies (7, 23) have demonstrated that the addition of extraneous superoxide dismutase (SOD) and catalase can inhibit the ability of normal neutrophils to damage hyphae of *A. fumigatus*. This has produced evidence that the respiratory burst (and the myeloperoxidase-halide system) is important in mediating neutrophil-mediated damage to the nonphagocytosable hyphae. However, by implication, it also suggests that SOD produced by the fungal hyphae itself may, if released into the extracellular environment in combination with catalase, have a role to play in abrogating the neutrophil oxidative burst. Thus, SOD may be a potential virulence factor, acting as an accessory

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to or in concert with the range of other putative virulence factors described for *Aspergillus* species.

Catalase has already been identified from members of the genus *Aspergillus* (9, 15, 27), and a Cu,Zn SOD has recently been isolated from *A. fumigatus* and found to occur in culture filtrates of mid-log-phase cultures (16). Indirect fluorescent-antibody tests and immunoelectron microscopic studies have demonstrated the presence of this enzyme in the cytoplasm and cell wall of *A. fumigatus* conidia and hyphae (13), and this enzyme is specifically recognized by sera from patients with aspergillosis (12). To date, there have been no reports of the identification of SODs from other members of the genus *Aspergillus*. In this paper, we describe the isolation and characterization of Cu,Zn SODs from *A. flavus*, *A. niger*, *A. nidulans*, and *A. ter reus*, together with a comparison of the equivalent enzyme from *A. fumigatus*. This study was designed to determine whether differences in the nature and behavior of the various *Aspergillus* SODs, which may affect their potential to act on neutrophil oxidative killing mechanisms, may correlate, in any way, with the apparent differences in the abilities of members of the genus *Aspergillus* to cause disease. In particular, we have attempted to determine whether a component of the known thermotolerance of *A. fumigatus* (relative to the other *Aspergillus* species) might be the ability of its Cu,Zn SOD to function more effectively at higher temperatures. Any such thermotolerance in the *A. fumigatus* Cu,Zn SOD might, in turn, positively affect its ability to perform a role as a putative virulence factor.

MATERIALS AND METHODS

Culture conditions and growth curves. Isolates of *A. flavus* NCPF 2208 (National Collection of Pathogenic Fungi, Mycological Reference Laboratory, Bristol, United Kingdom), *A. niger* NCPF 7162, *A. nidulans* NCPF 2020, and *A. terreus* NCPF 2026 were inoculated from water cultures onto Sabouraud agar plates and grown at 37°C until a profuse mycelial coverage of the plate was obtained (2 to 5 days). Conidial suspensions of each *Aspergillus* sp. in sterile distilled water were made from each plate, and approximately 2×10^8 conidia were used to inoculate 1-liter volumes of Sabouraud broth in 2-liter flasks which were then incubated on an orbital incubator (120 rpm) at 37°C for between 75 and 100 h. Mycelia were harvested by filtration, freeze-dried, and stored at −70°C as previously described (16), and culture filtrates were also retained.

Growth curves for each of the *Aspergillus* spp. were established by inoculating 2×10^8 conidia into six 2-liter flasks containing 1 liter of Sabouraud's broth. Flasks were then agitated (120 rpm) at 37°C for different periods (up to 168 h) and harvested as detailed above. All studies were performed in duplicate. The mycelia were then freeze-dried for 5 days, and the dry weight was determined.

Culture filtrates were refiltered through Whatman no. 1 paper (Whatman International Ltd., Maidstone, United Kingdom) and then concentrated fivefold on 1-kDa Microsep centrifugal concentrators (Flowgen Instruments Ltd., Sittingbourne, United Kingdom). The SOD activity of samples was detected by the method of Beauchamp and Fridovich (2), with a bovine erythrocyte SOD incorporated as a positive control. SOD activity in all assays was calculated on the basis that 1 U of purified enzyme is capable of causing 50% inhibition to the standard xanthine-xanthine oxidase system. All reagents for these assays were purchased from Sigma Chemical Co. Ltd., Poole, United Kingdom.

Purification of Cu,Zn SOD from *Aspergillus* spp. Mycelia harvested from mid- to late-log-phase cultures (the same time point from which the previously described *A. fumigatus* SOD was purified) were homogenized with 0.5-mm-diameter glass ball ballotini as previously detailed (16). The resulting homogenate was collected, and the beads were washed three times in a total volume of 150 ml of phosphate-buffered saline. This was pooled with the homogenate, and the mixture was centrifuged for 40 min at 4°C at 10,000 × *g*. The supernatant was dialyzed overnight against distilled water in dialysis tubing (size 11; Medicell Int. Ltd., London, United Kingdom), and 50 ml of the supernatant was electrophoretically separated on a Rotofor isoelectric focusing system (Bio-Rad) as previously described (16). The 20 fractions harvested from the Rotofor were then assayed for pH, protein content (by the Coomassie blue method [22]), and SOD activity. Fractions were also analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (see below).

The ratio of SOD to protein (in units per milligram) was determined for each Rotofor fraction. The contents of tubes containing the highest ratio of SOD to protein were pooled and concentrated on 1-kDa Microsep centrifugal concentrators (Flowgen) to a volume of approximately 2 ml. Subsamples (500 μl) of this concentrate were loaded onto a Superose 12 gel filtration fast protein liquid chromatography (FPLC) column (Pharmacia Biotech, St. Albans, United Kingdom) and eluted with 50 mM Tris buffer (pH 8.4) at a flow rate of 0.5 ml/min in 1-ml fractions. Protein content and SOD activity were monitored as described previously, and fractions were concentrated as required. The fractions with the highest SOD activity were concentrated and passed down the gel filtration column a total of four times.

SDS-PAGE and isoelectric focusing. SOD purification was monitored by SDS-PAGE with 10 and 15% polyacrylamide gels as previously described (10, 14). Protein bands were visualized with either Coomassie brilliant blue R-250 (11) or silver stain (Bio-Rad). Isoelectric focusing was also performed (14).

N-terminal amino acid sequencing of purified Cu,Zn SODs. Purified SOD (10 μg) from each of the *Aspergillus* spp. in disruption buffer (10) was loaded into each track of an aged multiwell SDS-PAGE gel (15% polyacrylamide) (16) and run at 150 V for 45 min. The gel was electrophoretically transferred (Western blot [immunoblot]) onto polyvinylidene difluoride membrane (Immobilon P; Millipore Corp., Watford, United Kingdom) (10). The blots were stained for 1 min with Coomassie brilliant blue and destained. Appropriate bands were subjected to N-terminal amino acid sequencing.

Characterization of the *Aspergillus* Cu,Zn SODs. All studies were undertaken with purified SOD (purified from at least two different batches of homogenate in the case of the enzyme from each species) and in duplicate, with the exception of the inhibitor assays and the temperature activity studies, which were performed in triplicate. To determine the effect of pH on SOD activity, the following buffer systems were used: pH 9.0 to 11.0 carbonate buffer (sodium carbonate, sodium bicarbonate [50 mM]) and pH 7.0 to 9.5 Tris HCl (50 mM). SOD activity was assayed with 2 to 5 μg of enzyme per assay.

The effect of various inhibitors on the activity of the *Aspergillus* Cu,Zn SODs was determined as previously described (16). One of the inhibitors, KCN, was also used at a final working concentration of 30 mM as a specific inhibitor of Cu,Zn SOD activity to measure the possible contribution to total SOD activity in cytoplasmic homogenate and culture filtrate made by other classes of SODs (e.g., Mn SOD).

To determine the resistance to heat inactivation of the *Aspergillus* SODs, samples containing between 2 and 5 μg of purified *A. flavus*, *A. niger*, *A. nidulans*, and *A. terreus* SOD, together with the previously purified enzyme from *A. fumigatus*, were incubated at 50 and 70°C for various periods (1 min to 1 h), assayed for residual SOD activity at room temperature, and compared with controls incubated at 25°C for the same periods. The relative activities of each of the five purified *Aspergillus* enzymes at 20 and 37°C were compared by setting up a dual incubation of identical standard assay tubes in addition to SOD-negative controls and assaying them after 0 and 5 min.

RESULTS

Growth curves and SOD production. The growth curve of *A. flavus* relative to detection of Cu,Zn SOD activity in culture filtrate is shown in Fig. 1a and demonstrates that the SOD activity in the culture filtrate increased with time. The other three species, *A. niger*, *A. nidulans*, and *A. terreus*, had virtually identical profiles (data not shown). The use of the specific Cu,Zn inhibitor, KCN, revealed no contribution to total SOD activity by Mn,Fe SOD. However, the ratio of SOD to total cytoplasmic protein fell with time. Figure 1b shows the data for *A. flavus* and is representative of the profile for *A. niger*, *A. nidulans*, *A. terreus*, and *A. fumigatus*, indicating that the expression of intracellular SOD is proportionately higher in the earlier stages of growth. The duplicate growth curves for each species, together with the relevant SOD assays, were highly replicable in these experiments.

Purification of the *Aspergillus* Cu,Zn SODs. The purification of the Cu,Zn SODs from the *Aspergillus* spp. involved three main steps: homogenization of mycelia with removal of cell debris and dialysis, followed by separation by liquid isoelectric focusing (Rotofor) and gel filtration FPLC. Figure 2a, c, e, and g demonstrate the pH, protein content, and SOD activity of the 20 Rotofor fractions for each *Aspergillus* sp. The rotofor profile of the *A. flavus* SOD was superficially indicative of a single form of the enzyme, in contrast to the broader spread seen in the Rotofor fractionation of the other species, which were more suggestive of the presence of isoenzymes. However, subsequent analysis of the purified Cu,Zn SODs by gel-based isoelectric focusing did not reveal the presence of isoenzymes (see below). For the subsequent purification, the fractions with

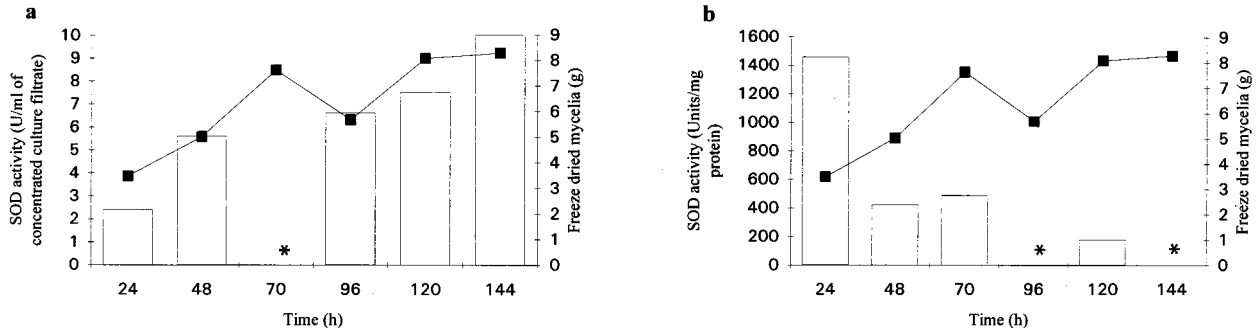

FIG. 1. Growth curve and SOD production in *Aspergillus* spp. as represented by the profile for *A. flavus*. (a) Detection of SOD in culture filtrate during mycelial culture in *A. flavus* (the line graph represents freeze-dried mycelia, and the histogram represents SOD activity in concentrated culture filtrate; (b) ratio of cytoplasmic mycelial SOD production against protein content during mycelial culture for *A. flavus* (the line graph represents freeze-dried mycelia, and the histogram represents SOD activity). *, not assayed. Profiles for the other species were virtually identical (data not shown).

the highest SOD/protein ratio were pooled and concentrated for additional purification by gel filtration FPLC. The purification profile of the first of each of the four sequential gel filtration runs is demonstrated in Fig. 2b, d, f, and h (fractions with the highest SOD/protein ratio were chosen in each case for sequential gel filtration). Table 1 illustrates the purification of the Cu,Zn SOD from *A. flavus*. The purification data for *A. niger*, *A. nidulans*, and *A. terreus* (data not shown) were very similar to those given for *A. flavus*. The specific activities for the enzyme from the four species were all relatively similar, ranging from 3,294 U/mg for *A. nidulans* through 3,322 U/mg for *A. niger* and 3,375 U/mg for *A. terreus* to 3,531 U/mg for *A. flavus.*

SDS-PAGE analysis (Fig. 3) demonstrates the purification to homogeneity of the Cu,Zn SOD from each species. The relative molecular masses of the enzymes, both reduced and nonreduced, ascertained from the SDS-PAGE analysis are summarized in Table 2, along with the pI values of the purified enzyme from each species. The reduced relative molecular masses are similar, the highest being 19.25 kDa for *A. nidulans* and the lowest being 17.5 kDa for *A. terreus*. The nonreduced molecular masses have a much wider variation, from 55 kDa for *A. nidulans* to 123 kDa for *A. flavus*. The pIs of the SODs ranged from 5.0 for *A. niger* to 5.7 for *A. flavus*. There was no evidence for the presence of pI-based isoenzymes in any of the species.

N-terminal amino acid sequences of the purified SODs. N-terminal amino acid sequencing of the *A. flavus*, *A. niger*, *A. nidulans*, and *A. terreus* SODs and comparison of the amino acid sequence with other known sequences showed extensive homology with Cu,Zn SODs from various sources (Table 3). There was no homology with Mn SODs or Fe SODs. The *Aspergillus* sequences demonstrated 100% homology with each other, except for the *A. terreus* SOD, which had one amino acid difference.

Effect of pH on SOD activity. The pH profiles of the SODs from the four species were essentially similar, with no clear and obvious differences over the range 7 to 11. pHs below 7 were not assayed, because the xanthine oxidase is inactive below pH 6.5. Above this pH, the xanthine oxidase is itself pH dependent, producing correspondingly different amounts of superoxide (the substrate for SOD). To define the pH dependence or otherwise of the SOD, the amount of substrate provided was controlled by varying the concentration of the xanthine oxidase added within defined limits.

Effect of known SOD inhibitors on purified *Aspergillus* enzymes. Potassium cyanide and diethyldithiocarbamate completely inhibited the activity of the Cu,Zn SODs from all four species at all working concentrations (data not shown). In contrast, SDS and guanidium chloride demonstrated no inhibition of activity of any of the SODs (data not shown). Sodium azide had a limited inhibitory effect, which was greatest at the highest working concentration in *A. flavus* and lowest in *A. terreus* (Table 4). *o*-Phenanthroline had little or no effect on any of the *Aspergillus* SODs at the lowest working concentration; however, at the highest working concentration, there was an inhibitory effect (up to 69% for *A. nidulans*). Finally, EDTA had a small inhibitory effect at the lowest working concentration on all four SODs. This inhibitory effect increased at higher concentrations up to a maximum of 45% inhibition for *A. nidulans* (Table 4).

Resistance to heat inactivation of the *Aspergillus* SODs and relative activities at 20 and 37°C. All of the *Aspergillus* SODs retained 100% activity after pretreatment for 1 h at 50°C. However, pretreatment at 70°C for 30 min destroyed all enzyme activity in the *A. flavus*, *A. niger*, *A. nidulans*, and *A. terreus* SODs. Under identical conditions, the previously described *A. fumigatus* SOD retained 100% activity (16).

The comparison of the relative activities of the purified *Aspergillus* SODs at 20 and 37°C is demonstrated in Table 5. All the SODs except the *A. fumigatus* enzyme showed reduced activity when assayed at 37°C compared with 20°C. Thus, the *A. flavus* and *A. niger* SODs retained 90 and 92% of activity at the higher temperature whereas the *A. nidulans* enzyme retained only 12% of activity and the *A. terreus* enzyme retained 70% activity.

DISCUSSION

We have previously isolated and characterized a Cu,Zn SOD from *A. fumigatus* (16). The methods used in this original study were directly applicable to the purification of Cu,Zn SODs from other members of the genus *Aspergillus*. On the basis of N-terminal amino acid sequence data and the inhibitory action of the known Cu,Zn SOD inhibitor diethyldithiocarbanate (1), the four newly described SODs are all clearly members of the Cu,Zn SOD group. The specific activities of the four *Aspergillus* SODs described here are broadly comparable, both between each other and with the previously described *A. fumigatus* Cu,Zn SOD (16). In addition Cu,Zn SOD activity is detectable in culture filtrates from all five species and the intracellular production of Cu,Zn SOD, relative to total cytoplasmic protein, is also similar, with the highest levels of SOD being present in early log phase. However, there are clearly

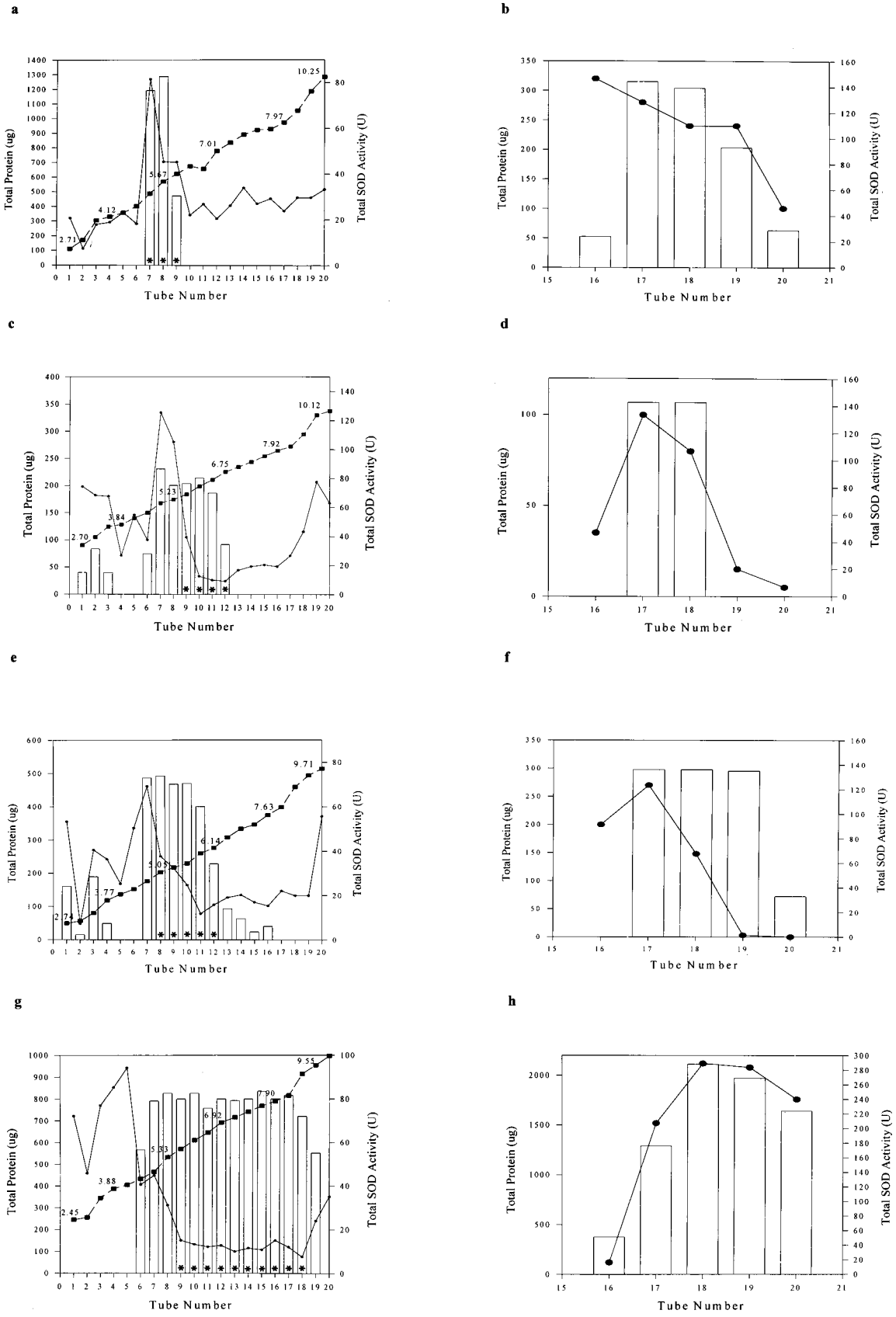

FIG. 2. Purification of the *A. flavus*, *A. niger*, *A. nidulans*, and *A. terreus* Cu Zn SODs. (a, c, e, and g) Rotofor liquid isoelectric focusing (■, pH gradient, ●, protein concentration, histogram, SOD activity of each fraction). Fractions used for subsequent purification are indicated by asterisks. (b, d, f, and h) gel filtration FPLC (●, protein concentration; histogram, SOD activity per fraction).

TABLE 1. Purification of the *A. flavus* Cu,Zn SOD[a]

Step	Total amt of protein (mg)	Total activity (U)	Sp act (U/mg of protein)	Yield (%)	Purification (fold)
Homogenization	512	9,204	17.9	100	1
Liquid isoelectric focusing	5.2	1,119	215	12.1	12
Gel filtration (first run)	1.2	429	357	4.6	20
Gel filtration (fourth run)	0.008	28.25	3,531	0.3	197.2

[a] The purification data for the other three species were similar (not shown).

TABLE 2. Relative molecular masses and isoelectric points of purified SODs from *Aspergillus* spp.

Species	Reduced relative mol mass (kDa)	Nonreduced relative mol mass (kDa)	pI
A. flavus	18.5	123	5.7
A. niger	18.0	69	5.0
A. nidulans	19.25	55	5.1
A. terreus	17.5	68.5	5.3
A. fumigatus[a]	19.5	95	5.9

[a] Data from reference 16.

significant differences between the four enzymes described here and the previously described *A. fumigatus* Cu,Zn SOD, particularly in the physical differences in nonreduced relative molecular mass and isoelectric point. Thus, the intact Cu,Zn SOD of *A. nidulans* has a significantly lower relative molecular mass than that of either *A. niger* or *A. terreus*, and the nonreduced forms of the enzymes in *A. fumigatus* and *A. flavus* were larger still, even though the reduced relative molecular masses of the five enzymes are within a few kilodaltons of each other. The Cu,Zn SODs isolated to date have all been shown to be homodimers, with each subunit being approximately 16 kDa in size, although they may be as large as 18 kDa (4, 26). We have previously hypothesized that the *A. fumigatus* Cu,Zn SOD may exist in its intact form as a tetramer (16). The relative molecular masses reported here for three of the four other *Aspergillus* species are suggestive of different intact enzyme structures, such as dimers and trimers. The much larger nonreduced relative molecular masses of the *A. fumigatus* and *A. flavus* Cu,Zn SODs are mirrored by their significantly higher isoelectric points relative to those of the enzymes from the other three species. The difference in pIs is indicative of differences either in the amino acid content or in glycosylation. In fact, the Cu,Zn SODs isolated thus far from fungi demonstrate approximately 68% homology over the whole molecule, so that with the obvious exception of the N-terminal amino acid sequences, there is clearly the possibility for amino acid differences among the five *Aspergillus* Cu,Zn SODs.

The full significance of such potential differences in structure and amino acid composition and glycosylation lies in their relative contributions to the biochemical characteristics of the *Aspergillus* enzymes. The *A. fumigatus* enzyme, in particular, would appear to behave differently from the other four enzymes. Thus, the *A. fumigatus* enzyme is not inhibited by the metal chelator EDTA, in contrast to the other *Aspergillus* Cu,Zn SODs. In addition, the *A. fumigatus* Cu,Zn SOD is much more resistant to heat inactivation than are the other four *Aspergillus* enzymes, surviving pretreatment at 70°C. Most significantly, the *A. fumigatus* enzyme retains 100% of activity at 37°C compared with 20°C. The latter is in marked contrast to the activities of the *A. terreus* and *A. nidulans* Cu,Zn SODs, which retain only 70 and 12%, respectively, of their 20°C activity at 37°C. Given that the specific activities of all five of the *Aspergillus* enzymes appear to be similar, this represents a significant difference in enzyme function. The resistance of the

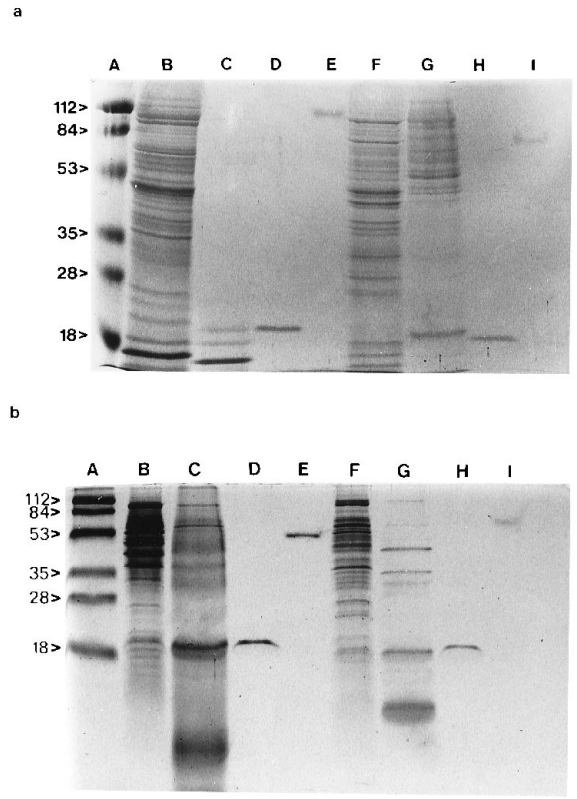

FIG. 3. Purification of *Aspergillus* SODs as determined with a silver-stained SDS-PAGE gel. (a) Lanes (10% polyacrylamide gel): A, Bio-Rad molecular mass markers (in kilodaltons); B, crude *A. flavus* mycelial antigen; C, pooled Rotofor fractions with greatest SOD/total-protein ratio; D, purified *A. flavus* SOD (reduced); E, purified SOD (nonreduced); F to I, *A. niger* mycelial antigen, Rotofor fraction, and pure SOD, respectively. (b) Lanes (15% polyacrylamide gel): molecular masses, mycelial antigens, Rotofor fractions, and purified SOD from *A. nidulans* and *A. terreus*, respectively, in same order as for panel a.

TABLE 3. N-terminal amino acid sequences of *Aspergillus* SODs and the most homologous proteins from the GenEMBL bank

SOD	Amino acid sequence (amino acid no.)	% Similarity[a] (total no. of amino acids)
Aspergillus flavus SOD[b]	VKAVAVLRGD (1–10)	100 (10)
Aspergillus terreus SOD	VKAVAVVRGD (1–10)	90 (10)
Schizosaccharomyces pombe Cu,Zn SOD	VRAVAVLRGD (1–10)	90 (10)
Neurospora crassa Cu,Zn SOD (*sod1*)[c]	VKAVAVVRGD (130–140)	90 (10)
Saccharomyces cerevisiae Cu,Zn SOD[c]	VQAVAVLKGD (130–140)	80 (10)
Onchocerca volvulus extracellular Cu,Zn SOD[c]	RAVAVLRGD (46–54)	80 (9)

[a] Percent sequence similarity in amino acid overlap.
[b] Sequences from *A. niger*, *A. nidulans*, and *A. fumigatus* are identical to this sequence.
[c] Derived from nucleotide sequences.

TABLE 4. Effect of various inhibitors on the activity of *A. flavus*, *A. niger*, *A. nidulans*, and *A. terreus* Cu,Zn SODs

Species	Inhibitor	% Inhibition of SOD activity at final working concn of inhibitor[a]			
		2.5 mM	5 mM	10 mM	30 mM
A. flavus	None (control)	0	0	0	0
	Azide	6.9	10.3	13.8	19
	o-Phenanthroline	0	0	0	20
	EDTA	5.5	11	18.2	32.7
A. niger	None (control)	0	0	0	0
	Azide	1.2	5.1	5.7	9.6
	o-Phenanthroline	0	0	0	50
	EDTA	6.7	12	17.2	29
A. nidulans	None (control)	0	0	0	0
	Azide	1.2	5.1	5.7	9.6
	o-Phenanthroline	4	8.5	26.5	69
	EDTA	6	10	22	45
A. terreus	None (control)	0	0	0	0
	Azide	0	2	2	4
	o-Phenanthroline	0	0	3.5	31.5
	EDTA	2.5	5	9	24

[a] Control with water gives 0% inhibition. The values for full activity (i.e., 0% inhibition) are equivalent to the specific activities given for the SOD from each species (see the text).

A. fumigatus Cu,Zn SOD to heat inactivation and its ability to retain full function at temperatures not normally encountered in the general environment are perhaps unsurprising in an organism which is frequently isolated from self-heating compost heaps in which temperatures often exceed 40°C (29). Although the resistance of the *A. fumigatus* Cu,Zn SOD to heat inactivation and its ability to retain full activity at 37°C have probably evolved in response to such environmental factors, the net result of these characteristics of the enzyme is that at body temperature, compared with the equivalent enzymes from the rarely pathogenic *A. nidulans* and *A. terreus*, the *A. fumigatus* Cu,Zn SOD will be significantly more effective at processing superoxide radicals. The enzymes from *A. flavus* and *A. niger* lose approximately 10% of their activity at 37°C and thus fall somewhere between the *A. fumigatus* enzyme and those from the other two members of the genus studied here. It is possible that the ability to retain full activity at 37°C seen in relation to the Cu,Zn SOD extends to other *A. fumigatus* enzyme systems, such as catalase, and for that matter to other enzymes, such as proteinases, which have been previously implicated in virulence. It is also of note that previous work on the ability of fungal pathogens to survive at 37°C has defined this characteristic in terms of a broad Tem^+ phenotype (as in the case of *Cryptococcus neoformans* [24]); our studies indicate that a component of this phenotype may be the ability of individual enzyme systems to retain full activity at 37°C.

TABLE 5. Relative activity of *Aspergillus* SODs at 20 and 37°C

Species	Activity of SOD (U/mg) at:		% Residual SOD activity at 37°C compared with 20°C
	20°C	37°C	
A. flavus	3,480	3,132	90
A. niger	3,338	3,071	92
A. nidulans	3,250	390	12
A. terreus	3,290	2,303	70
A. fumigatus	3,380	3,380	100

Cu,Zn SODs are usually found in the cytosol of eukaryotic cells, where they fulfill a housekeeping function, by protecting the cell against endogenous superoxide production. It is likely that this is the prime function of the *Aspergillus* Cu,Zn SODs described here in both the saprophytic (soil-dwelling) phase and that isolated from patients. However, we have previously demonstrated that the *A. fumigatus* Cu,Zn SOD is present in substantial quantities in the cell wall (13), and it is also released into culture filtrates, making it available to deal with exogenous sources of superoxide. Whether this extends to playing a role in attenuating the oxidative burst killing mechanisms of neutrophils remains hypothetical, but the apparent correlation between the relative activities of the *Aspergillus* Cu,Zn SODs at 37°C compared with 20°C and the pathogenicity of the five species studied suggests that this is an area worth further study. Such correlations within genera of pathogenic fungi have previously been used to support the role of putative virulence factors (17), and we are presently extending our studies to determine the relative susceptibilities to neutrophil killing of various *Aspergillus* spp. and the hypothetical role of their Cu,Zn SODs in attenuating this process. This work will also be extended to a study of the nature and relative abundance of catalase in *Aspergillus* spp. and the potential impact of this enzyme, in concert with the SOD, on the efficiency of neutrophil killing.

ACKNOWLEDGMENTS

This work was funded by The David and Frederick Barclay Foundation. We also acknowledge Johnson and Johnson, together with The Special Trustees of Guy's Hospital, for associated funding.

Our thanks to L. Packman, Department of Biochemistry, University of Cambridge, for N-terminal sequencing.

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Editor: T. R. Kozel