Detection of a Novel Catalase in Extracts of 
*Mycobacterium avium* and *Mycobacterium intracellulare*

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A novel class of catalase, which differs from the previously described M- and T-catalases of mycobacteria, was detected in strains of *Mycobacterium avium* and *M. intracellulare*. Designated A-catalase, this enzyme resisted inactivation at 68°C, was inactivated by 3-amino-1,2,4-triazole (aminotriazole), and exhibited no peroxidase activity. All of these properties distinguished the enzyme from T-catalase. The A-catalase exhibited a *Km* of 70 mM H₂O₂, which is between the upper and lower extremes of the ranges reported for T- and M-catalases, respectively. The A-catalase appeared to be more hydrophobic than M-catalase and did not react with antiserum to a representative sample of this class. The banding patterns of T- and M-catalases seen by polyacrylamide gel electrophoresis (PAGE) were essentially unaffected by the incorporation of sodium dodecyl sulfate (SDS) into the PAGE system, whereas the single band of A-catalase seen by PAGE without SDS resolved into as many as five bands in the presence of SDS; these bands were all of slower mobility than the original band. The banding pattern seen with SDS appeared to be related more to counterion charge effects than to molecular size increases that could be attributed to SDS complexed to the protein. It remains to be determined whether the multiple A-catalase bands reflect different proteins or different SDS micellar complexes of a single protein.

Two classes of catalase (EC 1.11.1.6) have been recognized previously in extracts of mycobacterial cells. Members of the T class are heat labile, are resistant to inactivation by 3-amino-1,2,4-triazole (aminotriazole), and exhibit Michaelis constants (*Km*) in the range of 3.1 to 6.8 mM H₂O₂. Members of the M class are heat stable, are inactivated by aminotriazole, and exhibit *Km* values in excess of 140 mM H₂O₂ (5, 15); in these respects, they resemble bovine liver catalase. T-catalases from different species of mycobacteria share serologic determinants with one another, as do the M-catalases (13–15). However, no serologic cross-reactivity has been demonstrated between members of the two classes. Quantitative studies on interspecies serologic cross-reactivity within each of these classes have provided the basis for techniques that have been useful in estimating evolutionary divergence of enzyme structure for taxonomic purposes, as well as for identification of individual strains (4, 13–17, 19).

Many species of mycobacteria produce both classes of catalase, and when this occurs, the M-catalase usually accounts for at least two-thirds of the total activity (15). A few species, notably those in the *Mycobacterium terrae* complex, produce only M-catalase. *M. tuberculosis* and *M. bovis* produce only T-catalase; *M. avium* and *M. intracellulare* produce T-catalase, but evidence has been presented for very small amounts of a heat-stable catalase in extracts of some cultures of these species (7, 8, 13, 17). T-catalase, but not M-catalase, exhibits peroxidase-like activity, which has provided the basis for a double-staining technique to distinguish between the bands of the two classes seen on polyacrylamide gel electrophoresis (PAGE) plates (18). Unlike bovine liver catalase (2), both classes of mycobacterial catalase retain their enzymatic activities in the presence of sodium dodecyl sulfate (SDS) (18). We recently undertook a survey of mycobacterial catalases by using PAGE in the presence and absence of SDS. The banding patterns of both M- and T-catalases from most species were essentially unaffected by the addition of SDS to the PAGE reagents. However, SDS caused the disappearance of the single heat-resistant catalase bands in extracts of *M. avium* and *M. intracellulare*; in their stead, bands of slower-migrating catalase appeared. The present study characterizes some of the properties of the newly recognized SDS-interactive mycobacterial enzyme, which we have designated A-catalase.

**MATERIALS AND METHODS**

**Bacterial strains.** Reference strains of 21 serovars of *M. avium* and *M. intracellulare*, as well as a number of strains of other mycobacteria from the Trudeau Mycobacterial Collection (TMC strains), were provided by Anna Tsang, National Jewish Hospital and Research Center, Denver, Colo., under a contract from the United States-Japan Cooperative Medical Sciences Program. The other cultures used in the present study were from the collection of this laboratory; their identities were confirmed by use of a diagnostic probability matrix (20).

**Preparation and standardization of catalases.** The large batches of catalases needed for characterization were prepared from sonic extracts by ammonium sulfate precipitation and ion-exchange chromatography on DEAE-Sephacel (Pharmacia) (15). Some samples were also subjected to molecular exclusion chromatography on Sephacryl S-200 (Pharmacia).

For the smaller crude catalase samples needed for survey purposes, the test strains were grown in single tubes containing 10 ml of medium prepared from Bacto Dubos Broth Base (Difco Laboratories) enriched with Bacto Dubos Oleic Albumin Complex (Difco) and 1% (wt/vol) glycerol. The cells were harvested, washed, and sonicated in the original screw-cap culture tube by using the cuphorn adapter to a model 185D sonifier (Heat Systems Ultrasonics, Inc.) (16). The assays for standardization of T- and M-catalases,
tolerance to 68°C and aminotriazole treatment, and determination of substrate kinetics have been described previously (15).

PAGE. Electrophoretic analyses were conducted in 7.5% polyacrylamide gels (pH 8.3) with and without SDS (6); catalase bands were visualized by the double stain that differentiates between catalases that have peroxidase activity and those that do not (18). Unless otherwise specified in Results, samples subjected to SDS-PAGE were preincubated with 1% SDS for 18 h at 22°C. No reducing agents were used.

Serologic studies. Serial titrations of antiserum against M. scrofulaceum M-catalase were carried out by the seroprecipitation method described previously (15). Titters are expressed as the reciprocal dilution of antiserum required to precipitate 10 mU of a given catalase from a standardized enzyme solution; immunologic distance (ImD) is expressed as 100 times the difference between the log titers of homologous and heterologous sera.

Additional serologic studies were conducted by mixing immunoglobulin G derived from 4 μl of antiserum to M. scrofulaceum M-catalase with 10 mU of different catalases and by applying the mixtures to the wells of a PAGE preparation. A positive serologic reaction prevents migration of the catalase into the PAGE plate.

RESULTS

When bovine liver catalase and crude, unfraccionated samples of sonic extracts of M. tuberculosis H37Rv, M. kansasii TMC 1201, and M. avium SIB-2 were subjected to PAGE without SDS, all three mycobacterial preparations, but not the bovine liver catalase, showed peroxidase-active T-catalase bands; the M. kansasii and M. avium preparations also exhibited nonperoxidatic (NP) catalase bands, as did the bovine liver catalase (Fig. 1A). When these preparations were treated with 1% SDS buffer and then subjected to PAGE with 0.1% SDS, the T-catalase bands of all three mycobacteria and the NP catalase band of M. kansasii were unchanged, but the bovine liver catalase band disappeared (Fig. 1B). The M-catalase band of M. kansasii overlapped the T-catalase band and cannot be recognized in Fig. 1B, but independent SDS-PAGE of these two catalases, separated from one another by ion-exchange chromatography as described below, confirmed their similar mobilities. However, the NP catalase band from M. avium, now designated A-catalase, resolved into bands of markedly decreased mobility, appearing near the top of the gel (Fig. 1B).

Crude sonic extracts from 12 more strains of serovars ascribed to M. avium and 16 strains of M. intracellulare serovars (17) were assayed for catalases that were resistant to aminotriazole and 68°C and were subjected to SDS-PAGE. A total of 25 extracts exhibited significant amounts of heat-resistant catalase, with ratios of aminotriazole-resistant T-catalase to heat-resistant A-catalase in the range of 6.2 to 127 U of T-catalase per unit of A-catalase (median ratio, 39); all of these crude extracts exhibited at least one slowly migrating A-catalase band in SDS-polyacrylamide gels. Crude extracts from three of the M. avium strains yielded ratios of aminotriazole-resistant T-catalase to heat-resistant A-catalase in the range of 204 to 327, and no definite A-catalase bands were seen by SDS-PAGE. The extract of one strain of M. avium demonstrated no aminotriazole-sensitive catalase nor any A-catalase band by SDS-PAGE.

Crude extracts of eight species of slowly growing mycobacteria were subjected to chloride gradient ion-exchange chromatography on DEAE-Sephal, and the fractions were assayed for catalase activity resistant to 68°C and aminotriazole. The T-catalases from M. asiaticum, M. avium, M. gordonae, M. kansasii, M. scrofulaceum, M. simiae, M. szulgai, and M. tuberculosis eluted in the range of 0.20 to 0.28 M chloride. The M-catalases from M. asiaticum, M. gordonae, M. kansasii, M. scrofulaceum, M. simiae, and M. szulgai eluted in the range of 0.29 to 0.32 M chloride. The A-catalase from M. avium eluted in 0.44 M chloride (Fig. 2). It was resistant to inactivation at 68°C but was inactivated by aminotriazole, as was M-catalase.

The M-catalase fractions obtained by DEAE ion-exchange chromatography with extracts of mycobacteria other than M. avium or M. intracellulare behaved the same upon PAGE whether in the presence or absence of SDS. However, the previously purified A-catalase fraction from M. avium resolved into multiple slowly migrating catalase bands in the presence of SDS but not in the absence of the detergent.

FIG. 1. Polyacrylamide gels of crude sonic extracts of mycobacteria without SDS (A) and with SDS (B). Lanes: 1, M. tuberculosis H37Rv; 2, M. kansasii TMC 1201; 3, M. avium SIB-2; 4, bovine liver catalase. Catalases are visualized by a double stain; the dark bands are T-catalases, exhibiting peroxidase activity; clear zones reflect simple catalatic destruction of peroxide by M-catalase (M. kansasii), A-catalase (M. avium), and mammalian catalase. The T- and M-catalases of M. kansasii almost completely overlap in panel B (see text). Migration is from top to bottom in the figure.
The contrast between the major effect of SDS on A-catalase and its lack of effect on the mobility of M-catalase in polyacrylamide gels suggested that the A-catalase was richer in hydrophobic regions. This hypothesis was further tested by comparing the effect of salt concentration on the hydrophobic binding of M-catalase from *M. scrofulaceum* TMC 1314 and A-catalase from *M. avium* SJB-2 to the polystyrene surfaces of microwells of a Nunc-II microtiter plate. The wells received 15 mU of catalase diluted in 100 µl of aqueous Na₂SO₄ of different concentrations. After being incubated for 2 h at room temperature, the contents of the wells were aspirated, with care to remove any droplets of fluid remaining on the surface. Then, without the wells being washed, H₂O₂ substrate in buffered albumin solution was added, and the destruction of the substrate was measured by a microwell assay (16). The affinity of the A-catalase for polystyrene was greater than that of M-catalase, and its dependence on electrolyte was less than that of M-catalase (Fig. 3); in analogy with hydrophobic-interaction chromatography, this result probably reflects the greater hydrophobicity of A-catalase.

To determine whether the effect of SDS on the mobility of A-catalase in polyacrylamide gels reflected an increase in its molecular size, a 500-mU sample of A-catalase in 0.1 ml of 0.1% SDS sample dilution buffer was applied to a 9.5-ml bed of Sephacryl S-200 in a 9-mm-diameter column and was eluted with 0.1% SDS buffer. The void volume of the column was 4.2 ml, and 0.15-ml fractions were collected. Aliquots (20 µl) of the fractions were assayed directly for catalase (0.1% SDS had no effect on the assay), and 50-µl aliquots of all active fractions were subjected to 0.1% SDS-PAGE. The bands of unfraccionated A-catalase seen by SDS-PAGE corresponded to a range of apparent molecular sizes from 125 to 265 kilodaltons, so clean separation of products on the SDS Sephacryl column would not be expected. However, we anticipated enrichment of the slower-moving band in the fraction eluting with the front fractions and enrichment of the faster bands in later fractions. No such enrichment was seen (Fig. 4). This result suggests that the low mobilities of the multiple bands seen by SDS-PAGE are not due to enzyme aggregation or to SDS-catalase complexes with significantly different molecular sizes, but the low mobilities

![FIG. 2.](http://iai.asm.org/) Comparative elution patterns of three classes of catalase in sonic extracts of *M. avium* and *M. scrofulaceum* by gradient chloride ion-exchange chromatography on DEAE-Sephal. T-catalase (T) is heat labile (68°C, 60 s) but resists aminotriazole (10 mg/ml, 2 h); A-catalase (A) and M-catalase (M) tolerate 68°C but are inactivated by aminotriazole.

When samples of partially purified A-catalase were diluted in 2% SDS buffer immediately before electrophoresis in a 0.1% SDS-7.5% PAGE system, five slow-moving SDS bands appeared, but the original NP catalase band seen with SDS-free buffer did not completely disappear. After preincubation of catalase in SDS buffer for 18 h or more, the original band did disappear. Substitution of Triton X-100 for SDS did not shift the position of the A-catalase band or cause the appearance of multiple bands. Catalase preparations preincubated in 0.1% SDS buffer gave the same PAGE pattern as those preincubated with 2% SDS. The time-related effects suggested that the multiple slow-moving bands of A-catalase in SDS were derived from the single faster-moving band that was seen without SDS, rather than from solubilization of some large complex that had failed to penetrate the gel in the absence of SDS. This hypothesis was confirmed by applying a sample of A-catalase to the entire width of a polyacrylamide gel without SDS. After electrophoresis, the NP catalase band was located by sequentially laying paper strips soaked in the catalase-staining reagents along the two edges of the gel; a strip of gel connecting the visualized parts of the band was then excised. The excised strip was macerated and subjected to extraction with electrophoresis buffer containing 1% SDS; the extract was then subjected to PAGE with SDS. The SDS-PAGE plate exhibited the multiple A-catalase bands, confirming that the single NP catalase band in the SDS-free gel was the source of the multiple slower-moving SDS bands.

![FIG. 3.](http://iai.asm.org/) Effect of concentration of Na₂SO₄ on hydrophobic binding of the two heat-stable mycobacterial catalase classes to polystyrene surfaces. The A-catalase (A) was isolated from *M. avium* SJB-2, and the M-catalase (M) was isolated from *M. scrofulaceum* TMC 1314.
FIG. 4. SDS-PAGE patterns of catalase-containing fractions from a molecular filtration column of A-catalase from \textit{M. avium} SJB-2, developed with 0.1% SDS in PAGE sample dilution buffer. The bar graph represents the amount of catalase determined by direct assay of 20-µl aliquots of the 150-µl fractions. The gel filtration bed consisted of 9.5 ml of Sephacryl S-200 in a 9-mm diameter column; the void volume \( (V_0) \) was 4.2 ml, as determined by dextran blue exclusion. \( KAV = (V_e - V_0)/(V_p - V_0) \), where \( V_e \) is the elution volume of a given fraction, \( V_0 \) is the void volume of the column, and \( V_p \) is the packed bed volume of that column.

may be due to charge effects related to counterion regions (10) associated with several coexisting micellar states of the catalase.

The Michaelis constants \((K_m\text{s})\) of mycobacterial T-catalases have previously been shown to fall in the range of 3.1 to 6.8 mM \( \text{H}_2\text{O}_2 \), whereas the \( K_m\)s of M-catalases exceeded 140 mM \( \text{H}_2\text{O}_2 \) (15). The A-catalase from \textit{M. avium} SJB-2 exhibited a \( K_m \) of 70 mM \( \text{H}_2\text{O}_2 \).

Aliquots of selected catalases were assayed in mixtures of 0.1 M mono-, di-, and tribasic sodium phosphate over a pH range of 5 to 11. The A-catalase from \textit{M. avium} SJB-2 exhibited a flat maximum response in the range of pH 7.5 to 10.5 and a shoulder at pH 6.5 (Fig. 5); the T-catalase from the same strain exhibited a well-defined maximum of pH 7.7. The M-catalase from \textit{M. scrofulaceum} TMC 1314 had maximum activity at pH 10.0 but exhibited a secondary shoulder in the range of pH 8 to 10. Bovine liver catalase exhibited a well-defined maximum at pH 8.2. The absence of a single, discrete pH optimum for heat-resistant mycobacterial catalase is in agreement with observations of heat-resistant catalase from \textit{Escherichia coli} (9), which also produces a second catalase that has peroxidase activity (1, 9).

The A-catalase of \textit{M. avium} SJB-2 was even more resistant to 68°C than the relatively resistant M-catalase of \textit{M. scrofulaceum} (Fig. 6); M-catalase showed a 50% decline in

FIG. 5. pH optima of mycobacterial and mammalian catalases. (A) T-catalase from \textit{M. avium} SJB-2; (B) A-catalase from \textit{M. avium} SJB-2; (C) M-catalase from \textit{M. scrofulaceum} TMC 1314; (D) bovine liver catalase.
activity after 400 s at 68°C, whereas A-catalase retained full activity. T-catalase is completely inactivated after only 60 s at 68°C (15).

Rabbit antiserum raised against M-catalase from M. scrofulaceum TMC 1314 was titrated by seroprecipitation (15) against homologous antigen, heterologous M-catalases from M. kansasii TMC 1201 and M. szulgai TMC 1328, and the A-catalase from M. avium SJB-2. The homologous titer of the serum was 1:1,120. The titer against M. kansasii M-catalase was 1:148, and the titer against M. szulgai M-catalase was 1:531, corresponding to ImD scores of 88 and 33, respectively. No evidence of seroprecipitation of the A-catalase from M. avium was seen with dilutions as low as 1:25. Immunoglobulin G corresponding to 4 μl of undiluted serum was mixed with 10 mU of M-catalase from M. scrofulaceum, M. terrae, and M. kansasii or with 10 mU of A-catalase from M. avium and applied to polyacrylamide gels without SDS; the three M-catalase preparations reacted with the antibody and failed to penetrate the gel, but the A-catalase migrated into the gel, unaffected by the antibody.

DISCUSSION

The A-catalase of M. avium and M. intracellulare is distinct from T-catalase in terms of a number of properties: lack of peroxidase activity, resistance to heat, sensitivity to aminotriazole, enzyme kinetics, and electrophoretic behavior in polyacrylamide gels with SDS. Although more similar to M-catalase, the A-catalase differs from M-catalase in terms of its (i) greater resistance to prolonged heating, (ii) elution profile in DEAE-Sephacel ion-exchange chromatography, (iii) hydrophobic nature (reflected in its response to SDS, seen by PAGE, and its binding to plastic surfaces at different salt concentrations), and (iv) failure to react with antibody to M-catalase from M. scrofulaceum. We reported previously that all M-catalases tested showed some cross-reactivity within the upper limit of detectable ImD scores of 200 (15). The ImD between T-catalases from M. avium and M. scrofulaceum is 55, and there is a good correlation between ImD scores of T- and M-catalases. The failure of the A-catalase to react with antibody to M. scrofulaceum M-catalase indicates a great difference in the protein structures of the two enzymes. All of these results lead us to conclude that A-catalase represents a novel third class of mycobacterial catalase.

We cannot yet explain the resolution of the single major slow-moving A-band, seen by SDS-PAGE of crude bacillary sonicates, into multiple bands when partially purified extracts were applied to SDS-polyacrylamide gels. Since all SDS bands of A-catalase are markedly less mobile than the parent band, the effect does not appear to be due to fractionation into subunits. The possibility that SDS caused the reassocication of active subunits or the aggregation of several whole molecules of enzyme was also considered, but the uniform distribution of all bands among the fractions of molecular filtration chromatography (Fig. 4) does not support this explanation. The effect of SDS in reducing the mobility of A-catalase in polyacrylamide gels could be the result of a large counterion charge in the field surrounding the protein-SDS micelles (10). The multiple bands in the purer products might reflect the presence of different molecules of A-catalase or multiple micellar states of a single protein resulting from partial stripping of competing mycobacterial lipid molecules from hydrophobic regions of the enzyme during the purification process. Further exploration of the multiple-band phenomenon will require preparation of large batches of cells, since the A-catalase represents a very small proportion of the total bacillary protein and since its tendency to bind to surfaces leads to severe losses during purification.

Lygren et al. (7) described multiple catalase bands with M. intracellulare, seen by SDS-PAGE, to which molecular sizes of 106 to 348 kilodaltons were ascribed. On the basis of the data we presented here, the seemingly larger catalase molecules probably represent the novel SDS complexes of A-catalase in that species, and their relative mobilities in SDS-polyacrylamide gels probably do not provide a basis for the estimation of their true molecular sizes.

Although we detected A-catalase only in strains of M. avium and M. intracellulare, it may also occur in some species that produce M-catalase. However, since such species tend to produce excessively greater amounts of M-catalase than of T-catalase and since T-catalase is found in M. avium in excessively greater amounts than A-catalase, the A-catalase in any strain that produces both A- and M-catalases would probably represent a very small proportion of the total heat-resistant NP enzyme and would thus be difficult to detect. We have initiated hydrophobic-binding chromatography studies of such possible mixtures.

It is premature to draw conclusions about the biological significance of A-catalase, but we can speculate on some possible avenues for further study. The apparent absence of an NP catalase in M. avium and M. intracellulare has been puzzling in light of the resistance of these organisms to isoniazid. Members of other species that have T-catalase but not M-catalase, such as M. tuberculosis, M. bovis, M. gastri, and M. xenopi (15), are inhibited by 1 μg or less of isoniazid per ml, and species that produce both T- and M-catalase tend to be resistant (20). Shoeb and colleagues (11, 12) have demonstrated that peroxidase, whether of eucaryotic or mycobacterial origin, catalyzes oxidation of isoniazid by H2O2 to yield toxic-free radicals and that NP catalase can inhibit the reaction. Thus A-catalase may play a role in the isoniazid resistance of M. avium and M. intracellulare, whereas M-catalase may provide protection to other species.

Although superoxide dismutase has been recognized in M. leprae, the presence of a catalase of mycobacterial rather than host origin has never been successfully demonstrated in this species (3, 7, 21). Methods that were used would probably have been capable of detecting a T- or M-catalase, had one been present, but the amounts of A-catalase found in mycobacterial extracts described in the present study were very small. A specific search with very large loads of partially fractionated M. leprae extracts by SDS-PAGE and by crossed immunoelectrophoresis (7) with a high-titered antibody to an A-catalase may be justified.

LITERATURE CITED