Targeting and Subcellular Localization of Toxoplasma gondii Catalase

IDENTIFICATION OF PEROXISOMES IN AN APICOMPLEXAN PARASITE

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Achim J. Kaasch and Keith A. Joiner‡

From the Section of Infectious Diseases, Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06520-8022

We sought to identify and characterize peroxisomes in the apicomplexan parasite Toxoplasma gondii. To initiate this process, we first cloned and sequenced the gene for T. gondii catalase (EC 1.11.1.6), a marker enzyme for peroxisomes in eukaryotic cells. The gene predicts a protein of 57.2 kDa and 502 amino acids, and has a strong homology to other eukaryotic catalases. A polyclonal antiserum raised against a glutathione S-transferase fusion protein recognized a single band with a molecular mass of 68 kDa by immunoblot. By immunofluorescence T. gondii catalase is present primarily in a punctate staining pattern anterior to the parasite nucleus. This compartment is distinguishable from other parasite organelles, namely micronemes, rhoptries, dense granules, and the apicoplast. Cytochemical visualization of catalase using diaminobenzidine precipitation gives a vesicular staining pattern anterior to the nucleus at the light level and round, vesicular structures with an estimated diameter of 100–300 nm by electron microscopy. T. gondii catalase has a putative C-terminal peroxisomal targeting signal in the last 3 amino acids (-AKM). Expression of T. gondii catalase in mammalian cells results in peroxisomal localization, whereas a construct lacking the targeting signal remains in the cytosol. Furthermore, addition of -AKM to the C-terminus of chloramphenicol acetyltransferase is sufficient to target this protein to peroxisomes. These results provide the first evidence for peroxisomes in Apicomplexan parasites.

Apicomplexan parasites such as Toxoplasma, Cryptosporidium, Plasmodium, and Eimeria are prevalent worldwide and cause disease in humans and livestock. Toxoplasma gondii is responsible for opportunistic infections in immunosuppressed individuals, in particular AIDS and transplant patients, and congenital infections in newborns. T. gondii is an obligate intracellular parasite that can survive and replicate inside many cell types, including activated macrophages. This suggests that the parasite has mechanisms to evade the macrophage respiratory burst. Previous studies have shown that the parasite is remarkably resistant to hydrogen peroxide and can quench released oxygen radicals. These phenomena are attributed to the high level expression of catalase and superoxide dismutase and their ability to decompose H2O2. Despite their importance, no further effort has been made to characterize these enzymes in detail.

Catalase (EC 1.11.1.6) is a marker enzyme of peroxisomes or microbodies. These subcellular organelles compartmentalize more than 50 different enzymes that intersect with a large variety of anabolic and metabolic pathways. In mammalian cells, these pathways include peroxide metabolism, β-oxidation of fatty acids, and ether phospholipid synthesis. Most peroxisomal enzymes are synthesized on free ribosomes and then post-translationally imported into peroxisomes through an evolutionarily conserved machinery. Several types of peroxisomal targeting signals (PTS) have been identified. PTS1, the C-terminal peroxisomal targeting signal, resides in the last 3 amino acids that share the following consensus motif: (S/T/A/K)X(S/T/A/K)X(S/T/A/K). These phenomena are attributed to the high level expression of catalase and superoxide dismutase and their ability to decompose H2O2. Despite their importance, no further effort has been made to characterize these enzymes in detail.

A well-characterized example of microbodies in protozoan parasites are the glycosomes of the Kinetoplastidae, e.g. Leishmania and Trypanosoma. These organelles, most prominent in the bloodstream form of these parasites, notably compartmentalize glycolytic enzymes but surprisingly do not contain catalase. Protein import into glycosomes is thought to be mediated by a similar mechanism as reported for mammalian peroxisomal import. In the phylum Apicomplexa, however, peroxisomes or related particles have never been defined.

In this study we report the cloning of T. gondii catalase, its subcellular localization in peroxisomes, and its targeting, mediated through a PTS1 signal. This is the first evidence for the existence of a peroxisomal compartment in any Apicomplexan parasite.

MATERIALS AND METHODS

Cell Culture and Growth of T. gondii—The RH strain of T. gondii was maintained by growth in monolayers of either African Green monkey (Vero) cells or human foreskin fibroblasts as described previously (7). Chinese hamster ovary (CHO) cells were cultured in α-minimum E-
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gle's medium supplemented with 3.5% fetal bovine serum, 2 mM l-glutamine, and penicillin/streptomycin at 37 °C in 5% CO₂ atmosphere.

**Cloning and Purification of T. gondii Catalase**—A catalase proximal heme-ligand signature (PROSITE, Swiss Institute of Bioinformatics) was found in two sequences of the EST data base of the Toxoplasma EST project (5). Both lambda phage clones (GenBank® accession numbers W9973 and W6349) were obtained from Genome Systems (St. Louis, MO), in vitro excised with VCSM13 (Stratagene, Menasha, WI) as helper phage and sequenced. The upstream sequence was obtained by 5'rapid amplification of cDNA ends (anchor primers: R1, R2, and R3 see Table I) and by RT-PCR with a degenerate primer (Fdeg) against a highly conserved motif in eukaryotic catalases and two specific reverse primers (R4 and R5). A second 5'rapid amplification of cDNA ends (anchor primers: R6, R7, and R9) revealed the missing upstream region. The sequence was confirmed by RT-PCR with specific primers against the 5'3'-untranslated regions (F4 and R0), and four independent clones were sequenced. For RT-PCR and rapid amplification of cDNA ends applications, RNA was isolated using TRIzol reagent and transcribed with Superscript II reverse transcriptase according to the manufacturer's instructions (Life Technologies, Inc.). DNA sequences were obtained by dyeoxy sequencing of both strands at the W. M. Keck Sequencing Center, Yale University School of Medicine. The strategy for determining the nucleotide sequence of T. gondii catalase is shown in Fig. 1.

**Production of Antibodies to a GST-Catalase Fusion**—The nucleotide sequence that codes for the C-terminal part of T. gondii catalase (aa 335–502) was amplified by PCR (primers ExF and ExR) and cloned in frame into the BamHI and XmaI site of the pGEX-4T-1 expression vector (Amersham Pharmacia Biotech). The resulting glutathione S-transferase (GST) fusion protein was expressed in the vector (Amersham Pharmacia Biotech). The resulting glutathione-S-methyl isopropyl-1-thio-

**Immunoblot with Anti-T. gondii Catalase**—About 5 × 10⁶ parasites (or an equivalent amount of host cell material) were collected by centrifugation, separated on a 10% SDS-polyacrylamide gel, and transferred onto nitrocellulose membrane. The membranes were probed with a 1:500 dilution of anti-T. gondii catalase antisera, followed by a 1:2000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG antibody (Calbiochem). The signal was visualized with the ECL kit (Amersham Pharmacia Biotech). As a control, incubation with preimmune serum at a 1:100 dilution was performed.

**Antibody Purification**—The rabbit antisera to T. gondii catalase was purified by adsorption to the 47-kDa band of the purified recombinant antigen on an immunoblot, as described previously (10). Alternatively, the antisera was affinity purified against the GST fusion protein coupled to cyanogen bromide-activated agarose (Sigma). The antibody was bound to the column in phosphate-buffered saline (PBS), pH 7.4, eluted with 0.2 M glycine, pH 2.8, and the pH of the eluate was immediately readjusted by adding 1 M Tris base.

**Immunofluorescence Assay**—The immunofluorescence assay (IFA) for intracellular parasites was performed as described previously (11), using 3% paraformaldehyde as fixative. Extracellular parasites were fixed in ice-cold acetone for 15 min and then processed similarly. Controls included incubation with preimmune serum, secondary antibody alone, and competition experiments with the purified GST fusion protein. The following dilutions of antibodies were used: fluorescein isothiocyanate (FITC)-linked goat anti-rabbit IgG (Calbiochem) at 1:500; rhodamine-linked goat anti-mouse (Roche Molecular Biochemicals) at 1:500; affinity purified rabbit anti-catalase at 1:5; murine monoclonal anti-rop3,4,3 (T3 4A7) at 1:250; murine monoclonal anti-GRa3 (T6 2H11) at 1:250; and murine monoclonal anti-MIC2 (D2R2) at 1:250. All monoclonal antibodies were generously provided by J. F. Dubremetz, Lille, France. The apoplastic was stained with 4',6-diamidino-2-phenylindole (12).

**For IFA on CHO cells, the same protocol as for intracellular parasites was applied. Antibody concentrations used were as follows: rabbit anti-chloramphenicol acetyltransferase antibody (5 Prime 3 Primary Inc., Boulder, CO) at 1:500; rabbit anti-catalase antisera at 1:250; and rabbit anti-SK antibody (Zymed Laboratories Inc., South San Francisco, CA) at 1:500.

**Cytological EM**—Diaminobenzidine (DAB) staining for catalase was performed according to established procedures (13). In brief, parasites were fixed for 15 min with 1.5% glutaraldehyde in 0.1 M Pipes buffer, pH 7.4, washed once in PBS, pH 7.4, and one time in Teorell-Stenhagen buffer (100 mM borate acid, 10 mM phosphoric acid, 2.5 mM citric acid brought to pH 10.5 by NaOH). Following a 1-h preincubation in DAB (2 mM in T/S) at room temperature, H₂O₂ was added to a final concentration of 0.15% for 1–3 h. After washing in T/S and 100 mM sodium cacodylate buffer, pH 7.4, the sample was fixed overnight with 2.5% glutaraldehyde in 100 mM sodium cacodylate at 4 °C and embedded in Epon (Electron Microscopy Sciences, Fort Washington, PA). Ultrathin 60–80 nm sections were mounted on Formvar-coated nickel grids (Electron Microscopy Sciences). The sections were not counterstained in order to facilitate recognition of the DAB precipitate. As a control, DAB incubations were done in the presence of 20 mM 3-amino-1,2,4-triazole or alternatively H₂O₂ was omitted. For light microscopy, the above procedure was performed on coverslips that were mounted and examined after the H₂O₂ incubation.

**Fig. 1. Sequencing strategy.** Catalase of T. gondii was cloned by a combination of techniques. The bars show the method used to determine the indicated part of the sequence. The 5'- and 3'-untranslated regions are shown by open boxes; the coding sequence is shown by the black box. The arrows denote the positions of the primers.
Fig. 2. Nucleotide sequence and predicted translation of *T. gondii* catalase. The *T. gondii* catalase sequence is available in GenBank™ (accession number AF136344). The underlined bases denote the position of the respective oligonucleotides in Table I.

**TABLE I**

| Underlined Bases | Position in Table I |
|------------------|---------------------|
| CAT ACT CAT CAT | CAT ACT CAT CAT |
| ACC ACC ACC ACC | ACC ACC ACC ACC |
| GAC GAC GAC GAC | GAC GAC GAC GAC |
| TCC TCC TCC TCC | TCC TCC TCC TCC |
| GAC GAC GAC GAC | GAC GAC GAC GAC |
| TCC TCC TCC TCC | TCC TCC TCC TCC |
| GAC GAC GAC GAC | GAC GAC GAC GAC |
| TCC TCC TCC TCC | TCC TCC TCC TCC |
Import Assays in Mammalian Cells—Chloramphenicol acetyltransferase (CAT) and *T. gondii* catalase expression in CHO cells was driven by the cytomegalovirus promoter. CAT-AKM was ligated into the HindIII and KpnI site (CAT-FHind, CATAKMKpn) of pcDNA3.1/Zeo(1) (Invitrogen, Carlsbad, CA). For a cytosolic control, CAT in the pcDNA3.1/Zeo/CAT vector (Invitrogen) was used. *T. gondii* catalase was cloned into NheI and BamHI sites of pcDNA3.1/Zeo(1) using NheCat, AKMBgl (full-length catalase), and CYPBgl (C-terminal truncation) as primers. CHO cells were transfected using SuperFect (Qiagen, Valencia, CA), following the manufacturer’s instructions. The cells were plated on coverslips 24–48 h after transfection, and IFA was performed 24 h later.

**RESULTS**

Analysis of the Catalase Sequence—The deduced amino acid sequence of *T. gondii* catalase encodes a protein of 502 residues with a predicted molecular mass of 57.2 kDa (Fig. 2). Evaluation of the protein sequence by BLASTP (14) shows a high degree of identity with other catalases (e.g., 53% identity with human catalase), whereas comparison of the *T. gondii* catalase nucleotide sequence with eukaryotic catalases reveals only minimal similarity. This suggests, along with additional data provided below (Fig. 4), that the deduced catalase sequence is derived from *T. gondii* and not from host cell contamination.

Further analysis confirms that this protein is indeed a catalase. Foremost, both catalase consensus patterns (15) are present: the active site signature at position 53–69 (consensus, (IF)X(RH)X4(EQ)RX2HX2(GAS)-(GASTF)-(GAST)) and at position 343–351 the proximal heme ligand signature (consensus, R-(LIVMFSTAN)-F-(GASTNP)YX3D(AST)-(QEH)) (Fig. 3).

**FIG. 3.** Sequence alignment for catalases of various species. Catalases from the following species are aligned with *T. gondii* catalase (NCBI accession number AF136344): *H. sapiens* (NCBI accession number 179950), *C. elegans*, *S. cerevisiae*. Dots in the sequence indicate that the residues are identical to the aligned *T. gondii* residues, whereas a dash denotes a gap. Symbols below the alignment show residues that bind the protein side chains of the heme by O, O indicates residues neighboring the active center; O indicates NADPH binding participants; ♦ denotes residues proposed to be involved in the catalytic mechanism; O shows residues in the environment of NADPH, ♦ indicates residues that build the substrate channel; ♦ denotes residues that are critical for tetramerization. The putative PTS1 signals are printed in bold, whereas the active site signature (aa 53–69) and the proximal heme-binding signature (aa 343–351) are underlined. The alignment was performed by ClustalW1.73.
to the human sequence with the exception of Thr-190 and Glu-448 (16).

The substrate H₂O₂ is thought to diffuse to the active site through an approximately 30-Å long channel (16). Residues that build the walls of the narrow part of this channel are identical to the human sequence (Val-105, Ala-106, Asp-117, Pro-118, Phe-142, Phe-143, Phe-150, Phe-153, Ile-154, Val-185, Leu-188, Gln-157, and Lys-158), suggesting that the range of potential substrates is similar (19).

Most catalases exist as homotetramers. Residues that are known to be involved in subunit interactions in *Proteus mirabilis* catalase are identical with the corresponding residues in *T. gondii* catalase (Leu-50, Asp-54, Phe-53, Asp-349, Arg-55, *P. mirabilis* alignment not shown) (16). Human and bovine catalases, which have a longer C terminus, also stabilize the subunit interactions with these additional amino acids.

*T. gondii* catalase contains a putative peroxisomal targeting signal (PTS1) at the C terminus. The last three residues of the protein (-AKM) match the consensus motif of C-terminal peroxisomal targeting signals ((S/T/A/G/C/N)-(R/K/H)-(L/I/V/M/A/F/Y)). This motif has been shown to be a PTS1, albeit a weak one, when linked to CAT and expressed in monkey kidney (CV1) cells (20).

**Anti-catalase Antiserum Recognizes a 63-kDa Protein**—Antiserum was raised to a fusion protein between GST and residues 335–502 of *T. gondii* catalase. This region of *T. gondii* catalase is comparatively divergent to human catalase and was therefore chosen as antigen.

As expected the antiserum recognizes the recombinant GST-catalase fusion protein migrating at 47 kDa (Fig. 4, 3rd lane). This antiserum recognizes a single band on immunoblot, when tested against whole parasites (Fig. 4, 1st lane). This protein migrates at 63 kDa, which is in reasonable agreement with the predicted size (57.2 kDa) of *T. gondii* catalase. The parasite protein expressed in CHO cells also migrates with the same *M*ᵣ (data not shown). No signal is observed with uninfected host cells (Fig. 4, 2nd lane) or with preimmune serum (data not shown), indicating that the band constitutes a *T. gondii* protein.

**Catalase Localizes to a New, Distinct Compartment Anterior to the Nucleus by IFA**—Anti-catalase antiserum recognizes a punctate, beaded structure anterior to the nucleus by immunofluorescence (Fig. 5). Additionally there is a weak but specific, diffuse signal throughout the parasite, which might correspond to a cytosolic pool of the protein. Colocalization
experiments show that the catalase localization is distinct from other parasite organelles, namely micronemes, rhoptries, dense granules, and the apicoplast (Fig. 6).

**Catalase Localization by Histocytochemistry**—Catalase was localized in *T. gondii* by histocytochemistry. Catalase can be visualized by precipitation of diaminobenzidine (DAB) under conditions of mild fixation, high substrate (hydrogen peroxide) concentrations, and high pH buffer. The precipitate can be seen in light microscopy as dark spots inside the parasite (Fig. 7c). Treatment with 20 mM 3-amino-1,2,4-triazole, a catalase inhibitor, abolishes the staining (data not shown).

By electron microscopy a specific reaction product appears in a juxtanuclear position as a round structure of about 100–300 nm (Fig. 7, a and b). It frequently possesses a core with a crystallloid shape. Reaction product in the mitochondrion is not specific for catalase, since it is still prevalent under inhibitor treatment or when the substrate hydrogen peroxide is omitted (data not shown).

**Putative PTS1 Is Necessary and Sufficient for Targeting to Mammalian Peroxisomes**—The functionality of the C-terminal -AKM motif as a peroxisomal targeting signal was tested by expressing *T. gondii* catalase in CHO cells, which provide a well defined peroxisomal compartment. Transiently expressed full-length *T. gondii* catalase localizes to particulate structures that show the same staining pattern as peroxisomes (Fig. 8, c and e). The truncated version of catalase, without the last three amino acids, yields a diffuse staining pattern (Fig. 8d), which is consistent with a predicted cytosolic localization.

To address the question whether the -AKM motif is sufficient to target proteins to peroxisomes, CAT was used as a marker protein. CAT resides in the cytosol and therefore has a diffuse staining pattern (Fig. 8b). Addition of -AKM to the C terminus (CAT-AKM) relocates the protein to particulate structures (Fig. 8a), suggesting a peroxisomal localization. To confirm targeting of CAT to peroxisomes we expressed CAT-SKL, which is known to reside in peroxisomes (data not shown).

**DISCUSSION**

Catalase is the characteristic marker enzyme of peroxisomes and is highly conserved across species. The deduced amino acid sequence of *T. gondii* catalase has typical features of eukaryotic catalases, e.g., residues that are known to be involved in heme binding, NADPH binding, tetramerization, and protein import are strongly conserved. This suggests a similar catalytic mechanism and localization in peroxisomes.

We addressed the latter by analyzing catalase distribution by the electron and light microscope. We identified a distinctive, vesicular compartment anterior to the nucleus that does not overlap with known organelles of the parasite. Since peroxisomal proteins reach their subcellular destination through a specific import mechanism that is mediated by evolutionarily conserved signals, we investigated the functionality of the PTS1-type import signal (-AKM) of *T. gondii* catalase. This PTS1 motif is necessary and sufficient to target *T. gondii* catalase and chloramphenicol acetyltransferase to mammalian peroxisomes. Although we have not identified a peroxisomal...
membrane, due to the difficulties in detection of DAB precipitate in counterstained sections, our data remain consistent with the presence of peroxisomes in T. gondii.

The enzymatic activity of catalase, the decomposition of \( \text{H}_2\text{O}_2 \), and therefore the protection from endogenously produced oxygen radicals are well established. One can also speculate about protection from exogenous \( \text{H}_2\text{O}_2 \), a mechanism that could potentially facilitate parasite survival during infection. However, in this case, a compartmentalization of catalase seems to be unfavorable, since \( \text{H}_2\text{O}_2 \) would have to diffuse through the parasite cytosol toward the enzyme. On the other hand, the observed pool of catalase in the parasite cytosol could be sufficient to clear exogenous hydrogen peroxide, whereas the peroxisomal pool might be needed to scavenge the \( \text{H}_2\text{O}_2 \) production of peroxisomal enzymes.

The biochemical role of peroxisomes in T. gondii has yet to be elucidated. Of particular interest will be to determine the contribution of peroxisomal enzymes in lipid synthesis or metabolism. The parasite seems to be deficient in its ability to synthesize cholesterol and selected phospholipids de novo\(^2\) and presumably acquires these components from the host cell. On the other hand, enzymes involved in fatty acid biosynthesis are imported into the T. gondii apicoplast, a chloroplast remnant present in many if not all Apicomplexan parasites (21, 22). Given the presence in T. gondii of genes for isocitrate lyase and malate synthase, two members of the glyoxylate cycle found in plant glyoxysomes, a role for T. gondii peroxisomes in fatty acid conversion in succinate and ultimately to glucose is also possible.\(^3\) Determining the relative contribution of these three pathways to lipid homeostasis within T. gondii will require a detailed understanding of the biochemical composition of the T. gondii peroxisomes and may ultimately provide insights into novel therapeutic approaches.

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