Evidence for Radical Formation at Tyr-353 in Mycobacterium tuberculosis Catalase-Peroxidase (KatG)*

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**Mycobacterium tuberculosis** KatG is a heme-containing catalase-peroxidase responsible for activation, through its peroxidase cycle, of the front line antituberculosis antibiotic isoniazid (isonicotinic acid hydrazide). Formation of Compound I (oxyferryl heme-porphyrin π-cation radical), the classical peroxidase intermediate generated when the resting enzyme turns over with alkyl peroxides, is rapidly followed by production of a protein-centered tyrosyl radical in this enzyme. In our efforts to identify the residue at which this radical is formed, nitric oxide was used as a radical scavenging reagent. Quenching of the tyrosyl radical generated in the presence of NO was shown using electron paramagnetic resonance spectroscopy, and formation of nitrotyrosine was confirmed by proteolytic digestion followed by high performance liquid chromatography analysis of the NO-treated enzyme. These results are consistent with formation of nitrosotryptophan by addition of NO to tyrosyl radical and oxidation of this intermediate to nitrotyrosine. Two predominant nitrotyrosine-containing peptides were identified that were purified and sequenced by Edman degradation. Both peptides were derived from the same *M. tuberculosis* KatG sequence spanning residues 346–356 with the amino acid sequence SPAGAWQYTAK, and both peptides contained nitrotyrosine residue 353. Some modification of Trp-351 most probably into nitrosotryptophan was also confirmed, nitric oxide was used as a radical scavenging reagent. Quenching of the tyrosyl radical generated in the absence of NO showed using electron paramagnetic resonance spectroscopy, and formation of nitrotyrosine was confirmed by proteolytic digestion followed by high performance liquid chromatography analysis of the NO-treated enzyme. These results are consistent with formation of nitrosotryptophan by addition of NO to tyrosyl radical and oxidation of this intermediate to nitrotyrosine. Two predominant nitrotyrosine-containing peptides were identified that were purified and sequenced by Edman degradation. Both peptides were derived from the same *M. tuberculosis* KatG sequence spanning residues 346–356 with the amino acid sequence SPAGAWQYTAK, and both peptides contained nitrotyrosine residue 353. Some modification of Trp-351 most probably into nitrosotryptophan was also found in one of the two peptides. Control experiments using denatured KatG or carried out in the absence of peroxide did not produce nitrotyrosine. In the mutant enzyme KatG(Y353F), which was constructed using site-directed mutagenesis, a tyrosyl radical was also formed upon turnover with peroxide but in poor yield compared with wild-type KatG. Residue Tyr-353 is unique to *M. tuberculosis* KatG and may play a special role in the function of this enzyme.

Bacterial catalase-peroxidases (KatGs)** are unusual in that in addition to typical peroxidase activity with broad specificity they also have high catalase activity (1, 2). KatG enzymes have little sequence homology with typical catalases but high homology to yeast cytochrome c peroxidase and ascorbate peroxidase, other Class I peroxidases (3). *Mycobacterium tuberculosis* KatG is of special interest to us because of its importance in the activation of isoniazid (INH), the first and still most important antituberculosis antibiotic used to treat tuberculosis. The catalytic mechanism of *M. tuberculosis* KatG is under investigation in the context of the activation of this prodrug and because of widespread resistance to INH that arises due to point mutations in the katG gene (4–7).

Our recent work demonstrated that the catalytic cycle of *M. tuberculosis* KatG includes a classical Compound I (oxyferryl [Fe(IV)=O] porphyrin π-cation radical) (8) that is catalytically competent, being reduced by a number of substrates including INH without evidence for stabilization of Compound II (8). It has also been shown that KatG Compound I decays quickly in the absence of exogenous substrates, consistent with an endogenous electron transfer pathway producing a tyrosyl radical (9, 10). The results of kinetic studies suggested that a unique radical is generated in a rate-limiting step presumably through oxidation of the phenolic ring of tyrosine by Compound I (9). Additional evidence in favor of this reaction pathway came from study of the KatG(Y229F) mutant in which formation of Compound II from Compound I is kinetically coupled to formation of tyrosyl radical (10). A similar pathway is responsible for initiation of the cyclooxygenase activity of prostaglandin-H synthase (PGHS) (11, 12).

Tyrosyl radicals are formed in other heme and non-heme proteins and enzymes such as myoglobin (13), photosystem II (14), ribonucleotide reductase (15), bovine catalase (16), turnip peroxidase (17), and prostaglandin-H synthase (18) from endogenous electron transfers to hypervalent metal centers. The tyrosyl radicals of PGHS, ribonucleotide reductase, and photosystem II are directly involved in their catalytic processes (19), while in the other examples, functional roles may be provided by the radical, but these have not yet been elucidated. The finding that in *M. tuberculosis* KatG the yield of tyrosyl radical upon turnover of the resting enzyme with a small excess of alkyl peroxide is great enough to suggest a potential functional role along with the evidence for a rapid reaction of the radical with small molecules, including the antibiotic INH (9), suggests that this radical might contribute to INH activation and or production of the bactericidal molecule known to require KatG activity in *M. tuberculosis* treated with the antibiotic.

Identification of the tyrosine residue that forms a radical in *M. tuberculosis* KatG is necessary for a more complete understanding of the catalytic cycle of this enzyme. We have approached this issue by application of a radical scavenging tech-

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§ The abbreviations used are: KatG, catalase-peroxidase; INH, isoniazid; PGHS, prostaglandin-H synthase; WT, wild-type; KatGY353F, Y353F mutant of KatG; PROL/NO, proline NONOate; PAA, peroxycetic acid; EPR, electron paramagnetic resonance; RFQ-EPR, rapid freeze-quench EPR; HPLC, high performance liquid chromatography; TPCK, 1-tosylamido-2-phenylethyl chloromethyl ketone; MALDI, matrix-assisted laser desorption ionization.
nique that takes advantage of the very rapid reaction between tyrosyl radical and nitric oxide, which has a rate near the diffusion limit (1–2 × 10^9 M^−1 s^−1) (20). Nitric oxide has been shown to quench the tyrosyl radicals formed during turnover of photosystem II (21) and in the enzymes ribonucleotide reductase (22), PGHS-1 (23), and PGHS-2 (24). Furthermore an initial reversibly formed nitrosyltyrosine intermediate is fused through the decomposition of the nitrosyltpeptide in the case of PGHS due to the peroxidase activity of this enzyme (24). The potential for similar reactions in KatG led to the present study.

A nitric oxide donor, proline NONOate (PROLI/NO), was used here to scavenge the tyrosyl radical during turnover of the resting enzyme with peroxide. Radical quenching as well as formation of nitrotyrosine was confirmed in these experiments. Peptide mapping and subsequent sequencing analysis demonstrated that nitrotyrosine was produced at tyrosine 353, a unique tyrosine found in the M. tuberculosis enzyme but not in other catalase-peroxidases.

EXPERIMENTAL PROCEDURES

Materials—PROLI/NO was purchased from Cayman Chemical Co. Proteinase K, TPCR-treated trypsin, trifluoroacetic acid, 3-nitrotyrosine, and sodium acetate were purchased from Sigma. All other chemicals were the highest purity grade available. Peroxoyacetic acid (20%) was treated with catalase, as previously described, to remove hydrogen peroxide (10).

M. tuberculosis KatG Preparation—Wild-type and mutant M. tuberculosis KatG were prepared from an overexpression system in Escherichia coli strain EM32 which contains the M. tuberculosis katG gene in the vector pKstII (a gift from Stewart Cole, Institut Pasteur, Paris, France). Enzyme purification and quantification were carried out according to previously published procedures (8) all in 20 mM potassium phosphate buffer, pH 7.2.

Enzyme Activity Assay—Peroxidase activity was measured spectrophotometrically in 50 mM sodium acetate buffer, pH 5.5, using tert-butyl hydroperoxide with 3-dianisidine forming absorbance at 460 nm (25). One unit of peroxidase activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of 3-dianisidine/min.

Catalase activity was measured according to a previously reported method (25).

Reaction of KatG with NO—NO donor PROLI/NO was dissolved in cold 10 mM NaOH immediately before use and is stable under alkaline conditions. At neutral pH, decomposition of 1 mol of PROLI/NO will yield 2 mol of NO. The concentration of PROLI/NO stock solution was evaluated based on the absorbance at 252 nm (ε = 8 400 M^−1 cm^−1). For experiments designed to follow the quenching of the EPR signal of tyrosyl radical, 50 μl of 100 mM KatG solution (pH 7.2) was injected into an EPR tube followed by addition of 2 μl of 2.5 mM alkaline PROLI/NO. PROLI/NO was allowed to decompose in the KatG solution for one half-life (~2 s at pH 7.2 and 25 °C) after which 50 μl of 300 μM peroxoyacetic acid (PAA) was added to initiate turnover of the resting (ferric) enzyme to Compound I, which is followed by tyrosyl radical (9). The samples were immediately immersed in liquid nitrogen and frozen. EPR spectra were subsequently recorded at 77 K using a Varian X-band spectrometer (9). Control experiments were performed under identical conditions but without addition of PROLI/NO.

Protein Nitration and HPLC Analysis of Nitrosyltyrosine-containing Peptides—A molar ratio of KatG/PAA:PROLI/NO = 1:6:3 was used to get maximum yields of nitrated protein. Briefly 150 μl of 100 mM KatG was mixed with a 3-fold excess of PROLI/NO in an Eppendorf tube; after 2 s of incubation at 37 °C, 150 μl of PAA (600 μM) was added to the mixture. The resulting reaction mixture was incubated for 15 min at 37 °C. Ultrafiltration using a 30-kDa molecular mass cut-off Centricon centrifugal filter device (Millipore Corp., Bedford, MA) was used to separate the enzyme from other components (five washes of retentate with 2 ml of 20 mM potassium phosphate buffer, pH 7.2).

Preliminary experiments were performed using Proteinase K for complete digestion of the NO-treated enzyme to ensure that optical density of nitrotirosine would not be compromised by interference from absorbance due to the presence of heme-containing peptide(s). The 3-nitrotyrosine-containing KatG was subjected to proteinase K digestion in 20 mM potassium phosphate buffer, pH 7.2 at 37 °C for 16 h) by the addition of 1:50 (w/v) Proteinase K. The digests were ultrafiltered (10-kDa cut-off Microcon centrifugal filter device) prior to HPLC separation. Peptides were separated by reverse-phase HPLC over a Zorbax SBC-18 column (4.6 × 250 mm). Buffer A was 0.1% trifluoroacetic acid, and buffer B was 0.1% trifluoroacetic acid in 80% acetonitrile. Elution was performed at a flow rate of 1.0 ml/min with a linear gradient from 0 to 60% B over 60 min and then 60% B to 100% B over 10 min followed by a series of washing steps. Absorbance was simultaneously monitored at 280 and 360 nm; the latter wavelength corresponds to an absorbance maximum of nitrotyrosine in acidic solution (26).

To further characterize peptides, fragments with strong absorbance at 360 nm were collected and dried using a Speed-Vac centrifuge and redissolved in either 0.1 N HCl or 0.1 N NaOH. UV-visible spectra were recorded using a 14NT UV-visible spectrophotometer (AVIV Biomedical, Inc.).

Tryptic Digestion of KatG and Peptide Mapping—TPCK-treated trypsin, which cleaves specifically at Arg-X and Lys-X bonds, was used to obtain reproducible peptide mapping results from the NO-treated enzyme. M. tuberculosis KatG was found to require denaturation to achieve complete digestion with trypsin. A solution of KatG at 50 mM ammonium bicarbonate buffer, pH 7.8, was made 6 μl in urea. After a 10-min incubation at 55 °C, the protein solution was diluted with buffer to lower the concentration of urea to 1.0 M. Proteolysis was started by the addition of 1.30 (w/v) TPCK-treated trypsin and allowed to proceed for 16 h at 37 °C. The reaction was stopped by the removal of trypsin through ultrafiltration using a 10-kDa cut-off filter. The tryptic fragments were separated by reverse-phase HPLC as described above except that the linear gradient was 0–50% B over 90 min at a flow rate of 0.5 ml/min. Peaks that showed strong absorbance at 360 nm were collected, concentrated, and further purified on a Zorbax SBC-8 column (4.6 × 250 mm) using a shallow gradient of 20–30% B over 40 min, which removed a small amount of co-eluted peptides that have no absorbance at 360 nm.

Peptide Sequencing and Mass Analysis—N-terminal Edman sequencing was carried out using an Applied Biosystems Proc 494 protein sequencing system, and MALDI-mass spectrometry was performed on a Perceptive Biosystems Voyager DE Pro in positive reflection mode, both at the Albert Einstein College of Medicine, Bronx, NY.

Construction, Expression, and Purification of the Y353F Mutant of KatG—E. coli strain UM262 (wecA katG: Tn10 pro leu rpsL hsdM hsdR) containing the p718 plasmid carrying KatG (27) carrying a target mutation was used for overexpression of both wild-type and mutated KatG proteins. Mutagenesis was performed on pKatII using the QuiChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). Pairs of complementary oligonucleotides 5′-1043 CTGGCGCGCTTGGCAAT1073-3′ and 5′-1043CC-1073CTCTTGCGGTTAATGGCCAAGCGCACGAAG-3′ were synthesized and purified by Qiagen) were designed to introduce the required mutation indicated in boldface to produce KatG(Y353F) (A to T mutation, Tyr→Phe replacement). Mutagenesis was performed according to the manufacturer’s protocol, and the reaction products were transformed into the E. coli XLI-Blue strain (Stratagene) for selection and amplification. Sequencing (Gene Wiz, Inc.) of the mutated katG gene confirmed that only the desired nucleotide substitution was present, and the mutated plasmid was then transformed back into E. coli strain UM262 for protein expression. Overexpression and purification of mutant KatG(Y353F) were achieved as previously described for WT-KatG (8).

RFQ-EPR and Optical Stopped-flow Spectrophotometry of KatG(Y353F)—Rapid freeze-quench EPR samples were prepared as previously reported using a System 1000 chemical/freeze quench apparatus (Update Instrument, Inc.) (9). Specifically wild-type or mutant KatG (50 μM final concentration) was mixed with 3-fold excess peroxoyacetic acid and aged for varying time periods. Frozen samples were packed into quartz EPR tubes in an isopentane bath at −130 °C. EPR spectra were recorded at 60 K using a Bruker ER 300 EPR spectrometer operating at X-band. Spin quantitation was performed by double integration of EPR signals and comparison to a CuII/EDETA standard in 50% ethylene glycol also examined at 77 K. The dilution of KatG by isopentane in the frozen samples was also taken into account (9).

Optical stopped-flow experiments were performed at 25 °C using a HiTech Scientific Model SF-61 DX2 apparatus equipped with a rapid scanning diode array spectrophotometer as published previously (9). The final concentrations of KatG(Y353F) mutant enzyme (50 μM) and PAA (150 μM) were the same as those used in the RFQ-EPR studies.

RESULTS

Quenching of KatG Tyrosyl Radical by Nitric Oxide—EPR spectroscopy was used to first monitor whether the tyrosyl radical formed upon reaction of resting KatG with alkyl peroxides could be quenched by NO generated from a donor such as
PROLI/NO. Fig. 1 shows the results of an experiment in which a 3-fold excess of peroxycetic acid was added to KatG in the presence and absence of the NO donor. Previous rapid freeze-quench EPR results demonstrated the formation of tyrosyl radical on a millisecond time scale under conditions similar to those used here with the maximum yield of radical achieved after ~5 s (16). Here the tyrosyl radical signal detected in the absence of NO (Fig. 1A) was completely quenched in the presence of NO. In the first protocol, peroxide was added to a mixture of the enzyme plus PROLI/NO that had been incubated for 2 s (Fig. 1B). The 2-s time interval corresponds to approximately one half-life of the donor decomposition rate when it is exposed to neutral pH. Therefore, the concentration of NO in the mixture upon addition of peroxide was estimated to be equal to 1 eqKatG subunit. The time interval during which enzyme was incubated with PAA was the same for both samples. Similar results were found when reversing the order of addition such that the same total amount of PROLI/NO was added immediately after the addition of a 3-fold excess of PAA to KatG.

Detection of Nitrotyrosine—Having demonstrated the quenching of the tyrosyl radical EPR signal, we proceeded to seek evidence for nitrotyrosine in the treated enzyme. The HPLC profile of Proteinase K digests of KatG treated with PROLI/NO and PAA showed several peptides with optical absorbance peaks both at 280 and 360 nm (Fig. 2A). The absorbance at 360 nm provided clues that nitrotyrosine was present. The fragment eluting at 17 min, which had the highest absorbance at 360 nm, also exhibited an optical spectrum similar to that of authentic nitrotyrosine. Furthermore, when this sample was examined at pH 10, an absorbance maximum was found at 430 nm. The shift in absorbance maximum is expected upon deprotonation of the phenolic hydroxyl group of nitrotyrosine (Fig. 2B) (26). Similar results were obtained with the other major fractions having an optical absorbance peak at 360 nm (data not shown). These results are consistent with the presence of nitrotyrosine in multiple peptides. Since Proteinase K is a nonspecific proteolytic enzyme, there is no specific information available from these HPLC profiles as multiple peptides could contain the same labeled residue.

In an effort to understand the specificity of the NO scavenging approach and to rule out nitrotyrosine formation in KatG that could occur due to reactions that do not involve tyrosyl radical, a series of control experiments were carried out. First of all, native or active enzyme was shown to be required for the formation of nitrotyrosine. For example, the protocol described above in which NO donor was added to KatG plus PAA was repeated using KatG that had been denatured either by heating for 5 min at 100°C (Fig. 3A) or by treatment with 6 M urea (data not shown). No nitrotyrosine was detected in the proteolytic digests of these samples. Likewise tryptic digests of KatG reacted with PAA in the absence of NO donor or with NO donor in the absence of PAA did not contain nitrotyrosine (Fig. 3, B and C). Nitrotyrosine was not observed in digests of KatG to which PROLI/NO was added 5 min after the addition of PAA during which time tyrosyl radical will have completely decayed (11) (data not shown). These results demonstrate that nitrotyrosine is found only when catalytically active KatG, PAA, and NO are present (Fig. 3D).

To test the effect of the concentrations of nitric oxide and PAA on the yield of protein nitration, varying amounts (3-, 6-, and 10-fold excess) of PROLI/NO in combination with 3-, 6-, and 10-fold excess of PAA were used in similar protocols. The labeling, tryptic digestion, and HPLC separation were carried out as described above. The same two major nitrotyrosine-containing peaks (as in Fig. 3D) were consistently detected in all digests (data not shown). Although 3-fold excess of PAA has been previously shown to be sufficient to generate a maximum yield of tyrosyl radical from resting KatG (9), in our labeling experiment, a maximum yield of nitrotyrosine was achieved using a 6-fold molar ratio of PAA and a 3-fold molar ratio of PROLI/NO (6-fold molar ratio of NO upon fully decomposing). Since the use of large excesses of NO did not produce any new significantly nitrated peptides, these results strongly suggested that a specific reaction, although possibly not at a unique tyrosine residue, was responsible for nitrotyrosine formation.

Using authentic 3-nitrotyrosine as an HPLC standard, we estimate that the maximum yield of nitrotyrosine was around 0.1–0.2 mol/mol of KatG protein subunit. The estimated yield of tyrosyl radical by direct measurement using quantitative RFQ-EPR was around 0.19 spin/heme, which was considered low due to rapid decay of the radical. This yield of nitrotyrosine then reflects a highly efficient scavenging of tyrosyl radical and conversion of the proposed nitroso intermediate to nitrotyrosine.

Identification of the Nitrated Tyrosine Residue—As shown in Fig. 4, the fractions eluting at 71 and 75 min showed absorbance maxima at both 280 and 360 nm, which indicated peptides containing nitrotyrosine. These peptides were collected, concentrated, and subjected to further purification using a C8 reverse-phase HPLC column. The twice purified peptides were subjected to sequencing analysis by automated Edman degradation. The results of a typical analysis are shown in Table I: peak 1 and peak 2 refer to the HPLC fractions eluted at 71 and 75 min, respectively. Surprisingly both nitrotyrosine-containing peptides had the same sequence, SPAGAWQYTAK, which represents residues 346–356 of M. tuberculosis KatG.

The eighth cycle of the sequencing reactions for both peptides, which should indicate residue Tyr-353, indicated a modified amino acid at this position. Making the assumption that the modified residue was nitrotyrosine, the phenylthiohydan-
toin derivative of authentic 3-nitrotyrosine was prepared and run in a separate mock sequencing protocol. This species was found to elute at 15.17 min under the conditions used in the analysis of the KatG peptides. The modified KatG residue eluted at exactly the same time as the phenylthiohydantoin derivative of 3-nitrotyrosine, which is evidence for the identification of this residue as nitrotyrosine. Furthermore mass analysis using the MALDI technique showed that the (MH)$^+$/H of the modified peptide (peak 1) was 1224, which is 45 mass units greater than the predicted mass of the unlabeled KatG tryptic peptide spanning residues 346–356. The additional mass of 45 is exactly equal to that expected for nitration of a residue at which hydrogen is replaced by a nitro group (mass = 46). We note here that residue Trp-351 in the peptide represented by peak 2 was also modified according to the sequencing profile of this fragment. The (MH)$^+$/H of this fragment is 29 mass units greater than that of peak 1, which could correspond to N-nitrosotryptophan (28). Assuming this is the product, the abundance of N-nitrosotryptophan was estimated to be 30% of the total nitrotyrosine based on a published extinction coefficient (28).

**Characterization of KatG Tyrosine-Phenylalanine Mutants—** KatG(Y353F) mutant enzyme was constructed by site-directed mutagenesis to examine radical formation in the absence of the tyrosine residue identified here. Characterization of the purified mutant enzyme showed no difference in catalase activity as compared with wild-type KatG, while the peroxidase activity of the mutant was about 40% lower than that in wild-type KatG. RFQ-EPR showed that radical still forms (Fig. 5A) when resting KatG(Y353F) mutant reacts with PAA. The EPR line shapes, that is the doublet and singlet signals observed at various time points in this mutant, closely resemble those reported for WT-KatG (9) and are ascribed to tyrosyl radical. However, the yield of tyrosyl radical in the mutant was very low compared with that found for WT-KatG (Fig. 5B). The maximum yield of tyrosyl radical was calculated to be 0.05 spin/heme in KatG(Y353F), which is about 73% less than that found in similar RFQ-EPR experiments on the wild-type en-

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**Fig. 2. Identification of nitrated peptides derived from KatG.** KatG was pretreated with a 3-fold excess of PAA and 1 molar eq. (relative to heme concentration) of PROLI/NO. **A**, reverse-phase HPLC profile (optical detection at 280 and 360 nm) of the Proteinase K digests of KatG. Nitrotyrosine has a characteristic absorbance at 360 nm at acidic pH. **B**, UV-visible absorbance of the peptide eluted around 29.5 min (indicated by the arrow in the HPLC profile), further purified, concentrated, and redissolved in 0.1 N HCl or 0.1 N NaOH.
zyme. This result demonstrates that while a pathway to tyrosyl radical formation still exists in the mutant enzyme oxidation of the new tyrosine residue occurs with significantly lower efficiency than in WT-KatG.

Based on sequence alignment and crystal structures of *Haloarcula marismortui* KatG, there are several conserved tyrosyl residues in *M. tuberculosis* KatG that might also be located near the heme center. To investigate their potential roles in radical formation, mutants Y98F, Y113F, Y155F, Y304F, Y426F, and Y229F were also constructed through site-directed mutagenesis. However, in RFQ-EPR experiments similar to those used here, none of these mutants with the exception of Y229F (see below) exhibited lower initial yield of tyrosyl radical doublet signal compared with wild-type KatG. Moreover all except Y229F also exhibited an evolution to a singlet species as observed for WT-KatG.

Optical stopped-flow experiments using KatG(Y353F) revealed the formation of Compound I under the conditions used to prepare the RFQ-EPR samples (3-fold excess PAA). This reaction, with a half-time of about 70 ms, suggests a second order rate constant for formation of Compound I similar to that in WT-KatG (9). Just as we reported for WT-KatG, no other intermediates accumulated during the interval following Compound I formation, although a single electron transfer producing tyrosyl radical would be expected to produce Compound II. We suggest that in the mutant as well as in wild-type enzyme Compound II is unstable and cannot be detected. Also the formation of Compound I at a rate similar to that of wild-type KatG allows for the rapid formation of tyrosyl radical in KatG(Y353F) as demonstrated in the RFQ-EPR experiments.

**DISCUSSION**

In previously published work (9), we presented evidence that suggested formation of a unique tyrosyl radical in *M. tuberculosis* KatG upon turnover of the resting enzyme with alkyl peroxides. Here our approach to the identification of the residue at which this radical is formed involved NO introduced...
under conditions known to generate the radical. A key feature of the success of this approach was the formation of nitrotyrosine, which allowed for confirmation of nitration in a single peptide at a unique tyrosine residue in KatG. The mechanism for production of nitrotyrosine in KatG was not directly probed, but other reports provide a background and precedent for reaction pathways we suggest to be relevant here. It was reported that tyrosyl radicals in ribonucleotide reductase, photosystem II, and prostaglandin-H synthase enzymes reacted with NO (22, 24, 29). In the case of the heme enzymes PGHS-1 and PGHS-2, formation of nitrotyrosine was proposed to occur through heme-catalyzed peroxidation of the nitrosotyrosine initially formed from NO and tyrosyl radical (23, 24). Our results demonstrate the likelihood of a similar reaction pathway in M. tuberculosis KatG, which most likely involves the following: 1) production of Compound I from the resting (ferric) enzyme and peroxyacetic acid followed by 2) formation of a protein-based tyrosyl radical due to a spontaneous process defined in previous work on wild-type and mutant KatG (9) followed by 3) quenching of this tyrosyl radical by NO released from the donor molecule and 4) heme-catalyzed oxidations ultimately producing a protein-based nitrotyrosine at residue Tyr-353. The secondary oxidation steps are possible due to the cyclic turnover of ferric heme to Compound I in the presence of the small excess of PAA used to initiate the first turnover in our experiments. Multiple turnover of the enzyme in the presence of excess peroxide was demonstrated for WT-KatG (9) and must still occur in the mutant Y353F since the oxidation reactions we assume to be operating produced nitrotyrosine in good yield.

Of the 21 tyrosine residues in M. tuberculosis KatG, nitrotyrosine was only found on residue 353. While many of the tyrosine residues would be solvent-accessible (according to sequence and predicted structural homology with the published three-dimensional crystal structures of other bacterial KatGs (30, 31)), the reaction mixture used here did not generate an increasing yield of nitrotyrosine even when NO was provided in a significant molar excess. Since no change in the pattern of labeling occurred in the presence of increasing excesses of NO, we suggest that the unique labeling represents efficient tyrosyl radical scavenging by NO and that reactions that could lead to nonspecific nitrotyrosine generation are not producing detectable side products.
Sequence alignment shows that tyrosine 353 corresponds to tryptophan 343 of H. marismortui KatG or tryptophan 328 of Burkholderia pseudomallei KatG, the side chains of which are located within the interior of the enzyme close to the heme group. The proximity suggests that tyrosine 353 is a reasonable locus for radical formation, although proximity alone is not the sole criterion for an electron transfer reaction pathway between Compound I and a tyrosine residue. The geometry of the phenolic ring and hydrogen bonding influence the redox potential of this group (32), and other factors may also be important for determining the stability of a protein-based tyrosyl radical.

Our results with the mutants Y98F, Y113F, Y155F, Y304F, and Y426F eliminated the likelihood that any of these residues stabilize the tyrosyl radical we observed in the wild-type enzyme (9), but KatG(Y229F) behaved differently. An interesting fact about residue Tyr-229 is that its homologues in H. marismortui and B. pseudomallei KatG are covalently incorporated into a three-amino acid adduct. In our earlier work on KatG(Y229F) (10), we suggested that the observed tyrosyl radical was likely to be formed on the same residue as in wild-type KatG. Whether Tyr-229 can also stabilize a radical under particular circumstances (yet not appear as nitrotyrosine in the present experiments) is currently under study since we believe that generation of the active site adduct involving residue 229, and methionine and tryptophan side chains, requires radical formation at one or more of these residues.

Although our current results strongly point toward residue 353 as a principal site for tyrosyl radical formation in the purified enzyme, we cannot rule out formation of radicals at other residues. Arguing against this idea, however, is the well-defined EPR spectrum of the tyrosyl radical doublet species described previously and simulated for wild-type KatG (9), which would not be expected if a combination of tyrosyl radicals on different residues contributed to the EPR signal (9).

We note here that modification of residue Trp-351 in one of the nitrotyrosine-containing peptides was consistent with N-nitrosotryptophan formation. The modification of Trp-351 in our experiments may or may not arise from reactions that involve a tryptophan radical since, for example, N-nitrosotryptophan is known to be produced using reagents such as N₂O₃, a nitrogen oxide that could be formed in the aerobic reaction after generation of Compound I, could be quenched by NO to yield an N-nitroso derivative.

It is very interesting that Tyr-353 is unique to M. tuberculosis KatG. The residue at this position is conserved as a tryptophan (or phenylalanine) in other catalase-peroxidase enzymes (National Center for Biotechnology Information BLAST tool) (34). Whether radical formation at this residue is responsible for a special function of KatG in Mycobacteria remains to be demonstrated.

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