**Analysis of Heme Structural Heterogeneity in Mycobacterium tuberculosis Catalase-Peroxidase (KatG)**

Salem Chouchane‡, Stefania Girotto‡, Sofia Kapetanaki§, Johannes P. M. Schelvis§, Shengwei Yu‡, and Richard S. Magliozzo¶

*From the ‡Department of Chemistry, Brooklyn College and the Graduate Center of the City University of New York, Brooklyn, New York 11210-2889 and the §Department of Chemistry, New York University, New York, New York 10003*

**Mycobacterium tuberculosis** catalase-peroxidase (KatG) is a heme enzyme considered important for virulence, which is also responsible for activation of the anti-tuberculosis pro-drug isoniazid. Here, we present an analysis of heterogeneity in KatG heme structure using optical, resonance Raman, and EPR spectroscopy. Examination of ferric KatG under a variety of conditions, including enzyme in the presence of fluoride, chloride, or isoniazid, and at different stages during purification in different buffers allowed for assignment of spectral features to both five- and six-coordinate heme. Five-coordinate heme is suggested to be representative of “native” enzyme, since this species was predominant in the enzyme examined immediately after one chromatographic protocol. Quantum mechanically mixed spin heme is the most abundant form in such partially purified enzyme. Reduction and reoxidation of six-coordinate KatG or the addition of glycerol or isoniazid restored five-coordinate heme iron, consistent with displacement of a weakly bound distal water molecule. The rate of formation of KatG Compound I is not retarded by the presence of six-coordinate heme either in wild-type KatG or in a mutant (KatG[Y155S]) associated with isoniazid resistance, which contains abundant six-coordinate heme. These results reveal a number of similarities and differences between KatG and other Class I peroxidases.

Bacterial catalase-peroxidases (KatG) are multimeric heme enzymes with 80–81-kDa subunits having high sequence homology in their N-terminal halves to cytochrome c peroxidase (CCP) and ascorbate peroxidase, especially in the distal and proximal heme regions (1). These enzymes fall into the category of Class I peroxidases, according to the system used by Welinder based on polypeptide structural parameters (2). In *Mycobacterium tuberculosis*, KatG is important for virulence due to its role in oxidative stress management (3, 4). KatG is also responsible for activation of the pro-drug isoniazid (isonicotinic acid hydrazide) (5–7), which has been in continual use since the early 1950s to treat tuberculosis infection (8). The mode of action of this old antibiotic is of current interest especially in the context of INH resistance mechanisms in clinically isolated strains, which appeared not long after TB therapy with this drug began (9). Various mutations in the gene (katG) that encodes this protein have more recently been correlated with drug resistance (10, 11), although identification of the structural or functional defects in the mutant enzymes remains underexplored. The elucidation of altered drug binding, altered heme binding, or other structural changes due to amino acid substitutions is only in very early stages of detailed study, most of which has been devoted to the commonly encountered mutant KatG(S315T) (12–17). Altered drug binding may be an important factor in drug resistance (18), rather than major changes in the enzyme’s activity as a catalase or peroxidase, although other mechanisms have been put forth, including effects involving superoxide-initiated reactions (19).

Although catalase-peroxidases from various microorganisms have been purified and characterized, the relationship between the catalytic function of wild type or mutant enzymes and the heme structural features such as coordination number or spin state has not been defined. Understanding such relationships is required if drug resistance mechanisms in the growing number of mutant KatG enzymes are to be characterized. Spectroscopic characterization of recombinant *M. tuberculosis* KatG and mutant S315T reported elsewhere showed that the purified enzyme contained a mixture of five-coordinate (5-c) and six-coordinate (6-c) high spin (HS) heme iron as well as a large component of low spin (LS) heme (12). Our earlier results (20) suggested that the majority heme iron species in purified overexpressed *M. tuberculosis* KatG was the 5-c HS form. A recent report on overexpressed KatG from the cyanobacterium *Synechocystis* (21) demonstrated the presence of 5-c HS and 6-c high and low spin heme iron in this enzyme. In the present investigation, optical, resonance Raman, and EPR spectroscopy were used to investigate the heterogeneity of heme iron species in wild type *M. tuberculosis* KatG and two mutants (S315T and Y155S), both identified as drug-resistant mutants (11, 22–24). Our results reveal that a 5-c heme form of KatG may be isolated but that coordination and spin state changes are unavoidable during handling of the enzyme under a variety of conditions. Similar issues have appeared in the literature over the years concerning yeast CCP (25–28).

The initiation of the peroxidase cycle, evaluated by determination of the rate of formation of Compound I, is not altered greatly for wild-type KatG samples varying in their content of 6-c heme iron. In KatG[Y155S], formation of 6-c HS heme is facilitated and predominates in the purified enzyme, although it is not inhibited compared with wild-type KatG in its rate of Compound I formation either. Interestingly, this mutation...
causes a significant change in heme structure in the pure enzyme, whereas the common mutation S315T found in INH-resistant bacteria does not.

**EXPERIMENTAL PROCEDURES**

**Materials**—E. coli UM262 (pKAT II) (overexpression system containing M. tuberculosis katG gene) was a gift from Stewart Cole (Institut Pasteur, Paris). Mutagenesis was performed as reported elsewhere (18). All reagents were from Sigma-Aldrich. Commercial peroxyacetic acid contains hydrogen peroxide, which was removed by incubation of PAA (10 mM) for 1 h with bovine liver catalase (780 units/ml) in 20 mM phosphate buffer, pH 7.2, followed by removal of the enzyme by ultrafiltration.

**Purification of M. tuberculosis KatG**—KatG was isolated and purified from E. coli strain UM262 (KatG minus) expressing the M. tuberculosis katG gene or a mutated gene for preparation of KatG[Y155S] and KatG[S315T]. The bacteria were grown at 37 °C in LB medium containing ampicillin (100 μg/ml) plus the heme biosynthetic precursor, δ-aminolevulinic acid (300 μM), which eliminated the large proportion of heme-deficient enzyme isolated in its absence. Cells were cultured for various time periods (routinely 6–8 h and up to 20 h, as indicated). The enzyme was purified by fast protein liquid chromatography according to a published procedure (29), using either 20 mM potassium phosphate buffer (pH 7.2) or 20 mM triethanolamine-HCl (pH 7.8) (TEA-Cl). Sodium chloride gradients were generally used for enzyme elution from ion exchange media (Amersham Biosciences Q-Sepharose FastFlow, MonoQ), whereas ammonium sulfate from 1 M down to 0 M was used in the gradient for elution of KatG from phenyl-Sepharose media (Amersham Biosciences phenyl-Sepharose). Optical spectroscopy was performed using a model NT14 UV-visible spectrophotometer (Aviv Associates, Tokiwood, NJ) interfaced to a personal computer running 1DS software. All spectra were recorded for enzyme in potassium phosphate buffer, pH 7.2, at 25 °C, except where noted otherwise.

Solid sodium chloride, fluoride, or formate was added in large excess (up to 1 M) to solutions of KatG for examination of spectroscopic changes. Ferric KatG-NO was prepared by exposing the enzyme to NO gas under anaerobic conditions, monitoring the complete formation of the complex according to optical features (Soret at 420 nm, a and β bands at 570 and 536 nm). No evidence for reduction to the ferrous enzyme was found under these conditions.

**Regeneration of 5-c KatG—Reduction of KatG samples containing a high proportion of 6-c heme, using a small excess of sodium dithionite under anaerobic conditions, led to the formation of ferrous KatG (confirmed according to its optical spectrum in Table 1) (29, 30)). Exposure to air immediately converted ferrous KatG to the ferric enzyme, based on optical spectra (29). Recovery of 5-c enzyme was also attempted by adding glycerol (60%) to KatG in phosphate buffer (31, 32) or by titration with isoniazid.

Reconstitution of heme-deficient KatG yielded a mixture of specifically and nonspecifically bound heme (both 6-c and 5-c), and this technique was not pursued for evaluation of structural changes in the enzyme.

**Electron Paramagnetic Resonance—Low temperature (5–6 K) EPR** spectra were obtained at X-band using a Varian E-12 spectrometer interfaced to a personal computer and equipped with a liquid helium cryostat and Heli-Tran liquid helium transfer system (Advanced Research Systems, Inc., Allentown, PA). Data acquisition made use of WinEPR software (20). Assignment of g values was accomplished using difference spectra and/or simulation for a large collection of spectra recorded under varying conditions of pH, temperature, and/or microwave power. In this way, the gα and gβ partners could be isolated when multiple signals were present. Quantitative EPR of low spin heme was based on signal intensity of one rhombic low spin heme species. EPR was performed by double integration of signals recorded at 42 K, using the LS myoglobin-mercaptoethanol complex as a spin standard. This sample was prepared using commercial ferric horse heart myoglobin in potassium phosphate buffer, pH 7.0, by the addition of 1% β-mercaptoethanol (33). Final heme concentration in the standard was based on the Soret absorbance of the starting aquometmyoglobin (34), assuming complete conversion to the LS form.

**Resonance Raman Spectra of KatG**—Resonance Raman spectra were obtained using a single spectrograph (TriAx 550, JY/Horiba) and a liquid nitrogen cooled CCD detector (Spectrum One, JY/Horiba) with a UV-enhanced 2048 × 512-pixel chip (EVE). The Rayleigh scattering was removed using a 406.7-nm holographic notch filter (Kaiser Optical). The samples were excited with the 406.7-nm line from a Kr+ laser (Coherent, I-302). Samples were placed in a spinning cell under an N2 atmosphere and kept at 6 ± 2 °C during the experiments. Enzyme concentration was 40 μM, and the laser power incident on the sample was 10 mW. Background correction of spectra was performed by subtracting a polynomial function from the data. The vibrational modes were labeled according to Ref. 35, and the modes were assigned following published analyses (36, 37). To determine relative peak intensities and positions, a curve-fitting program was used to simulate experimental spectra using Lorentzian line shapes. A bandwidth of 12.5 cm−1 was used for the simulation of the ν3 region bands.

**RESULTS**

**Optical Spectroscopy**—The absorption spectrum of M. tuberculosis KatG isolated and fully purified in TEA-Cl2 for this work is characterized by a Soret peak at 408 nm and a CT1 band at 629 nm (Table 1), as reported previously (20). Other features of the spectrum, which are not discussed further, include a shoulder near 545 nm and a maximum at 505 nm (the CT2 bands, respectively). The wavelength maxima for the Soret and CT1 bands are very close to those reported elsewhere for overexpressed M. tuberculosis KatG prepared in other buffers, either at pH 7.0 or 8.0 (15, 30, 38). These maxima, which are most often associated with 6-c heme in peroxidases, however, were found to be notably different from those seen early in the KatG purification protocol, an observation suggesting that published spectra did not represent “native” KatG and that a more detailed investigation was warranted.

The position of the Soret peak and the CT1 band (as well as absorbance ratios at specific wavelengths defined below) were used to provide clues about iron coordination number and possibly spin state, similar to reports on CCP (27, 32). Whereas the purity of the enzyme varies throughout purification, our approach, in which the enzyme was monitored from the first through the last chromatographic procedure, was considered viable, since KatG always represented the majority protein (according to SDS-PAGE, not shown).

Table I summarizes the optical wavelength maxima for KatG recovered and examined during and after purification. The

| Table I | Absorption maxima in the optical spectrum of M. tuberculosis KatG after each step in the purification protocol |
|---------|--------------------------------------------------------------------------------------------------|
| **Soret** | CT1 |
| TEA-Cl buffer (20 mM, pH 7.8) | 380, 405 | 642–645 |
| Step 1* | 380, 405 | 642–645 |
| Step 2 | 408 | 630 |
| Step 3 | 408 | 629 |
| Phosphate buffer (20 mM, pH 7.2) | 380, 405 | 642–645 |
| Step 1 | 380, 405 | 644 |
| Step 2 | 380, 405 | 642 |
| Step 3 | 380, 405 | 642 |

*Step 1, enzyme collected from first anion exchange chromatography; 2, enzyme collected from phenyl-Sepharose chromatography using reverse ammonium sulfate gradient; 3, enzyme collected from second anion exchange chromatography. Spectra were recorded in 20 mM TEA-Cl, pH 7.8.

2 TEA and other cationic amine buffers are recommended for use with strong anion exchangers such as FastFlow Q-Sepharose and MonoQ (Amersham Biosciences).
**Table II: Absorption maxima and optical ratios sensitive to coordination state in various forms of *M. tuberculosis* KatG**

| KatG form                        | Absorption maxima | $A_{404}/A_{380}$ | $A_{614}/A_{645}$ |
|----------------------------------|-------------------|-------------------|-------------------|
| Fresh (partially purified)       | 380, 404, 642–645 | 1.19              | 0.90              |
| Pure                             | 380, 405, 642     | 1.44              | 0.92              |
| After storage (AS)               | 408, 630, 642     | 1.72              | 1.02              |
| AS reduced/reoxidized            | 380, 405, 642     | 1.45              | 0.85              |
| AS + 60% glycerol                | 380, 405, 640     | 1.44              | 0.92              |

* Shoulder.

The spectra are offset for presentation purposes.

**Fig. 1.** Electronic absorption spectra of fresh partially purified KatG, KatG examined after 3 weeks’ storage, and KatG after reduction/reoxidation. Inset, expanded spectra showing CT1 bands. The spectra are offset for presentation purposes.

The spectrum of the enzyme changes significantly during purification in TEA but much less so in phosphate buffer. Catalytic reactions stimulated in the presence of amine-containing buffer may be partly responsible for these changes, as reported for CCP (39). Enzyme having a Soret peak at or near 405 nm with a shoulder at 380 nm and a CT1 band at or above 640 nm may be considered to represent mainly 5-c HS heme, whereas peaks at 408 and 630 nm indicate abundant 6-c HS heme. The purity index ($A_{380}/A_{404}$) for KatG prepared in TEA-Cl was 0.65–0.7, whereas for KatG prepared in potassium phosphate buffer, the ratio was consistently lower (0.47–0.5). This observation is not due to differences in purity but demonstrates the greater abundance of 6-c heme when TEA buffer is used. A greater Soret extinction coefficient occurs for 6-c HS heme, as noted, for example, in CCP (32).

Also given (Table II) are absorbance ratios in the Soret and CT1 band regions that vary with the proportion of 6-c, relative to 5-c heme. For these ratios, higher values correspond to greater abundance of 6-c HS heme (27).

Close inspection of the CT1 band for the fresh enzyme after the first chromatography protocol during numerous preparations provided evidence that the enzyme even at this stage could be a mixture of at least two components, since shoulders red- and blue-shifted from 640 nm could occasionally be seen (not shown).

In order to evaluate the stability of 5-c heme in KatG, the enzyme recovered after partial purification (step 1) in potassium phosphate buffer was stored for 3 weeks at 4 °C. During this time, the proportion of 6-c HS enzyme increased (Fig. 1 and Table II), and higher $A_{404}/A_{380}$ and $A_{614}/A_{645}$ ratios were found.

We wanted to ensure that the length of the period for bacte-rial overexpression of KatG had not allowed changes in structure before isolation of the enzyme. To address this issue, we compared partially purified (step 1) KatG isolated from cells grown for 6 or 20 h after induction of enzyme overexpression. No differences in optical spectra were found following elution from the first chromatographic column (not shown). This result demonstrates that if any structural changes occur during overexpression, they do not continue during the extended period from 6 to 20 h. For this reason, we suggest that the characteristics of the fresh, partially purified enzyme represent or closely resemble a “native” structure.

In order to determine whether 6-c heme could be converted back to a 5-c form, we evaluated the effects of reducing and reoxidizing KatG, the effect of glycerol, and the effect of organic ligand binding. The first approach was based on the behavior of certain reversibly formed 6-c LS hemichromes identified in hemoglobin that can be converted back to high spin methemoglobin by reduction/reoxidation (40). Glycerol has been shown to modify the coordination state of heme iron in CCP (31, 32). For KatG, these two methods produced changes in optical spectra indicating conversion toward 5-c enzyme (Fig. 1, Table II).

**Fig. 2.** Electronic absorption spectra of fresh partially purified KatG and this enzyme in the presence of 1 M NaCl or NaF. Inset, expanded spectra showing CT1 bands. The spectra are offset for presentation purposes.

The reoxidation results also demonstrate the instability of KatG Compound III (oxyferrous KatG) as reported previously based on optical stopped-flow kinetic measurements (20), in which it had been generated from the ferric enzyme in the presence of excess hydrogen peroxide (31).

The addition of INH and analogous ligands to 6-c KatG regenerated 5-c enzyme (not shown), confirming earlier reports (15, 18). Overnight dialysis to remove INH restored the spectrum of the starting, 6-c enzyme. No optical changes were detected when INH was added to the fresh, partially purified enzyme.

Whereas formation of 6-c heme due to water coordination under different conditions was considered the most likely origin of the heterogeneity in KatG heme structure, the potential formation of a 6-c KatG-chloride complex during purification
was also investigated. Chloride binding to heme iron was considered possible because it is used in a relatively high concentration (0.3 M) to elute the enzyme from ion exchange media, and chloride binding to heme iron in CCP has been reported (26). However, no significant differences were found in the optical spectrum of KatG when the first chromatography procedure was performed using a potassium phosphate concentration gradient (at pH 6.5) in place of the usual sodium chloride gradient for protein elution (not shown). This demonstrates that exposure of the enzyme to 0.3 M sodium chloride for periods of 20–24 h does not produce heme structural changes. This also suggests that the enzyme eluted from the first column can be assigned to a “native” form with more confidence. We then deliberately attempted to produce a chloride complex in fresh partially purified KatG using higher concentrations of sodium chloride (1 M).4 Here, a Soret peak at 408 nm without the shoulder at 380 nm, a new shoulder around 358 nm, and a CT1 band at 640 nm (Fig. 2) were found. Whereas the changes in optical features suggest formation of 6-c iron, the shift in the CT1 band is a small fraction of that seen for 6-c HS KatG anion complexes (KatG-fluoride or KatG-formate (Table III and Fig. 2), and only a small shift in the Soret band occurred. For comparison, in CCP plus chloride, the Soret peak occurs at 413 nm, and the CT1 band occurs at 640 nm (26). 

Fluoride binding to fresh, partially purified enzyme was also examined. Interestingly, two CT1 bands are found in the optical spectrum, one around 615 nm (typical of 6-c HS anion complexes) and another close to 650 nm (Fig. 2, inset). In contrast to this, the KatG-fluoride complex prepared under the same conditions, using KatG that contained abundant 6-c heme, exhibited a single CT1 band at 616 nm (not shown). These results are consistent with a low affinity for fluoride in enzyme that lacks a specific water molecule in the distal pocket required for stabilization of the ligand (41).

In order to evaluate the amount of LS iron in KatG, we used optical difference spectroscopy (and EPR presented below) based on the report of an endogenous LS KatG form exhibiting peaks at 540 and 570 nm (12). No new maxima could be detected between 530 and 580 nm in difference spectra recorded for enzyme containing abundant 5-c heme (data not shown). We also examined the first derivative of the optical spectrum of pure KatG and did not identify features indicating the presence of bands due to LS iron. Furthermore, the well-formed CT1 band and the ratio of absorbances at 570 nm compared with 640 nm in the optical spectrum of KatG argue against the presence of LS heme in the pure enzyme. This ratio correlates well with the relative abundance of 6-c LS heme according to inspection of the data for Synechocystis KatG, CCP, and ascorbate peroxidase presented in Ref. 21. Also, when LS heme is abundant enough to contribute a feature near 570 nm in spectra of these Class I peroxidases, the Soret peak is usually found at or above 410 nm (12, 21), in contrast to the significantly lower Soret wavelengths for partially purified and pure KatG presented above. No 6-c LS heme was found in the optical spectra of KatG at pH 10.

The optical spectrum of purified KatG(S315T) was characterized by features close to those of the wild type enzyme lacking 6-c heme (Soret peak at 403 nm and a CT1 band at 642 nm), whereas for KatG[Y155S] the spectrum resembled that of 6-c wild type enzyme (not shown).

**EPR Spectra**—EPR spectroscopy was applied to confirm and extend the results described above wherever possible. The partially purified enzyme (from potassium phosphate buffer) had an EPR spectrum dominated by a rhombic species (signal r1) with other features at g = 6.0 and −5.6 assigned to signal r2. Samples of pure KatG exhibit some weak feature at g = 2 that may be due to LS heme (Fig. 3, inset). The EPR spectrum of the purified enzyme was recorded after storage of a sample in potassium phosphate buffer for 3 weeks at 4°C (Fig. 3). Here, the changes indicate increased abundance of 6-c heme and loss of 5-c heme, consistent with the optical and Raman results (see below).

| Table III |
| --- |
| Absorption maxima of fresh partially purified KatG in the presence of weak field ligands |
| Ligand | Absorbance maxima |
| --- | --- |
| KatG | 405* |
| NaCl | 408 |
| NaF | 405 |
| CHONa | 406 |

* Optical changes with chloride, fluoride, and formate addition to KatG were very slow. A very high concentration of these ligands was used to effect optical changes in a reasonable time frame and to achieve maximal conversion of the enzyme into a new form.
Experimental conditions were the same as in Fig. 3 at 5 K.

... plus excess NaCl.

... chloride being facilitated in the presence of 1M sodium chloride.

... Raman spectroscopy (see below) do not suggest chloride coordination under these conditions and are more consistent with water coordination being facilitated in the presence of 1M sodium chloride.

The g values for signals r1 and r2 fit the “rule of thumb” definition for the presence of intermediate spin iron used by many authors in analyses of rhombic EPR signals in other heme proteins and enzymes (42–45) in that the \( g_{av} = (g_x + g_y)/2 \) values fall between 4 and 6. The high \( g_z \) value for signal r1, however, is just beyond the range usually associated with rhombic EPR signals thought to represent quantum mechanically mixed spin (QS) heme and may be best assigned to 5-c QS heme. The \( g_{av} \) value, as well as the \( g_x \) and \( g_y \) values for signal r2, fall within the range of those assigned to 6-c QS heme species in Class III peroxidases (45, 46). Thus, some agreement is found between our results for KatG and observations in Class III peroxidases containing 5- and 6-c QS heme (see “Discussion”).

The EPR spectrum of the purified mutant KatG[Y155S] is also shown (Fig. 5). Here, the predominant signal represents a 6-c HS species assumed to have water as the sixth ligand by analogy to similar EPR signals reported for lignin peroxidase and other heme proteins (47, 48). This mutant enzyme was considered useful to examine Compound I formation starting from an endogenous 6-c resting form of the enzyme (see below). EPR spectra of the purified mutant KatG[Y315T] (not shown) were nearly identical to the pure wild-type enzyme and were not analyzed further.

**Resonance Raman Spectra of KatG**—We turned to Raman spectroscopy to help define the heterogeneity observed in the optical and EPR spectra of KatG described above. Here, we will be focusing on the \( v_3 \) bands (spin and coordination state markers) in the high frequency region for evidence in support of coordination number and spin state assignments (36, 37). The \( v_3 \) frequencies for the different forms of KatG are summarized in Table IV. The resonance Raman spectrum of *M. tuberculosis* KatG purified in TEA-Cl buffer was previously reported to contain a \( v_3 \) band centered at 1490 cm\(^{-1}\) (20) assigned to 5-c HS heme, whereas a mixture of high and low spin forms was described in another report for KatG purified and examined in Tris-Cl buffer (12). Here, the fresh partially purified enzyme (from phosphate buffer) exhibited two bands of nearly equal intensity; the 1495 cm\(^{-1}\) band corresponds to a 5-c HS species, whereas the 1503 cm\(^{-1}\) band can be assigned either to a 6-c LS or a 5-c QS species (Fig. 6). A band near 1487 cm\(^{-1}\) also contributes to the breadth of the \( v_3 \) region, indicating the presence of a low abundance component due to 6-c heme (but not low spin heme). The relative abundance of these species based on deconvolution of the bands is 8% 6-c HS (or QS) form (1487 cm\(^{-1}\)), 38% 5-c HS form (1495 cm\(^{-1}\)), and 54% 5-c QS form (1503 cm\(^{-1}\)). The Raman spectrum of pure KatG contains higher intensity near 1487 cm\(^{-1}\) associated with 6-c heme (Fig. 6) and a decrease to 30% in the proportion of the band at 1503 cm\(^{-1}\). This is consistent with optical and EPR spectra, which also revealed a greater abundance of 6-c heme in the pure enzyme. Enzyme examined after storage showed...
further loss of 5-c QS heme and small increases in 6-c heme (but not LS heme), whereas the central band remained relatively constant in intensity (not shown). No increased intensity due to 6-c LS species was detected in the high frequency region of spectra for KatG examined at pH 10.

The high frequency region of resonance Raman spectra of pure KatG in Hepes buffer (not shown) was indistinguishable from that in potassium phosphate. This suggests again that formation of the 6-c species involves a molecule of water rather than coordination of a buffer component.

A ν3 band near 1503 cm⁻¹ has been assigned elsewhere to the unusual QS state heme in Class III peroxidases (44, 46, 49, 50). However, a similar ν3 band was assigned to 6-c LS heme in KatG containing LS heme signatures in EPR and/or optical spectra (12), whereas a nearby band at 1505 cm⁻¹ was assigned to 6-c LS heme in Synechocystis KatG (21). Here, we prefer to assign the 1503 cm⁻¹ band to 5-c QS heme based on the lack of LS species in the optical spectrum and the near absence of LS heme in the EPR, contrasted by the high proportion of this feature in the Raman spectrum. Furthermore, and most importantly, the relative intensity at 1503 cm⁻¹ decreases on going from the partially purified to the pure enzyme and finally to enzyme examined after storage for a few weeks, whereas the EPR data indicate some increase in LS components, the other form that may give rise to the 1503 cm⁻¹ band in such samples.

The lowest frequency ν3 band (1487 cm⁻¹) is assigned to 6-c heme, and its high frequency suggests QS rather than HS heme (44, 46, 49, 50). Formation of 6-c QS heme from 5-c QS heme is reasonable, since the heme distortions thought to be required for stabilization of the mixed spin state are not removed upon conversion to a 6-c form, at least in Class III peroxidases (45).

In order to obtain a reference marker for 6-c HS KatG, the Raman spectrum of KatG-fluoride was examined. Here, a band typical for a 6-c HS peroxidase anion complex was found (1482 cm⁻¹), although residual ν3 bands due to the starting enzyme were evident. Incomplete conversion of the fresh enzyme to the 6-c fluoride complex was also found in EPR and optical spectra described above.

The resonance Raman spectrum of partially purified KatG was also examined in the presence of a large excess of NaCl. Here, no increase in intensity was found at a frequency indicating formation of a typical 6-c HS anion complex. Instead, a broad ν3 feature centered at 1495 cm⁻¹ with no resolved feature at 1503 cm⁻¹ was seen, along with an increase in intensity around 1487 cm⁻¹ (according to deconvolution estimates). These observations demonstrate that high concentrations of sodium chloride reduce the abundance of the 5-c QS species and may increase the abundance of 6-c QS heme, the latter based on similarities to 6-c QS peroxidase-benzhydroxamic acid complexes (41, 45). These results are also in agreement with the EPR data in that an increase in coordination number was suggested, although an axial signal characteristic of 6-c HS anion complexes was not found for similar samples. Removal of excess NaCl by extensive dialysis restored the Raman spectrum (and the optical spectrum) of the "native" enzyme (not shown).

Rate of KatG Compound I Formation—In earlier work on purified M. tuberculosis KatG (20), the rate of Compound I formation from the resting enzyme and peroxides was found to be significantly slower than that for horseradish peroxidase, and we considered that a reason for this could be the presence of 6-c heme iron in KatG. We therefore measured the rate constants for Compound I formation from fresh KatG (partially purified) and the same enzyme after storage for 4 weeks. The values for KatG examined weeks after purification were somewhat greater than that for the fresh enzyme (6.5 × 10⁹ M⁻¹ s⁻¹ versus 5.6 × 10⁸ M⁻¹ s⁻¹). Whereas these rates are lower than those reported previously for the purified enzyme, the results indicate that the presence of 6-c heme iron species is not inhibitory in the reactions leading to Compound I formation in KatG.

We also monitored the effects of peroxide (PAA and CPBA) on the heterogeneity of KatG starting from partially purified enzyme and enzyme that had been stored for a few weeks. The optical spectra of the starting forms were recovered in both cases after the spontaneous return to the ferric state that occurs when excess peroxide is consumed, with no observable changes due to heme degradation. In the case of the fresh enzyme, resonance Raman results demonstrated that the 6-c form is found immediately after cycling of the enzyme with peroxide, whereas the features assigned to 5-c KatG, including the QS heme, slowly return after a longer time period has elapsed (not shown).

The KatG mutant enzyme, KatG[Y155S], which is nearly completely 6-c HS in its resting state according to optical and EPR spectra, did not show a decreased rate of Compound I formation compared with the wild-type enzyme. In contrast to these results, ferric KatG-NO was strongly inhibited in Compound I formation under similar conditions. For example, kobs for Compound I formation, using 10-fold excess PAA, was 1.23 s⁻¹ for pure KatG and 0.08 s⁻¹ for KatG-NO.

**DISCUSSION**

The aims of this study include characterization of heme structural heterogeneity in M. tuberculosis KatG and possibly definition of a "native" form of this enzyme isolated from overexpression in E. coli. Our approach has relied, in part, on examination of KatG after only partial purification and within 36 h of isolation from the E. coli overexpression culture system. We reported earlier (20) that pure KatG seemed to be stable after purification, when, instead, the use of TEA-Cl buffer for its purification at that time had accelerated the loss of 5-c, or what we now consider "native" enzyme. Summarized in Fig. 7 are the different forms of KatG and the processes and factors that allow for interchange between them. The apparent "evolution" of 6-c species from the 5-c form is due to coordination of a molecule of water to iron and is apparently unavoidable. The water ligand is weakly bound and dissociates in ferrous KatG and under other conditions (e.g. by the addition of glycerol or organic ligands like INH). One interesting hypothesis concerning the accumulation of the specifically bound water molecule in 6-c heme may be from basal level catalytic turnover of the enzyme, which produces 2 mol of water in the active site from...
the decay of Compounds I and II.

The 6-c form is converted back to 5-c enzyme upon binding of INH, although this is nearly fully reversed during overnight dialysis to remove the drug. This reversal is considered significant, because it may be evidence for permanent modification of the enzyme that prevents reestablishing the heme pocket structure found in the fresh partially purified enzyme. Relevant to this point is the finding that pure KatG isolated in TEA-Cl buffer, having the optical spectrum characteristic of abundant 6-c heme, exhibited a molecular weight (using matrix-assisted laser desorption ionization mass spectrometry) within 0.04% of that calculated for the KatG dimer (based on translation of the M. tuberculosis katG gene (51); two runs, average mass 162,540 ± 167 kDa; calculated mass 162,470 kDa). This may be taken to indicate that if permanent modification of KatG occurred and was responsible for maintaining 6-c heme, the modification is not large enough to yield a detectable change in the overall mass of the enzyme.

In the absence of coordination to iron of distal residues, the occupancy and architecture of water in the distal pocket of KatG is considered an important feature producing heterogeneity in KatG and other peroxidases. This water may or may not be directly coordinated to heme iron and may participate in hydrogen bonding with the side chains of distal residues such as INH (55). Nevertheless, in the case of INH binding to KatG, water coordination in the 5-c fresh enzyme is not induced, and dissociation of water from iron in the 6-c enzyme occurs. The origin of this opposite effect is not understood at this time, and little is known about the details of INH binding to KatG other than the proximity between heme iron and nitrogen sites of the ligand in the complex (15, 57). The effects of high sodium chloride also remain poorly understood at this time.

Other results presented here support the finding of the QS heme in M. tuberculosis KatG. The structural origin of this spin state in Class III peroxidases, which arises when intermediate spin iron (S = 3/2) is present along with high spin iron, may reside in deformations of the heme macrocycle, with saddling considered important but not a sole requirement for the QS state (44). Thus, there is no a priori reason for the QS spin state not being accessible in a Class I peroxidase; nor can we state that KatG possesses a known feature that would predict its presence. Although the Raman spectrum of fresh KatG exhibits a v1 band analogous to that found in Class III peroxidases containing QS heme (44–46), we have not found an accompanying EPR signal that closely resembles the axial one assigned to 5-c QS heme (in barley peroxidase) (44). Rhombic EPR signals with gav values below 6 have often been assigned to QS heme (42, 43), but the r1 signal of KatG representing the predominant species in the fresh enzyme has a high g1 value more consistent with 5-c HS heme. The correlation between gav values and spin state may even be of questionable reliability, since, for example, magnetic susceptibility measurements reported in a recent investigation of R. capsulatus cytochrome c′ were most consistent with high spin iron (59) despite the rhombic EPR spectrum meeting the accepted criteria for QS heme in this protein. Therefore, the most cautious interpretation of our results that takes into account the Raman and EPR observations on KatG is that close structural analogs, both of which are 5-c, represent QS heme at room temperature and HS heme in frozen solution at 5 K. Little additional insight into the assignment of spin states in KatG is found by examination of optical spectra, since CT1 bands may overlap for the various forms in question, and KatG under the conditions we have employed here is apparently always a mixture of species. Ongoing studies of KatG, including low temperature resonance Raman spectroscopy, are expected to shed more light on this matter. We also note that temperature-dependent structural changes that alter spin state and coordination number have been documented for peroxidases (60–62), warning against making strict correlations between the present Raman and EPR results for KatG. Nevertheless, we have found general agreement between room temperature and frozen solution results when following changes in coordination number observed in KatG. The assignment of the r1 signal as the predominant species in partially purified KatG to a 5-c HS species, while more satisfying in terms of the nature of the EPR spectrum, would mean that temperature- or freezing-induced structural changes favored a shift in the spin state distribution from 3/2 toward 5/2.

Another aim here was to attempt to correlate heme struc-
tural heterogeneity with functional or mechanistic changes in the peroxide cycle of KatG. This was approached through the measurement of the rate of Compound I formation for KatG samples containing 5-c or 6-c enzyme as a majority species and a mutant enzyme, in which 6-c heme was present in high abundance. If we assume that the similarity between KatG and CCP fluoride complexes extends to the structures in the 6-c species having water bound and that the presence of this water is responsible for the slow formation of Compound I in “aged” CCP as reported previously (39), similar behavior for aged KatG would be predicted. Instead, no inhibition of Compound I formation is a factor contributing to structural modification surrounding the heme in KatG, an issue we are actively addressing.

Acknowledgments—We thank Irina Kovatch and Nicolas Carrasco for assistance in data collection.

REFERENCES

1. Zamocky, M., Regelbruger, G., Jakopitsch, C., and Ohinger, C. (2001) FEBS Lett. 492, 177–182
2. Welander, K. G., Mauro, J. M., and Norskov-Lauritsen, L. (1992) Biochem. Soc. Trans. 20, 347–348
3. Collins, D. M. (1996) Trends Microbiol. 4, 426–430
4. Pym, A. S., Domenech, P., Honore, N. S., Jong, J. J., Berite, V., and Cole, S. T. (2001) Mol. Microbiol. 40, 879–889
5. Zhang, Y., Heym, B., Allen, B., Young, D., and Cole, S. (1992) Nature 358, 581–583
6. Zhang, Y., Garbe, T., and Young, D. (1993) Mol. Microbiol. 8, 521–524
7. Heym, B., and Cole, S. T. (1997) Int. J. Antimicrob. Agents 8, 61–70
8. Robitzek, E. H., and Selikoff, I. J. (1952) Am. Rev. Tuberc. 65, 402–410
9. Malechek, G., Cohn, M. L., and Schaeffer, W. B. (1954) Am. Rev. Tuberc. 70, 852–872
10. Musser, J. M., Kapur, V., Williams, D. L., Kreiswirth, B. N., van Sooeling, D., and van Embden, J. D. (1996) J. Infect. Dis. 173, 196–202
11. Rouze, D., DeVito, J. A., Li, Z., Beyer, H., and Morris, S. L. (1996) Mol. Microbiol. 22, 583–592
12. Lukat-Rodgers, G. S., Wengenack, N. L., Rusnak, F., and Rodgers, K. R. (2000) Biochemistry 39, 9884–9993
13. Lukat-Rodgers, G. S., Wengenack, N. L., Rusnak, F., and Rodgers, K. R. (2001) Biochemistry 40, 7149–7157
14. Wengenack, N. L., Uhl, J. B., Amand, A. L., Tomlinson, A. J., Benson, L. M., Naylor, S., Kline, B. C., Cockerill, F. R. 3rd, and Rusnak, F. (1997) J. Infect. Dis. 176, 722–727
15. Wengenack, N. L., Todorovic, S., Yu, L., and Rusnak, F. (1998) Biochemistry 37, 15825–15834
16. Wengenack, N. L., Lopes, H., Kennedy, M. J., Tavares, P., Pereira, A. S., Moura, I., Moura, J. J., and Rusnak, F. (2000) Biochemistry 39, 11508–11513
17. Wengenack, N. L., and Rusnak, F. (2001) Biochemistry 40, 8990–8996
18. Yu, S., Chouchane, S., and Magliozzo, R. S. (2002) Protein Sci. 11, 58–64
19. Wengenack, N. L., Heard, H. M., and Rusnak, F. (1999) J. Am. Chem. Soc. 121, 9748–9749
20. Chouchane, S., Lippai, I., and Magliozzo, R. S. (2000) Biochemistry 39, 9975–9983
21. Heering, H. A., Indiani, C., Regelebruger, G., Jakopitsch, C., Ohinger, C., and Smulevich, G. (2002) Biochemistry 41, 9237–9247
22. Heym, B., Alzari, P. M., Honore, N., and Cole, S. T. (1996) Mol. Microbiol. 15, 285–295
23. Haas, W. H., Schilke, K., Brand, J., Amthor, B., Weyer, K., Fourie, P. B., Bretzel, G., Sticht-Greh, V., and Bremer, H. J. (1997) Antimicrob. Agents Chemother. 41, 1801–1803
24. Marttila, H. J., Soini, H., Eerola, E., Vyshevskaya, E., Vyshevskiy, B. I., Otten, T. F., Vaaleny, A. V., and Viljanen, M. K. (1998) Antimicrob. Agents Chemother. 42, 2443–2445
25. Smulevich, G., Evangelista-Kirkup, R., English, A., and Spero, T. G. (1986) Biochemistry 25, 4426–4430
26. Venotani, T. and Anni, H. (1996) J. Biol. Chem. 271, 9547–9554
27. Vistelius, L. B., Huang, M., and Erman, J. E. (1999) Biochemistry 38, 4283–4288
28. Ferrer, J. C., Ring, M., and Mauk, A. G. (1991) Biochem. Biophys. Res. Com. 176, 1469–1472
29. Marcinkeviciene, J. A., Magliozzo, R. S., and Blanchard, J. S. (1995) J. Biol. Chem. 270, 22290–22295
30. Johnson, K., Froland, W. A., and Schultz, P. G. (1997) J. Biol. Chem. 272, 19854–19860
31. Smulevich, G., Mantini, A. R., English, A. M., and Mauro, J. M. (1989) Biochemistry 28, 5058–5064
32. Fento, A. E., Sono, M., Elenegra, E. A., McLeay, D. E., Goodin, D. B., English, A. M., and Dawson, J. H. (1999) J. Inorg. Biochem. 76, 165–174
33. Magliozzo, R. S., and Peisach, J. (1993) Biochemistry 32, 8446–8456
34. Antonini, E., and Brunotti, M. (1971) Hemoglobin and Myoglobin in Their Reactions with Ligands, North-Holland Co., Amsterdam
35. Ahe, M., Kitagawa, T., and Kyogoku, Y. (1976) J. Chem. Soc. 8456, 4524–4536
36. Choi, S., and Spero, T. G. (1983) J. Am. Chem. Soc. 105, 3683–3692
37. Choi, S., Lee, J. J., Wei, Y. H., and Spero, T. G. (1985) J. Am. Chem. Soc. 105, 3692–3707
38. Hillar, A., and Loewen, P. C. (1995) Arch. Biochem. Biophys. 323, 438–446
39. Anni, H., and Venotani, T. (1998) Blood Cells Mol. Dis. 27, 437–449
40. Peisach, J., Blumberg, W. E., Wittenberg, B. A., Wittenberg, J. B., and Kampa, L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 934–939
41. Howes, B. D., Rodriguez-Lopez, J. N., Smith, A. T., and Smulevich, G. (1997) Biochemistry 36, 1532–1543
42. Fuji, S., Yoshimura, T., Kamada, H., Yamaguchi, K., Suzuki, S., Shibara, S., and Takakawa, S. (1995) Biochem. Biophys. Acta 1251, 161–169
43. Ikeda-Saito, M., Hori, H., Hara, M., Prince, R. C., Pickering, I. J., George, N. G., Sanders, C. R., H. Lutz, R. S. McElvee, J. C., and Mattera, R. (1992) J. Biol. Chem. 267, 22543–22552
44. Howes, B. D., Chadwick, C. B., Dixon, M., Ginn, K. G., Marzocchi, M. P., Ma, M. J., Zhang, J., Shelnutt, J. A., and Smulevich, G. (1999) Biochemistry 38, 478–482
45. Indiani, C., Feis, A., Howes, B. D., Marzocchi, M. P., and Smulevich, G. (2000) J. Inorg. Biochem. 79, 269–274
46. Forster, K., Blumberg, W. E., Ogawa, S., Rachmilewitz, E. A., and Oltz, R. (1971) J. Biol. Chem. 246, 3342–3355
47. Anderson, L. A., Renganathan, V., Chiu, A. A., Loehr, T. M., and Gold, M. H. (1985) J. Biol. Chem. 260, 6080–6087
49. Feis, A., Howes, B. D., and Smulevich, S. (1998) J. Raman Spectrosc. 29, 933–938
50. Nissum, M., Feis, A., and Smulevich, G. (1998) Biospectroscopy 4, 355–364
51. Heym, B., Zhang, Y., Poulet, S., Young, D., and Cole, S. T. (1993) J. Bacteriol. 175, 4255–4259
52. Hillar, A., Peters, B., Pauls, R., Loboda, A., Zhang, H., Mauk, A. G., and Loewen, P. C. (2006) Biochemistry 39, 5866–5875
53. Regelsberger, G., Jakopitsch, C., Furtmüller, P. G., Rueker, F., Switala, J., Loewen, P. C., and Obinger, C. (2001) Biochem. Soc. Trans. 29, 99–105
54. Neri, F., Kok, D., Miller, M. A., and Smulevich, G. (1997) Biochemistry 36, 8947–8953
55. Aitken, S. M., Turnbull, J. L., Percival, M. D., and English, A. M. (2001) Biochemistry 40, 13980–13989
56. Itakura, H., Oda, Y., and Fukuyama, K. (1997) FEBS Lett. 412, 107–110
57. Todorovic, S., Juranic, N., Macura, S., Rusnak, F. (1999) J. Am. Chem. Soc. 121, 10962–10966
58. Yamada, Y., Fujigawa, T., Sato, T., Igarashi, N., and Tanaka, N. (2002) Nat. Struct. Biol. 9, 691–695
59. Tsan, P., Caffrey, M., Daku, M. L., Cusanovich, M., Marion, D., and Gans, P. (2001) J. Am. Chem. Soc. 123, 2231–2242
60. Andersson, L. A., Renganathan, V., Loehr, T. M., and Gold, M. H. (1987) Biochemistry 26, 2258–2263
61. Evangelista-Kirkup, R., Crisanti, M., Poulos, T. L., and Spiro, T. G. (1985) FEBS Lett. 190, 221–226
62. Feis, A., Marzocchi, M. P., Paoli, M., and Smulevich, G. (1994) Biochemistry 33, 4577–4583
63. Nissum, M., Neri, F., Mandelman, D., Poulos, T. L., and Smulevich, G. (1998) Biochemistry 37, 8680–8687
64. Maltempo, M. M., Ohlsson, P. I., Paul, K. G., Petersson, L., and Ehrenberg, A. (1979) Biochemistry 18, 2935–2941
Analysis of Heme Structural Heterogeneity in *Mycobacterium tuberculosis* Catalase-Peroxidase (KatG)
Salem Chouchane, Stefania Girootto, Sofia Kapetanaki, Johannes P. M. Schelvis, Shengwei Yu and Richard S. Magliozzo

*J. Biol. Chem. 2003, 278:8154-8162.*  
doi: 10.1074/jbc.M208256200 originally published online December 28, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M208256200

Alerts:  
• When this article is cited  
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 63 references, 11 of which can be accessed free at [http://www.jbc.org/content/278/10/8154.full.html#ref-list-1](http://www.jbc.org/content/278/10/8154.full.html#ref-list-1)