Mode of Binding of the Tuberculosis Prodrug Isoniazid to Heme Peroxidases

BINDING STUDIES AND CRYSTAL STRUCTURE OF BOVINE LACTOPEROXIDASE WITH ISONIAZID AT 2.7 Å RESOLUTION

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Isoniazid (INH) is an anti-tuberculosis prodrug that is activated by mammalian lactoperoxidase and Mycobacterium tuberculosis catalase peroxidase (MtCP). We report here binding studies, an enzyme assay involving INH, and the crystal structure of the complex of bovine lactoperoxidase (LPO) with INH to illuminate binding properties and INH activation as well as the mode of diffusion and interactions together with a detailed structural and functional comparison with MtCP. The structure determination shows that isoniazid binds to LPO at the substrate binding site on the distal heme side. The substrate binding site is connected to the protein surface through a long hydrophobic channel. The acyl hydrazide moiety of isoniazid interacts with Phe422 O, Gln423 O, and Phe254 O. In this arrangement, pyridinyl nitrogen forms a hydrogen bond with a water molecule, W-1, which in turn forms three hydrogen bonds with Fe3+, His109 Nε2, and Gln105 Nε2. The remaining two sides of isoniazid form hydrophobic interactions with the atoms of heme pyrrole ring A, Cβ and Cγ atoms of Glu258, and Cγ and C6 atoms of Arg255. The binding studies indicate that INH binds to LPO with a value of 0.9 × 10⁻⁶ M for the dissociation constant. The nitro blue tetrazolium reduction assay shows that INH is activated by the reaction of LPO–H₂O₂ with INH. This suggests that LPO can be used for INH activation. It also indicates that the conversion of INH into isonicotinoyl radical by LPO may be the cause of INH toxicity.

Heme-containing peroxidases constitute a large group of enzymes that catalyze the oxidation of halides, pseudohalides, and organic aromatic molecules with the help of hydrogen peroxide, leading to products with a wide range of antimicrobial activities. Peroxidases can be broadly divided into two superfamilies. One of these consists of mammalian peroxidases comprising lactoperoxidase (LPO), myeloperoxidase (MPO), eosinophil peroxidase (EPO), and thyroid peroxidase, which will be termed hereafter as mammalian peroxidase superfamily II (1). The other distinct class of heme peroxidases consists of enzymes from plants, fungi, and bacteria, which will be designated hereafter as superfamily I (2). The most distinguishing feature between the peroxidases of the two families pertains to the association of the prosthetic heme group, which is covalently linked in mammalian peroxidases (3, 4), whereas it is non-covalently attached with the protein in non-mammalian peroxidases (5–8). The enzymes of two superfamilies share low amino acid sequence identities. Furthermore, mammalian heme peroxidases are not structurally homologous to non-mammalian heme peroxidases. Despite these strong differences, the enzymes from two superfamilies show identical modes of peroxidatic action. The residues, histidine and arginine, in the substrate binding site on the distal side of the heme of mammalian peroxidases are positioned in a manner similar to that of catalytically active histidine and arginine residues in plant and fungal peroxidases (3–8). Therefore, all of the heme peroxidases support similar formation of the high valent compound I intermediate as well as the occurrence of subsequent steps of the catalytic process. At the same time, several remarkable differences have also been observed in the specificities of substrates that result in a rich diversity of functions across the peroxidase superfamilies. In view of this, it is particularly of great interest to determine the specificities of aromatic substrates by examining the stereochemical arrangements of the activation domains. So far, crystal structures of two mammalian peroxidases (MPO (3) and LPO (4)), two plant peroxidases (horseradish peroxidase isozyme C (5) and ascorbate peroxidase (APX) (6)), two fungal peroxidases (Arthromyces ramosus peroxidase (7) and cytochrome C peroxidase (CcP) (8)), and one Mycobacterium tuberculosis catalase peroxidase (MtCP) (9) have been determined. Although MtCP is a bifunctional heme-dependent enzyme with a primary catalase activity, its catalytic activity as a peroxidase is comparable with those of other monofunctional heme peroxidases (10). It is pertinent to mention here that this is beneficial because MtCP efficiently...
converts isoniazid (isonicotinic acid hydrazide (INH)) into a biologically active form (11), but the emergence of M. tuberculosis strains that are resistant to the first line drugs represents a serious challenge to control the disease. Thankfully, the three-dimensional structure of MtCP has been reported recently (9), which provided useful information about the shape and nature of the hydrophobic channel and the substrate binding site on the distal side. However, the mode of binding of INH to MtCP is not yet known due to lack of direct data on the structure of the complex of MtCP with INH. Fortunately, a few structures of the complexes of INH with other heme peroxidases, including the present structure, have just become available, and indeed they added valuable information for understanding the mode of binding of INH to MtCP. It may be emphasized here that the substrate binding site on the distal side in LPO appears to be a very suitable site for aromatic compounds; hence, it appears to be a preferred model to design aromatic ligands for further therapeutic examination. Unlike plant peroxidases, whose ligand binding sites are situated very close to the surface of the protein, the site in LPO as in MtCP is buried deep in the protein core (4, 9). Therefore, the mode of binding of INH to LPO can provide useful details of many common features of the mode of binding of INH to MtCP. Recently, the presence of LPO has been reported in human airways (12), implying a possible functional role of LPO in the defense against respiratory infections. In addition to general defense against bacterial infection, LPO may have similar binding properties as well as a similar mode of enzyme activity as that of catalase peroxidase in converting INH to active species, leading to protection against M. tuberculosis infection in the respiratory system. We present here a detailed analysis of the crystal structure of the complex of LPO with INH and provide a revealing comparison with the structure of MtCP and other heme peroxidases as well as an insight into INH toxicity.

**Experimental Procedures**

**Protein Purification**—Fresh bovine milk was collected from the Indian Veterinary Research Institute (Izatnagar, India). The samples were skimmed and separated from fat. These were diluted twice with 50 mM Tris-HCl (pH 7.8). Cation exchanger CM-Sephadex C-50 (7 g/liter) equilibrated in 50 mM Tris-HCl, pH 7.8, was added stirred slowly for about 1 h with a mechanical stirrer. The gel was allowed to settle, and the solution was decanted. In order to remove the unbound proteins, protein-bound gel was washed with an excess of 50 mM Tris-HCl (pH 8.0). The washed gel was loaded on a CM-Sephadex C-50 (Amersham Biociences) column (10 × 2.5 cm) and equilibrated with 50 mM Tris-HCl, pH 8.0. The elution of LPO was done with a linear gradient of 0.0–0.5 M NaCl using the same buffer. The protein fractions with an Rz value of 0.79 and above were pooled and concentrated using an Amicon ultrafiltration cell. The concentrated protein sample was passed through a Sephadex G-100 column (100 × 2 cm) using 50 mM Tris-HCl buffer, pH 8.0. The elution was done at a flow rate of 6.0 ml/h. The fractions with an Rz value of 0.9 and above were pooled and dialyzed against deionized water, lyophilized, and stored at 253 K.

**Detection of INH in Crystal**—In order to confirm the presence of INH in the crystals that were used for x-ray intensity data collection, the crystals were washed with distilled water and dissolved in the buffer containing 50 mM Tris-HCl, pH 8.0. 1 M NaCl solution prepared in the same buffer was added to make it 1:1 (v/v) with the crystal solution. This solution was ultrafiltered using a membrane with a cut-off of 1 kDa. The presence of INH in the filtrate was observed by an NBT reduction assay with a minor modification (15). The reaction mixture was prepared with 0.2 mM NBT in 0.05 M phosphate buffer at pH 7.0. LPO, H2O2, and INH were added to the above mixture. Scavengers for oxygen free radicals superoxide dismutase and benzene were added after the reaction was initiated. The changes in A566 were recorded continuously using a double beam spectrophotometer (Beckman Instruments, Inc.). The final estimated volume of the reaction mixture was 3.0 ml. All of the above experiments were performed at 298 K.

**Crystalization of LPO with INH**—The purified samples of protein were dissolved in 0.05 M Tris-HCl buffer (pH 8.0) to a concentration of 25 mg/ml. INH was dissolved in the same buffer at a concentration of 1 mg/ml. Another solution containing 0.2 M ammonium iodide and 20% (w/v) polyethylene glycol 3350 was prepared for the reservoir. 6 μl of protein-ligand solution was mixed with 6 μl of reservoir solution to prepare 12 μl of drops for the hanging drop vapor diffusion method using 24-well Limbro plates. The rectangular dark brown-colored crystals measuring up to 0.4 × 0.4 × 0.3 mm3 were obtained after 5 days.

**X-ray Intensity Data Collection and Processing**—The x-ray intensity data were collected at 283 K using an MAR345 imaging plate scanner mounted on a Rigaku RU-300 rotating anode x-ray generator operating at 50 kV and 100 mA. The complete data were collected to 2.7 Å resolution using one crystal. The data were processed and scaled with DENZO and SCALEPACK (16), respectively. The crystals belong to monoclinic space group P21 with one molecule in the asymmetric unit. The results of the data collection and processing statistics are given in Table 1.

**Structure Determination and Refinement**—The structure was determined with molecular replacement method using the
Crystal Structure of Lactoperoxidase Complex with Isoniazid

FIGURE 1. Stereoview of the observed \(|F_o| - |F_c|\) difference Fourier density (countered at 2.5\(\sigma\)) for the INH molecule prior to inclusion of INH in the model. This figure was drawn using PyMol (49).

FIGURE 2. Plot showing INH binding to LPO. The different concentrations (1–20 \(\mu\)M) of INH were incubated with 5 \(\mu\)M LPO for 12 h to make sure that the equilibrium was achieved. The binding of INH was detected by taking absorbance of LPO (heme) at 412 nm. The data were fitted using SigmaPlot version 8.0 (20). The absorbance of LPO (heme) without INH (\(A_o\)) and with INH (\(A\)) were measured. The dissociation constant (\(K_d\)) of LPO with INH was determined from the non-linear fit curve.

FIGURE 3. NBT reduction by the product of the reaction LPO-H\(_2\)O\(_2\) with INH. The reaction mixture consisted of 0.2 mM NBT, 33 \(\mu\)g of LPO, and 100 \(\mu\)M H\(_2\)O\(_2\) in 3.0 ml of 0.05 M phosphate buffer (pH 7.0) at 298 K. The reaction was initiated by adding 9.6 mM INH. The change in absorbance at 560 nm showed an approximate value of \(K_d\) for LPO binding INH is on the order of 10\(^{-5}\) M. This value indicates a stronger binding of INH to LPO than those of salicylhydroxamic acid (7 \(\times\) 10\(^{-5}\) M) (21) and benzylhydroxamic acid (3 \(\times\) 10\(^{-2}\) M) (21), but it is comparable with the binding constant of INH with MtCP (1.4 \(\times\) 10\(^{-6}\) M) (22). The corresponding value for INH binding to horseradish peroxidase isozyme C is on the order of 10\(^{-5}\) M (23).

RESULTS AND DISCUSSION

Analysis of INH Binding to LPO—Because of the relatively small spectral changes, a concentration of 5 \(\mu\)M LPO was incubated with varying concentrations of INH (from 20 to 1 \(\mu\)M) for 12 h at 4 °C. The binding of INH to LPO was confirmed by heme absorbance at 412 nm. The non-linear fitting curve was obtained from the absorbance data for depleting concentrations of INH using SigmaPlot version 8.0 (20). The dissociation constant for LPO with INH was estimated from the non-linear fitting curve (Fig. 2). The approximate value of \(K_d\) was found to be 0.9 \(\times\) 10\(^{-6}\) M. This value indicates a stronger binding of INH to LPO than those of salicylhydroxamic acid (7 \(\times\) 10\(^{-5}\) M) (21) and benzylhydroxamic acid (3 \(\times\) 10\(^{-2}\) M) (21), but it is comparable with the binding constant of INH with MtCP (1.4 \(\times\) 10\(^{-6}\) M) (22). The corresponding value for INH binding to horseradish peroxidase isozyme C is on the order of 10\(^{-5}\) M (23).

INH as a Substrate of LPO—As seen from Fig. 3, the reduction of NBT as indicated by absorption at 560 nm showed a clear solution with correlation coefficient of 62.6% and an \(R\) factor of 35.7%. The refinement was carried out using the program CNS (17). The \(|2F_o| - |F_c|\) and \(|F_o| - |F_c|\) maps were calculated to adjust the protein molecule in the density maps using the program O (18). Several cycles of restrained positional refinement with individual \(B\)-factors and several rounds of simulated annealing from 3000 to 300 K allowed the correct tracing of the flexible loops, where the conformations were markedly different from the initial model. At the end of this stage, the \(R_{cryst}\) and \(R_{free}\) factors dropped to 0.257 and 0.291, respectively. Both Fourier \(|2F_o| - |F_c|\) and difference Fourier \(|F_o| - |F_c|\) maps computed at this stage clearly indicated the presence of an isoniazid molecule in the substrate binding site on the distal side in lactoperoxidase (Fig. 1). The initial coordinates of the isoniazid structure were taken from the studies of Bhat et al. (19) and were fitted into the very characteristic observed electron density in the \(|F_o| - |F_c|\) difference Fourier map. Further rigid body refinement and several rounds of positional and restrained individual \(B\)-factor refinement were carried out. Additional peaks above 3\(\sigma\) in \(|F_o| - |F_c|\) and 1.5\(\sigma\) in \(|2F_o| - |F_c|\) electron density maps were observed, which were interpreted as a heme moiety, one SCN\(^-\) ion, 10 carbohydrate residues from four glycan chains, seven iodide ions, and a calcium ion. These maps were also used to determine the positions of 300 water molecules by applying the usual criteria of hydrogen bonding with protein atoms or another water molecule and \(B\) factors up to 50 Å\(^2\). After the final round of calculations with 3000 K simulated annealing coupled with positional refinement and restrained individual \(B\)-factor refinement, the \(R_{cryst}\) and \(R_{free}\) factors converged to 0.205 and 0.232, respectively. Both Fourier \(|2F_o| - |F_c|\) and difference Fourier \(|F_o| - |F_c|\) electron density maps were calculated to adjust the protein molecule in the density maps using the program CNS (17). The \(|2F_o| - |F_c|\) and \(|F_o| - |F_c|\) maps were calculated to adjust the protein molecule in the density maps using the program O (18). Several cycles of restrained positional refinement with individual \(B\)-factors and several rounds of simulated annealing from 3000 to 300 K allowed the correct tracing of the flexible loops, where the conformations were markedly different from the initial model. At the end of this stage, the \(R_{cryst}\) and \(R_{free}\) factors dropped to 0.257 and 0.291, respectively. Both Fourier \(|2F_o| - |F_c|\) and difference Fourier \(|F_o| - |F_c|\) maps computed at this stage clearly indicated the presence of an isoniazid molecule in the substrate binding site on the distal side in lactoperoxidase (Fig. 1). The initial coordinates of the isoniazid structure were taken from the studies of Bhat et al. (19) and were fitted into the very characteristic observed electron density in the \(|F_o| - |F_c|\) difference Fourier map. Further rigid body refinement and several rounds of positional and restrained individual \(B\)-factor refinement were carried out. Additional peaks above 3\(\sigma\) in \(|F_o| - |F_c|\) and 1.5\(\sigma\) in \(|2F_o| - |F_c|\) electron density maps were observed, which were interpreted as a heme moiety, one SCN\(^-\) ion, 10 carbohydrate residues from four glycan chains, seven iodide ions, and a calcium ion. These maps were also used to determine the positions of 300 water molecules by applying the usual criteria of hydrogen bonding with protein atoms or another water molecule and \(B\) factors up to 50 Å\(^2\). After the final round of calculations with 3000 K simulated annealing coupled with positional refinement and restrained individual \(B\)-factor refinement, the \(R_{cryst}\) and \(R_{free}\) factors converged to 0.205 and 0.232, respectively. The refined coordinates of the lactoperoxidase complex with INH have been deposited in the Protein Data Bank (Protein Data Bank code 3I6N). The statistics of data collection and refinement are listed in Table 1.

RESULTS AND DISCUSSION

Analysis of INH Binding to LPO—Because of the relatively small spectral changes, a concentration of 5 \(\mu\)M LPO was incubated with varying concentrations of INH (from 20 to 1 \(\mu\)M) for 12 h at 4 °C. The binding of INH to LPO was confirmed by heme absorbance at 412 nm. The non-linear fitting curve was obtained from the absorbance data for depleting concentrations of INH using SigmaPlot version 8.0 (20). The dissociation constant for LPO with INH was estimated from the non-linear fitting curve (Fig. 2). The approximate value of \(K_d\) was found to be 0.9 \(\times\) 10\(^{-6}\) M. This value indicates a stronger binding of INH to LPO than those of salicylhydroxamic acid (7 \(\times\) 10\(^{-5}\) M) (21) and benzylhydroxamic acid (3 \(\times\) 10\(^{-2}\) M) (21), but it is comparable with the binding constant of INH with MtCP (1.4 \(\times\) 10\(^{-6}\) M) (22). The corresponding value for INH binding to horseradish peroxidase isozyme C is on the order of 10\(^{-5}\) M (23).

INH as a Substrate of LPO—As seen from Fig. 3, the reduction of NBT as indicated by absorption at 560 nm showed...
that the oxidation of INH by LPO occurred at the expense of H$_2$O$_2$. A proportional increase in the rate of reduction of NBT was clearly observed with increasing concentrations of INH. LPO and INH or H$_2$O$_2$ was omitted from the reaction mixture, the reduction of NBT did not occur. Thus, it clearly showed that the product of the reaction of LPO-H$_2$O$_2$ with INH was essential to cause the reduction of NBT.

**Quality of the Model**—The final model, as summarized in Table 1, represents a well defined molecule of lactoperoxidase consisting of residues 1–595, a prosthetic heme group, one molecule of prodrug isoniazid, and 300 water oxygen atoms. The overall mean $B$-factor for all atoms is 43.4 Å$^2$, which is slightly higher than the values reported for other lactoperoxidase structures (24–26). However, there is no apparent reason for this increase. The geometry of the protein molecule is close to ideal values with root mean square deviations of 0.01 Å and 1.8° from the standard values for bond lengths and angles, respectively. The quality of the model was further checked using PROCHECK (27), which showed that 86.3% of the non-Gly and non-Pro residues were present in the most favored regions of the Ramachandran plot (28). The MolProbity score (29) of the protein structure indicates a score in the 92nd percentile.

**Overall Structure**—The overall folding of the polypeptide chain of LPO in the complex with INH is essentially similar to that of its native structure (PDB code 3GC1) with a root mean square shift of 0.6 Å for the C$^\alpha$ atoms. The molecular organization of LPO with bound INH at the distal site is shown with $\alpha$-helices as a cylindrical representation (Fig. 4). The INH molecule in the substrate binding site on the distal heme side is oriented with its pyridine ring toward the heme iron, whereas the acyl hydrazide moiety occupies the distant position near the entrance to the substrate binding site. The distances of the farthest (amino nitrogen N3 of the hydrazide moiety) and the nearest (pyridinyl nitrogen N1) atoms of INH from heme iron are 8.6 and 4.0 Å, respectively. These values indicate that INH occupies a more central position in the substrate binding site when compared with those of salicylhydroxamic acid (SHA; 8.1 and 3.0 Å) (26), benzylhydroxamic acid (BHA; 8.8 and 3.3 Å) (26), and acetyl salicylic acid (ASA; 10.4 and 4.6Å) (26). The pyridine ring of INH is essentially planar, whereas the acyl hydrazide moiety is considerably non-planar. The plane of the acyl hydrazide group is oriented with respect to the plane of the pyridine ring at 82.1°. The corresponding values for INH in the complexes with CcP, APX, W41A, and H42A are 35.2, 50.5, 25.1, and 9.0°, respectively. In the crystals of native INH, the angle between the two planes was reported to be 18.1° (19).

**Table 1**

| Parameter                        | Value   |
|----------------------------------|---------|
| **Data collection**              |         |
| Space group                      | P2$_1$  |
| Unit cell dimensions             |         |
| a (Å)                            | 54.4    |
| b (Å)                            | 80.5    |
| c (Å)                            | 77.7    |
| $\beta$ (degrees)                | 102.6   |
| No. of molecules in the unit cell| 2       |
| $V_o$ (Å$^3$/Da)                 | 2.6     |
| Solvent content (%)              | 52      |
| Resolution range (Å)             | 75.8–2.7|
| Total no. of measured reflections| 38,436  |
| No. of unique reflections        | 17,002  |
| Completeness of data (%)         | 93.7 (96.7) |
| $R_{free}$ (%) (for all data)    | 23.2    |
| $R_{cryst}$ (%) (for all data)   | 20.5    |
| Protein atoms                    | 4817    |
| Calcium ion                      | 1       |
| Isoniazid (1) atoms              | 10      |
| Thiocyanate (1) atoms            | 3       |
| GlcNAc residues (8) (N-linked)    | 112     |
| Man residues (2) (N-linked)       | 22      |
| Water oxygen atoms               | 300     |
| Root mean square deviation in bond lengths (Å) | 0.01 |
| Root mean square deviation in bond angles (degrees) | 1.9 |
| Root mean square deviation in dihedral angles (degrees) | 15.1 |
| Average $B$-factor (Å$^2$)       | 43.4    |
| All atoms                        | 43.4    |
| Protein atoms                    | 41.9    |
| Water atoms                      | 51.2    |
| Ligand atoms                     | 31.5    |
| Wilson                            | 45.3    |
| Ramachandran plot                |         |
| Residues in the most favored regions (%) | 86.3 |
| Residues in the additionally allowed regions (%) | 11.4 |
| Residues in the generously allowed regions (%) | 2.3 |
| MolProbity score in percentile   | 92      |
| Protein Data Bank code           | 3I6N    |

*a* Number in parentheses indicates number of molecules.

**FIGURE 4**. The overall folding of the protein shown as a schematic diagram, where $\alpha$-helices are indicated as cylinders and numbered. Helix $H_{2a}$ (red) is a unique element of the structure of LPO. The two anti-parallel $\beta$-strands are drawn as arrows. Heme iron (Fe) is shown in blue. Residues involved in the hydrogen bonding with INH are also shown. Two water molecules, W-1 and W-89, are indicated as part of the hydrogen-bonded network. An important salt bridge between Glu$^{130}$ and His$^{426}$ that works as an anchor for the loop 421–430 is also shown. The dotted lines indicate hydrogen bonds.
hydrophilic center, whereas the fourth side is formed by the heme moiety. The most important and distinctive character of the substrate specificity in LPO is contributed by Gln423, Phe422 O, and Pro424. These residues are lined up favorably because of the unique conformation of loop Lys420–Phe431, of which residues, Leu421-Phe422 O-Gln423–Pro424 make one of the walls of the internal face of the substrate binding site with Phe422 O and Gln423 providing the most needed recognition element for the substrates. The corresponding loop, Arg405–Ile413, in MPO adopts an entirely different conformation of which residues Leu406-Phe407-Val410 make the corresponding side of the wall of the substrate binding site, which offers Phe407 for interactions at the time of entrance to the substrate binding site. Remarkably, the corresponding loop, Lys310–Ile317 in MtCP displays a similar character as that of lactoperoxidase with Thr314-Ser315-Ile317 making the corresponding face of the substrate binding site of which residues Thr314 and Ser315 participate in the interactions with the substrate. In contrast, in plant and fungal peroxidases, the situation is completely different, with a shallow cleft in which Arg186 offers a mixture of C40, C40, and NH3 for initial recognition. The resemblance of catalytic regions in LPO and MtCP indicates that these two enzymes have similar characteristics at the substrate recognition site for interactions with ligands, whereas in this regard plant and fungal peroxidases differ considerably.

**Binding of INH**—LPO has been shown to catalyze the oxidation of a variety of aromatic donor compounds (30–40), including INH (10), with the help of H2O2. A similar catalytic mechanism has also been described for horseradish peroxidase isozyme C enzyme (10). The binding of INH has also been reported with CcP (41) and APX (41). The solution studies using an NBT reduction assay indicated the presence of INH in the crystals, which was subsequently confirmed by crystal structure determination through the observation of a characteristic electron density for INH. It was located in the substrate binding site on the distal heme side. In the native structure, the site is filled with six water molecules, W-1, W-2’, W-3’, W-4’, W-5’, and W-6’ (Fig. 5). The identical water molecules are replaced when ASA interacts with LPO, whereas W-2 and W-3’ remain and participate in the hydrogen-bonded network at the binding site.

These data indicate that a considerable adjustment in the relative orientations of the planes of pyridine ring and acyl hydrazide moiety can occur upon binding to peroxidases at the distal heme ligand binding site. The plane of the pyridine ring of INH is inclined at 17.9° with respect to the plane of the heme moiety. The corresponding values in the INH complexes with CcP, APX, W41A, and H42A are 39.0, 28.7, 25.1, and 9.0° respectively. The corresponding values for SHA, BHA, and ASA in their respective complexes with LPO are 15.7, 6.2, and 70.7°, respectively. These values reveal interesting relationships between the positions of aromatic rings and the relative inclinations between the planes of aromatic rings and heme moiety. When the aromatic rings are located in the proximity of the hydrophilic center, the planes are relatively more parallel than those when it is located away from the hydrophilic center. This indicates that the substrate binding site on the distal heme side is narrower at the hydrophilic center and gradually broadens toward the entrance from the hydrophobic channel. This also shows that INH can be oriented to fit in the ligand binding sites of peroxidases.

**Substrate Specificity**—Lactoperoxidase shows a high degree of substrate specificity for aromatic compounds. The substrate binding site on the distal heme side in LPO is situated deep in the core of the protein. The binding site is connected to the surface of the protein through a long hydrophobic channel, the length of which is more than 20 Å as measured from Pro234 Ca to the nearest atom of heme moiety. The channel is composed of residues Pro234, Pro236, Phe254, Phe380, Phe381, Phe422, and Pro424. At the entrance of the substrate binding site, on one side, it provides partners for the formations of hydrogen bonds through Phe422 O and Gln423, whereas on other side, it has Arg255 C7 and C8 and Glu256 Cl and C9 atoms that provide van der Waals interactions. Facing the entrance directly, it has a Ile317 in MtCP displays a similar character as that of lactoperoxidase with Thr314-Ser315-Ile317 making the corresponding face of the substrate binding site of which residues Thr314 and Ser315 participate in the interactions with the substrate. In contrast, in plant and fungal peroxidases, the situation is completely different, with a shallow cleft in which Arg186 offers a mixture of C40, C40, and NH3 for initial recognition. The resemblance of catalytic regions in LPO and MtCP indicates that these two enzymes have similar characteristics at the substrate recognition site for interactions with ligands, whereas in this regard plant and fungal peroxidases differ considerably.

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**Substrate Specificity**—Lactoperoxidase shows a high degree of substrate specificity for aromatic compounds. The substrate binding site on the distal heme side in LPO is situated deep in the core of the protein. The binding site is connected to the surface of the protein through a long hydrophobic channel, the length of which is more than 20 Å as measured from Pro234 Ca to the nearest atom of heme moiety. The channel is composed of residues Pro234, Pro236, Phe254, Phe380, Phe381, Phe422, and Pro424. At the entrance of the substrate binding site, on one side, it provides partners for the formations of hydrogen bonds through Phe422 O and Gln423, whereas on other side, it has Arg255 C7 and C8 and Glu256 Cl and C9 atoms that provide van der Waals interactions. Facing the entrance directly, it has a Ile317 in MtCP displays a similar character as that of lactoperoxidase with Thr314-Ser315-Ile317 making the corresponding face of the substrate binding site of which residues Thr314 and Ser315 participate in the interactions with the substrate. In contrast, in plant and fungal peroxidases, the situation is completely different, with a shallow cleft in which Arg186 offers a mixture of C40, C40, and NH3 for initial recognition. The resemblance of catalytic regions in LPO and MtCP indicates that these two enzymes have similar characteristics at the substrate recognition site for interactions with ligands, whereas in this regard plant and fungal peroxidases differ considerably.

**Binding of INH**—LPO has been shown to catalyze the oxidation of a variety of aromatic donor compounds (30–40), including INH (10), with the help of H2O2. A similar catalytic mechanism has also been described for horseradish peroxidase isozyme C enzyme (10). The binding of INH has also been reported with CcP (41) and APX (41). The solution studies using an NBT reduction assay indicated the presence of INH in the crystals, which was subsequently confirmed by crystal structure determination through the observation of a characteristic electron density for INH. It was located in the substrate binding site on the distal heme side. In the native structure, the site is filled with six water molecules, W-1, W-2’, W-3’, W-4’ W-5’ and W-6’. The binding of INH displaces four water molecules, W-2’, W-4’ W-5’ and W-6’ (Fig. 5). The identical water molecules are replaced when ASA interacts with LPO, whereas SHA and BHA replace W-1 instead of W-6’. The water molecule W-1 occupies a position in the center between the positions of aromatic rings and the relative inclinations of heme-iron and the His109 Nε atom. INH is involved in extensive interactions with protein atoms from all sides. The hydrazide nitrogen atom N3 forms two hydrogen bonds with Gln423 (Gln423 Oε1–N3 = 3.1 Å) and Phe422 (Phe422 O–N3 = 3.3 Å), whereas the carbonyl oxygen atom of the acyl hydrazide
moiety interacts with Phe$^{254}$ O through a bridging water molecule, W-89. On the opposite side to this, the pyridine ring N1 atom is hydrogen-bonded to water molecule W-1 (N1--W-1 = 2.7 Å), which in turn is hydrogen-bonded to heme iron, Gin$^{105}$ N$^{ε2}$, and His$^{109}$ N$^{ε2}$. The other atoms of the pyridine ring form several van der Waals contacts (distances of <4.2 Å) with atoms of the heme pyrrole rings from one side, whereas C$^{γ}$ and C$^{β}$ atoms of Arg$^{255}$ and C$^{β}$ and C$^{γ}$ atoms of Glu$^{258}$ interact from the remaining sides. Overall, INH is held in the substrate binding site by several hydrogen-bonded interactions from two opposite ends, whereas van der Waals forces stabilize it from the other two opposite sides in the perpendicular direction.

Comparisons of INH Binding in Various Heme Peroxidases—In addition to the present complex of INH with LPO, the structures of INH complexes with other heme peroxidases, such as APX (41) and CcP (41), including NMR studies on horseradish peroxidase isozyme C (23), are available. The INH complexes with two mutants of ascorbate peroxidase, W41A and H42A have also been reported (41). The substrate binding sites on the distal side in plant and fungal peroxidases are situated very close to the surface of the protein (5–8), whereas those in mammalian peroxidases are located deep inside the molecule. The substrate binding site on the distal side of M. tuberculosis catalase peroxidase is also deeply buried in the protein core. The substrate binding site in mammalian peroxidases is connected through a long hydrophobic channel that guides the passage of the ligand to the substrate binding on the distal side (Fig. 6a). In this context, the passage to the substrate binding site on the distal heme side in the M. tuberculosis catalase peroxidase that apparently is the target for the prodrug INH resembles very closely that of lactoperoxidase (Fig. 6b), whereas in plant and fungal peroxidases, the substrate binding site on the distal heme side is located near the protein surface (Fig. 6c). In order to describe the ligand binding site in mammalian heme peroxidases, it has been divided into five subsites (26). In this nomenclature, the heme group is designated as subsite S1, which provides mainly hydrophobic interactions; next to it is the hydrophilic center designated as subsite S2, which consists of active site residues, such as His$^{109}$, Gin$^{105}$, and water molecule W-1; the surface opposite the heme group is defined as subsite S3, having C$^{γ}$ and C$^{β}$ atoms of Arg$^{255}$; the surface below Arg$^{255}$ is called subsite S4, which provides C$^{β}$ and C$^{γ}$ atoms of Glu$^{258}$ for hydrophobic interactions; and finally the residues Phe$^{422}$, Gin$^{423}$, and Pro$^{224}$ at the entrance are part of the subsite S5. One of the most important differences among the substrate
binding sites of various heme peroxidases has been observed in subsite S5. The key residues that are involved in the recognition of INH are Gln423 and Phe422 in LPO, Arg184 and Ser185 in CcP, Arg172 and Ser173 in APX, and Thr314 and Ser315 in MtCP. It may be mentioned here that Ser315 in MtCP is one of the hotspots of mutations that have been identified in INH-resistant strains (9).

As revealed by the structure of the complex of LPO with INH, the hydrazide moiety is in contact with subsite S5, whereas the pyridinyl nitrogen atom interacts with the residues of subsite S2. In contrast, INH is oriented in the opposite direction in the complexes of CcP (41) and APX (41). As reported earlier, different orientations were observed for various aromatic ligands, such as SHA, BHA, and ASA, when complexed with LPO (26). Similarly, the structures of SHA and BHA complexed with other peroxidases indicated more than one orientation of aromatic ligands in the substrate binding sites on the distal side (42–45). These observations indicate that the size and chemical nature of the binding sites in peroxidases on the distal side allow the substrates of the size of INH to be able to orient in more than one way. Therefore, the substrates, such as INH, are in dynamic equilibrium in the binding site of peroxidases. There have also been observations suggesting that the substrate orientations could also be influenced by the introduction of H₂O₂ (46). In this regard, it may be understood that the observed orientation of INH in the complex of LPO-INH is the first step of recognition of the substrate by the protein, which may undergo further changes in the orientations. However, resulting resistance to INH does not arise.

Conclusions—Prodrug isoniazid is activated by lactoperoxidase in a manner similar to that of catalase peroxidase endogenous to M. tuberculosis. It is indeed remarkable to observe that the substrate binding sites on the distal side in both of these enzymes are situated deeply in the protein cores. The buried substrate binding sites are connected to the surface of the protein through a well formed hydrophobic channel. In contrast, the substrate binding sites in plant and fungal heme peroxidases are virtually exposed to the surfaces of these proteins. Due to the long hydrophobic channels, which are rich with aromatic residues, the aromatic substrates with an attached hydrophilic moiety, such as isoniazid, is expected that the substrate diffuses into the hydrophobic channel with the aromatic moiety in the forefront. Upon entering the substrate binding site, the same orientation of isoniazid as is maintained during diffusion is captured initially by the substrate binding site through interactions of Phe422 and Gln423 with the hydrazide moiety of INH. Because of the similarities of hydrophobic channels and hydrophilic residues at the entrance in both enzymes, LPO and MtCP, a similar mode of binding may occur. It may be mentioned here that in plant and fungal peroxidases, a similarly long hydrophobic channel is not present to induce any orientation in the substrate molecule. Notably, the binding constants of LPO and MtCP for isoniazid are comparable, whereas those of plant and fungal peroxidases show relatively low values. Recent findings have indicated that human airways contain a complete and functional LPO antimicrobial system, which is effective against a variety of respiratory pathogens. It has also been shown that...
isoniazid binds to LPO and converts it into an active species similar to that produced by catalase peroxidase. The structural studies further define the mode of binding and the process of diffusion of isoniazid through the hydrophobic channel to the site of substrate binding on the distal side. Because LPO is present in the human airways and is capable of binding to isoniazid for oxidation, the administration of dry powders of isoniazid through inhaling will convert INH into active species for the treatment of airway tuberculosis. The fundamental understanding of the process of diffusion, mode of binding, and mechanism of action gained through these studies further suggests that the isonicotinoyl radical can be produced by the reaction of LPO-H2O2 with isoniazid. Using a structure-based approach, new ligands can be designed in place of isoniazid for producing isonicotinoyl radical-like species to overcome the drug resistance. The possibility of lactoperoxidase substituting for the catalase peroxidase activity is also established. It is also pertinent to mention here that the presence of LPO and H2O2 at certain sites may be the basis of INH toxicity as a result of long term INH administration.

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Crystal Structure of Lactoperoxidase Complex with Isoniazid